PAPEL DE LA LEPTINA Y SU RESISTENCIA EN EL SÍNDROME DE OVARIO POLIQUÍSTICO Y LA DIABETES GESTACIONAL

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Memoria presentada por Dª. Teresa Vilariño García para optar al grado de Doctor por la Universidad de Sevilla

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CERTIFICAN:

Que la Tesis Doctoral titulada "**PAPEL DE LA LEPTINA Y SU RESISTENCIA EN EL SÍNDROME DE OVARIO POLIQUÍSTICO Y LA DIABETES GESTACIONAL**" realizada por Dª. Teresa Vilariño García para optar al grado de Doctor, ha sido llevada a cabo bajo nuestra dirección.

V.º B.º
Los Directores

Dr. Víctor Sánchez Margalet Dr. Antonio Pérez Pérez

A mi familia

"Si en medio de las adversidades persevera el corazón con serenidad, con gozo y con paz, esto es amor"

Santa Teresa de Jesús

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ABREVIATURAS

AHA,: Asociación Estadounidense del Corazón

AQP, Aquaporina

ATPIII, National Cholesterol Education Program Adult Treatment Panel III

BCR, receptor de células B

DMG: Diabetes Mellitus Gestacional

EGF: Factor de crecimiento epidérmico

HDL, Lipoproteína de alta densidad

IDF, Federación Internacional de Diabetes

IMC, Índice de masa corporal

JAK-STAT Janus kinase/signal transducer and activator of transduction)

LEPR, Receptor de Leptina

MAPK, Mitogen-Activated Protein Kinase

NHLI, Instituto Nacional del Corazón, los Pulmones y la Sangre

PAI-1, Inibidor del Activador del Plasminógeno

PCOS, Síndrome de Ovario Poliquístico

STAR, signal transduction and activation of RNA metabolism

TCR, receptor de células T

TNF, Factor de Necrosis Tumoral

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INTRODUCCIÓN

1.SINDROME DE RESISTENCIA A LA INSULINA-SINDROME METABÓLICO.

El síndrome metabólico se define como un complejo conjunto de factores relacionados entre sí, entre los que se encuentra la obesidad, y que contribuyen en diferente medida a aumentar el riesgo de padecer enfermedad cardiovascular y diabetes. Se postula además el posible papel de la resistencia a la insulina como factor de enlace entre todos los factores de riesgo que constituyen dicho síndrome [1]

En 1998, la WHO (OMS) sentó las bases de la definición de Síndrome Metabólico, entendiéndolo como un estado de intolerancia a la glucosa o diabetes mellitus y/o Resistencia a la insulina con presencia de al menos 2 de 5 estados relacionados a continuación:

1.- Disminución de la capacidad de regulación de la glucemia o diabetes según los siguientes criterios: (los valores de la **tabla** 1)

Table 1. Values for diagnosis of diabetes mellitus and other categories of hyperglycaemia

	Glucose concentration (mmol l^{-1} (mg dl^{-1}))		
	Whole blood		Plasma[a]
	Venous	Capillary	Venous
Diabetes Mellitus:			
Fasting	≥6.1 (≥110)	≥6.1 (≥110)	≥7.0 (≥126)
or			
2-h post glucose load	≥10.0 (≥180)	≥11.1 (≥200)	≥11.1 (≥200)
or both			
Impaired Glucose Tolerance (IGT):			
Fasting concentration (if measured)	<6.1 (<110)	<6.1 (<110)	<7.0 (<126)
and			
2-h post glucose load	≥6.7 (≥120) and <10.0 (<180)	≥7.8 (≥140) and <11.1 (<200)	≥7.8 (≥140) and <11.1 (<200)
Impaired Fasting Glycaemia (IFG):			
Fasting	≥5.6 (≥100) and <6.1 (<110)	≥5.6 (≥100) and <6.1 (<110)	≥6.1 (≥110) and <7.0 (<126)
2-h (if measured)	<6.7 (<120)	<7.8 (<140)	<7.8 (<140)

[a]Corresponding values for capillary plasma are: for Diabetes Mellitus, fasting ≥7.0 (≥126), 2-h ≥12.2 (≥220); for Impaired Glucose Tolerance, fasting <7.0 (<126) and 2-h ≥8.9 (≥160) and <12.2 (<220); and for Impaired Fasting Glycaemia ≥6.1 (≥110) and <7.0 (<126) and if measured, 2-h <8.9 (<160).

For epidemiological or population screening purposes, the fasting or 2-h value after 75 g oral glucose may be used alone. For clinical purposes, the diagnosis of diabetes should always be confirmed by repeating the test on another day unless there is unequivocal hyperglycaemia with acute metabolic decompensation or obvious symptoms.

Glucose concentrations should not be determined on serum unless red cells are immediately removed, otherwise glycolysis will result in an unpredictable underestimation of the true concentrations. It should be stressed that glucose preservatives do not totally prevent glycolysis. If whole blood is used, the sample should be kept at 0–4 °C or centrifuged immediately, or assayed immediately.

Tabla 1. Criterios diagnósticos de alteraciones del metanolsm

2. - Resistencia a la insulina

3. Presión arterial elevada ≥160/90 mmHg

4. Triglicéridos plasmáticos elevados (≥1.7 mmol/L; 150 mg/dL) y / o bajo HDL-colesterol (<0.9 mmol/L, 35 mg/dL hombres; <1.0 mml/L, 39 mg/dL mujeres)

5. Obesidad central (hombres: relación cintura-cadera>0.90; mujeres: índice de cintura a cadera >0.85) y / o IMC >30 kg/m^2.

6. Microalbuminuria (tasa de excreción urinaria de albúmina ≥ 20 µg/min o relación albúmina: creatinina ≥ 20 mg/g) [2].

La definición de Síndrome Metabólico siguió evolucionando según los criterios diagnósticos postulados por las diferentes organizaciones, la propia WHO (OMS) en 1998, los criterios ATPIII (National Cholesterol Education Program Adult Treatment Panel III) en 2001 y la Federación Internacional de Diabetes (FID) con la Asociación Estadounidense del Corazón (AHA) y el Instituto Nacional del Corazón, los Pulmones y la Sangre (NHLI) en 2005. Mientras la OMS basaba su definición en la presencia de alteración para regular la glucemia y resistencia a la insulina, la ATP III postula que no es necesario la demostración de resistencia a la insulina *per se*, sino la presencia de al menos 3 de los siguientes 5 factores para establecer el diagnóstico:

1.- Obesidad abdominal (que a su vez está íntimamente ligada a la Resistencia a la insulina)

2.- Elevación de los niveles séricos de triglicéridos

3.- Bajos niveles de HDL-colesterol

4.- Hipertensión

5.- Alteración de la glucosa en ayunas o diabetes mellitus tipo 2[1]

En 2005, la IDF como la AHA / NHLBI, intentaron conciliar las diferentes definiciones clínicas, aunque mantuvieron diferencias relacionadas con la circunferencia de la cintura.

La IDF eliminó el requisito de resistencia a la insulina de la OMS, pero hizo necesaria la obesidad abdominal como uno de los 5 factores necesarios en el diagnóstico, con especial énfasis en la medición de la cintura como una herramienta de detección simple; el resto de los criterios fueron esencialmente idénticos a los provistos por ATP III.

Por su parte la AHA/NHLBI modificó los criterios de ATPIII y con respecto a los de IDF no obligaba a la presencia de obesidad abdominal como factor de riesgo requerido, asumiendo sus otros 4 factores. Finalmente, IDF y AHA / NHLBI acordaron que la obesidad abdominal no debería ser un requisito previo para el diagnóstico, pero que es 1 de 5 criterios, por lo que la presencia de 3 de 5 factores de riesgo constituye un diagnóstico de síndrome metabólico. Esto daría como resultado la definición común que se muestra en la tabla 2[1].

La importancia de unificar criterios diagnósticos que definan la situación de Síndrome Metabólico radica en su condición de problema de salud pública, íntimamente ligado a la evolución de los hábitos de vida hacia el sedentarismo y por tanto, existiendo una posibilidad de prevención sobre el mismo a través de la intervención en salud para minimizar los diferentes factores de riesgo que lo componen.

El síndrome metabólico supone 5 veces más riesgo de desarrollar diabetes mellitus tipo 2. Los factores de riesgo metabólico más ampliamente reconocidos son la dislipidemia aterogénica, la presión arterial elevada y la glucosa plasmática elevada. Además, las personas con estas características comúnmente manifiestan un estado protrombótico y un estado proinflamatorio. La mayoría de las personas con el síndrome metabólico tienen obesidad abdominal y resistencia a la insulina.

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Table 1. Criteria for Clinical Diagnosis of the Metabolic Syndrome

Measure	Categorical Cut Points
Elevated waist circumference*	Population- and country-specific definitions
Elevated triglycerides (drug treatment for elevated triglycerides is an alternate indicator†)	≥150 mg/dL (1.7 mmol/L)
Reduced HDL-C (drug treatment for reduced HDL-C is an alternate indicator†)	<40 mg/dL (1.0 mmol/L) in males; <50 mg/dL (1.3 mmol/L) in females
Elevated blood pressure (antihypertensive drug treatment in a patient with a history of hypertension is an alternate indicator)	Systolic ≥130 and/or diastolic ≥85 mm Hg
Elevated fasting glucose‡ (drug treatment of elevated glucose is an alternate indicator)	≥100 mg/dL

HDL-C indicates high-density lipoprotein cholesterol.

*It is recommended that the IDF cut points be used for non-Europeans and either the IDF or AHA/NHLBI cut points used for people of European origin until more data are available.

†The most commonly used drugs for elevated triglycerides and reduced HDL-C are fibrates and nicotinic acid. A patient taking 1 of these drugs can be presumed to have high triglycerides and low HDL-C. High-dose ω-3 fatty acids presumes high triglycerides.

‡Most patients with type 2 diabetes mellitus will have the metabolic syndrome by the proposed criteria.

Tabla-2. Alberti KGMM, et al [1]

En el año 2009 K.G.M.M. Alberti y col. [1] proponen una definición de Síndrome metabólico que homogeniza los criterios de las organizaciones anteriormente señaladas. Definiéndolo como aquella situación de intolerancia a la glucosa o diabetes mellitus y/o resistencia a la insulina acompañado de dos o más de los criterios propuestos por la OMS en 1998. Se basa principalmente en los criterios propuestos en 2005 por la Federación Internacional de Diabetes (IDF) con la Asociación Estadounidense del Corazón (AHA) y el Instituto Nacional del

Corazón, los Pulmones y la Sangre (NHLI) y recogidos en la tabla considerando la importancia de establecer claros los puntos de corte del diámetro de cintura según grupos de edad, sexo o etnia [1]

La resistencia a la insulina se entiende como una disminución de la habilidad de la insulina para activar la captación de glucosa y su utilización. Las células β pancreáticas incrementan la producción de insulina como respuesta compensatoria a la hiperglucemia, generándose una situación de hiperinsulinemia [3,4]. Es bien sabido que la obesidad provoca resistencia a la insulina e hiperinsulinemia compensatoria. En los individuos obesos se diferencian dos tipos de distribución de grasa: grasa subcutánea y grasa visceral intraabdominal. Varios estudios clínicos demuestran la contribución de la acumulación de grasa visceral al desarrollo de trastornos metabólicos, incluida la intolerancia a la glucosa y la hiperlipidemia [3,5], demostrándose que la obesidad visceral presenta mayor prevalencia de síndrome de resistencia a la insulina que la acumulación subcutánea de grasa [4]. Aunque se postulan diferentes mecanismos que llevan al estado de resistencia a la insulina, Matsuzawa et al (2011) [4] propone que uno de ellos se basa en que los tejidos adiposos secretan directamente múltiples moléculas bioactivas (adipoquinas), como el factor de necrosis tumoral a (TNF-α), **la leptina** y el inhibidor del activador del plasminógeno 1 (PAI-1), que desempeña un papel importante en el desarrollo de trastornos metabólicos.

1.1.- la inflamación como base fisiopatológica del síndrome de resistencia a la insulina y la obesidad.

La inflamación es la forma de manifestarse de muchas enfermedades. Se trata de una respuesta inespecífica frente a las agresiones del medio, y está generada por los agentes inflamatorios. La respuesta inflamatoria ocurre solo en tejidos conectivos vascularizados y surge con el fin defensivo de aislar y destruir al agente dañino, así como reparar el tejido u órgano dañado. Se considera por tanto un mecanismo de inmunidad innata, en contraste con la reacción inmune adaptativa, específica para cada tipo de agente infeccioso [6].

Cuando la inflamación se mantiene durante un tiempo prolongado (semanas o meses), se habla de inflamación crónica, en la que coexisten el daño tisular y los intentos de reparación, en diversas combinaciones [6]. Puede producirse por mantenimiento de la inflamación aguda (si no se resuelve la causa), o bien empezar de manera progresiva y poco evidente, sin las manifestaciones de la inflamación aguda. Este segundo caso es el responsable del daño tisular de algunas de las enfermedades humanas más invalidantes, como la artritis reumatoide, la aterosclerosis [7], la tuberculosis [8] o la fibrosis pulmonar [9]. Además, es importante en el desarrollo del cáncer [10–12], campo en el que nuestro grupo ha trabajado, en especial en el cáncer de mama [11] y en enfermedades que anteriormente se consideraban exclusivamente degenerativas, como el Alzheimer [6].

Entre las causas de la inflamación crónica se pueden distinguir:

- Infecciones persistentes
- Exposición prolongada a tóxicos
- Enfermedades mediadas por el sistema inmune

En algunas enfermedades en las que la respuesta inmunitaria se produce de manera exagerada o inapropiada en relación al agente desencadenante, la inflamación crónica juega un papel importante en el aspecto patológico de las mismas. En estos casos, como la respuesta inmune está sobredimensionada, no produce beneficio, sino daño.

En este tipo de enfermedades, se suelen producir brotes repetidos de inflamación, por lo que se pueden observar características mixtas de la inflamación aguda y crónica.

Características

Mientras que la inflamación aguda se caracteriza por la aparición de cambios vasculares, edema e infiltración de neutrófilos, la inflamación crónica presenta las siguientes características distintivas:

- infiltración con células mononucleares: macrófagos, linfocitos y células plasmáticas;

- destrucción de tejidos, debido a la persistencia del agente o de las células inflamatorias;

- intentos de reconstrucción, reemplazando el tejido dañado con tejido conectivo, con proliferación de vasos (angiogénesis) y, sobre todo, fibrosis. El crecimiento de vasos sanguíneos (angiogénesis) y linfáticos, es estimulado por factores de crecimiento como (factor de crecimiento endotelial vascular) VEG, producidos por macrófagos y células endoteliales.

Los mecanismos precisos que enlazan la inflamación con la obesidad y complicaciones asociadas permanecen todavía sin establecer completamente. Durante los últimos años, diversos estudios han propuesto que la obesidad podría ser un desorden inflamatorio. Además, este estado inflamatorio, ha sido propuesto como nexo entre la obesidad y desórdenes asociados, como son la resistencia insulínica, los desórdenes cardiovasculares y el síndrome metabólico [13]. Siguiendo esta teoría, se ha asumido de forma general, que la inflamación es una consecuencia de la obesidad; sin embargo, algunos estudios recientes, han sugerido que la inflamación podría ser realmente una posible causa de la obesidad [14]. La respuesta inflamatoria iniciada en el tejido adiposo blanco, produce una situación crónica a nivel sistémico, generando un círculo vicioso, el cual finalmente conduce a resistencia insulínica, ateroesclerosis y alteraciones propias del síndrome metabólico [13,15] .El papel de la inflamación con la leptina y la obesidad en este círculo vicioso lo hemos revisado en una publicación que incluimos como **primer artículo** en la presente Tesis Doctoral.

1.2. Papel de la leptina en la inflamación

La leptina es una hormona producida por el tejido adiposo no sólo con un importante papel central en el control del metabolismo energético [6], sino también con múltiples efectos pleiotrópicos en diferentes sistemas [16,17]. Una de estas importantes funciones de la leptina es un papel regulador en la interfase entre el metabolismo energético y el sistema inmunológico, siendo la pieza

fundamental del nuevo campo de estudio del inmunometabolismo. El receptor de leptina se expresa en todas las células del sistema inmunitario, tanto del sistema innato como el adaptativo. La leptina es una de las adipocinas responsable del estado inflamatorio en la obesidad que predispone no sólo a la diabees tipo 2, el síndrome metabólico y enfermedad cardiovascular, sino también enfermedades autoinmunes y alérgicas. La leptina es un importante mediador del estado inmunosupresor en estados de malnutrición. Hemos revisado el papel central de la leptina en el inmunometabolismo en una publicación que incluimos como **segundo artículo** en la presente Tesis Doctoral.

En el caso de la mujer, el síndrome metabólico se asocia a dos circunstancias fisiopatológicas que se relacionan con la leptina y la resistencia a la insulina como elemento común: el síndrome de **ovario poliquístico** y la **diabetes gestacional** [18–20], que son las 2 patologías sobre las que hemos buscado objetivos experimentales.

2.SÍNDROME DE OVARIO POLIQUÍSTICO. PAPEL DE LA LEPTINA

El síndrome de ovario poliquístico (PCOS) es una de las manifestaciones clínicas en la mujer con síndrome metabólico. PCOS es la alteración endocrino-metabólica más común en la mujer y la causa más importante de infertilidad anovulatoria. PCOS afecta al 10-15% de las mujeres en edad reproductiva [21]. Se caracteriza por hiperandrogenismo, oligo-anovulación crónica y morfología ovárica poliquística [22]. Sin embargo, la etiología del PCOS no está completamente establecida. PCOS está considerado como una patología multifactorial con anormalidades genéticas y metabólicas que incluyen la resistencia a la insulina y la leptina [23–25]. Además, la desregulación de diferentes adipocinas, incluyendo la leptina podrían jugar un papel relevante en la patogénesis del PCOS [26]. Algunos estudios han mostrado aumentos en los niveles circulantes de leptina en mujeres con PCOS [27,28]. Un estudio incluso ha confirmado niveles aumentados de leptina en mujeres obesas con PCOS,

mientras que los niveles de adiponectina están disminuidos [29]. En este sentido, como las mujeres con PCOS suelen tener sobrepeso y obesidad, las alteraciones de la hiperleptinemia podrían venir producidas por el exceso de masa grasa, y compartir el riesgo de desarrollo de diabetes [30]. De hecho, los niveles aumentados de leptina se correlacionan con la resistencia a la insulina, las alteraciones metabólicas, la infertilidad e incluso el riesgo cardiovascular del PCOS, y podría contribuir al desarrollo del mismo [24]. Los niveles elevados de leptina podrían suponer uno de los mecanismos que favorecieran la producción de andrógenos, ya que los niveles de leptina aumentados en sangre se asocian con niveles elevados de testosterona [31].

Existen diferencias entre aquellas pacientes PCOS con obesidad o sobrepeso y las que presentan normopeso. Las primeras se caracterizan por un estado metabólico y de hiperandrogenismo empeorado, con menstruaciones y rendimiento ovulatorio inferior y peores tasas de embarazo [32]. La obesidad, tal como se ha expuesto anteriormente, produce por sí misma, alteraciones de la maduración del ovocito [33–35] y parece que, incluso, pueden ocurrir cambios moleculares en ovocitos de aspecto normal en mujeres con PCOS [36]. De hecho, parece que modificaciones en la composición del líquido folicular en la obesidad podrían alterar el desarrollo del ovocito, así como del folículo, incluyendo la granulosa, células del cúmulo y de la teca [37]. Dichas alteraciones incluirían incrementos en los niveles de insulina, glucosa y lactato, proteína C reactiva, y niveles hormonales de gonadotropina coriónica [38,39] y leptina [40]. La influencia de la obesidad en la fertilidad de las mujeres con PCOS también se evidencia por el hecho de que las medidas de intervención encaminadas a mejorar los hábitos de vida consiguiendo una pérdida de peso se muestran eficaces ya que se consiguen mejoras en las alteraciones metabólicas, hiperandrogenismo, ovulación y fertilidad en general [32]. Estos conceptos se han incluido en un artículo de revisión del papel de la leptina en la patología del embarazo incluido en esta Tesis [41], como **tercer artículo**

Sin embargo, la literatura científica no aclara si la leptina favorece directamente la síntesis de andrógenos o si la hiperleptinemia del PCOS (Fig. 1) sería un marcador de resistencia a una acción de la leptina que sería favorecedora de la

función ovárica, incluyendo la síntesis de estrógenos, lo que supone una de las preguntas de investigación de la presente Tesis Doctoral.

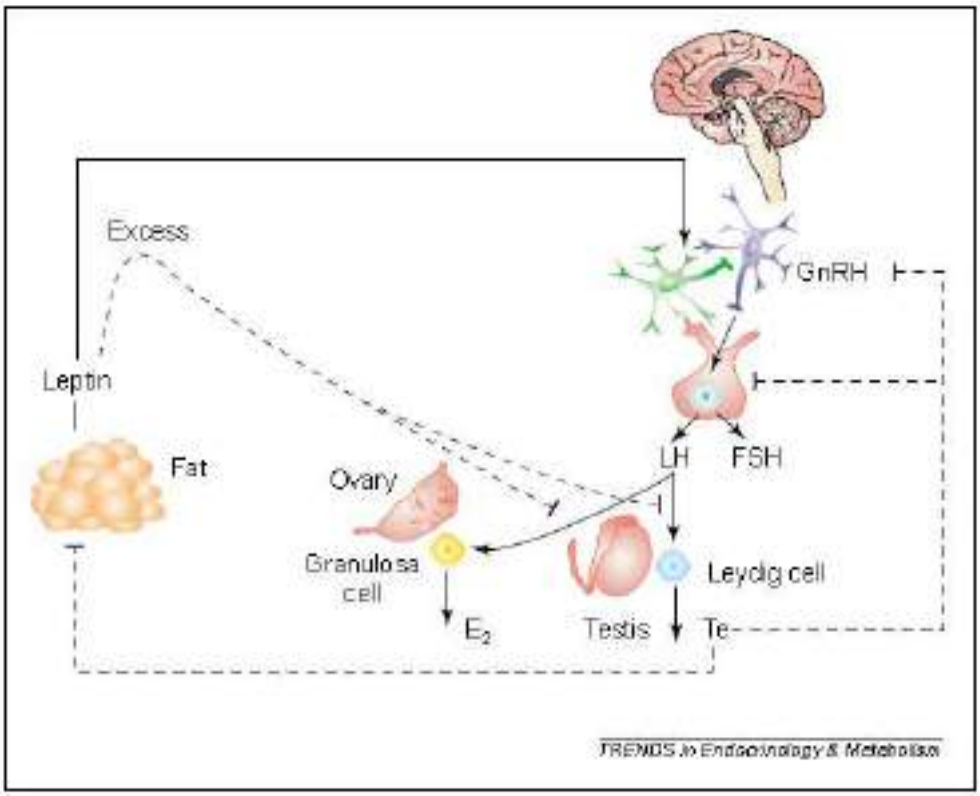

Figura 1. Regulación de la leptina de la secreción de gonadotropinas y la función gonadal [42]

3. DIABETES GESTACIONAL. PAPEL DE LA LEPTINA

3.1. LEPTINA Y PLACENTA

El crecimiento fetal depende de la habilidad de la placenta para aportar nutrientes adecuados para las necesidades del feto, que van aumentando conforme avanza la gestación. El citotrofoblasto velloso es una población de células progenitoras que producen células hijas para proveer la expansión del citotrofoblasto con el aumento del área de superficie placentaria, así como la expansión de las columnas de citotrofoblasto que contienen las células destinadas a invadir la decidua materna [43]. La placenta crece exponencialmente a lo largo del primer y segundo trimestre, pero el crecimiento se ralentiza hacia el término de la

gestación [44]. Por tanto, el crecimiento de la placenta, en especial al principio de la gestación es un requisito para una interfase de alta capacidad de transporte.

En 1997, la leptina fue descrita como una nueva hormona placentaria en humanos [45] por la aparición en la evolución de un enhancer de expresión placentario a partir de los primates [46]. De hecho, durante el embarazo, los niveles circulantes de leptina están aumentados por la producción en las células trofoblásticas [47]. Por eso, tras el parto, los niveles circulantes de leptina vuelven a la normalidad [48].

Para producir la señal intracelular que desencadena su función, la leptina debe unirse a su receptor (LEPR) [49]. Hay 6 isoformas diferentes de LEPR (a-f) (Fig. 2) producidas por splicing alternativo del ARN [50]. La única isoforma capaz de activar por completo las vías de señalización del LEPR es LEPRb (isoforma larga), mientras que las otras isoformas cortas tienen truncado el dominio intracelular y sólo activan parcialmente la señalización, o incluso carecen del dominio transmembrana y son solubles. Todas se expresan en el trofoblasto [51].

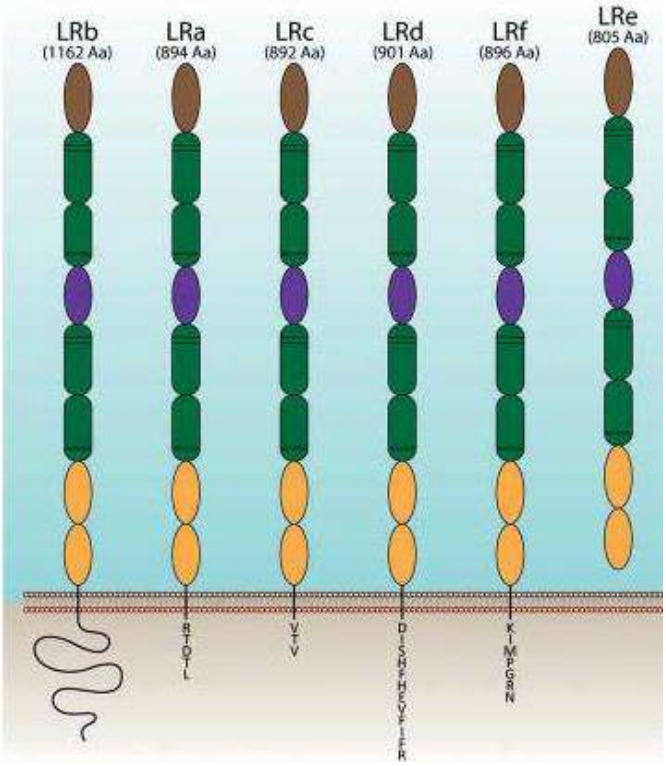

Figura 2. Isoformas del receptor de leptina [6]

La activación de LEPRb resulta en la activación de varias vías de señalización (Figura 3), que incluyen la vía JAK-STAT (Janus kinase/signal transducer and activators of transduction), la vía MAPK (mitogen-activated protein kinase), así como la vía PI3K (phosphatidylinositol 3-Kinase) [52]. Hay evidencias de interambio materno-fetal de leptina a través de la membrana [53]. Sin embargo, no sabemos cuál de los receptores media este transporte de leptina a través de la placenta.

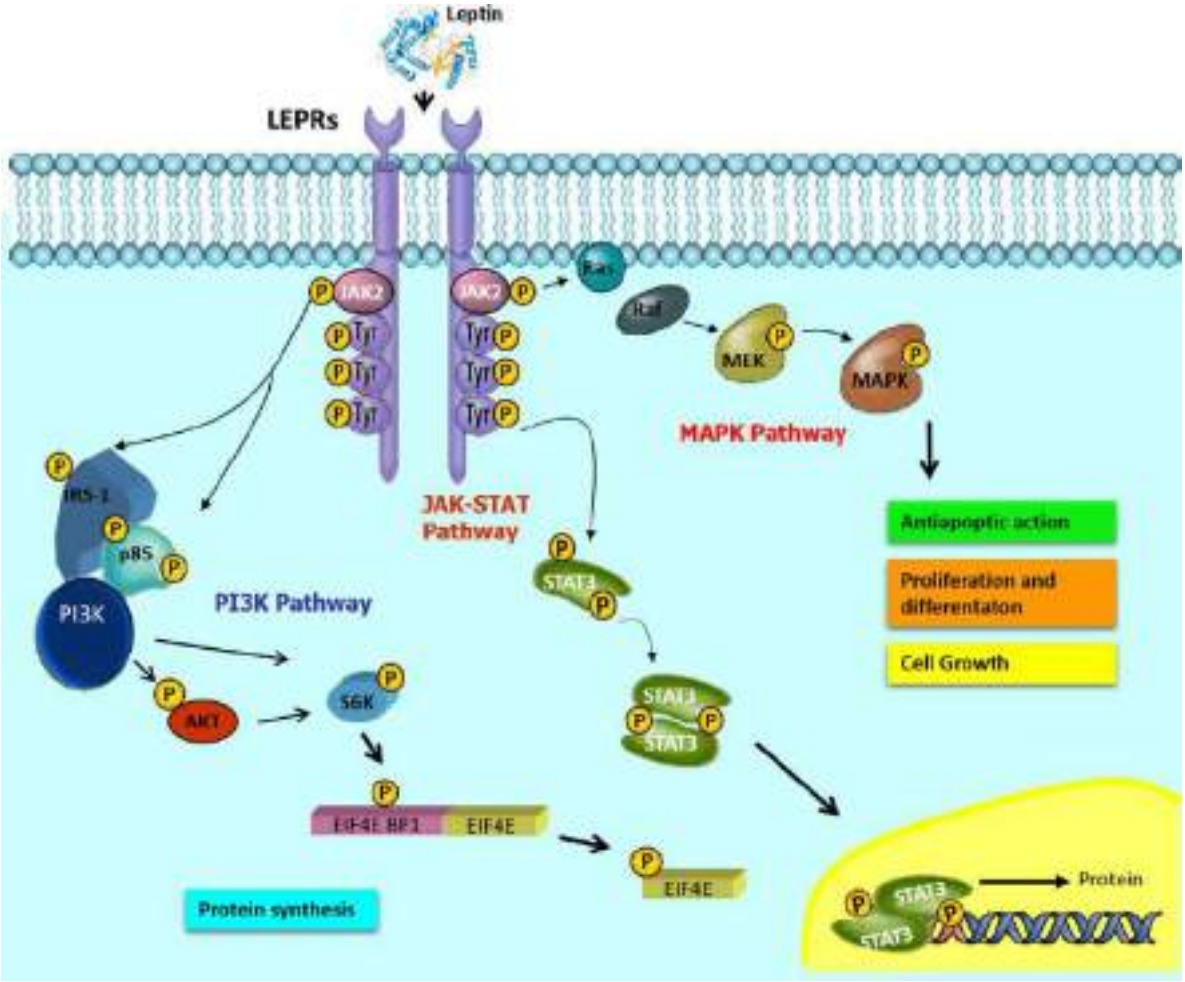

Figura 3. Vías de señalización del receptor de leptina [54]

Entre los efectos fisiológicos de la leptina en la placenta se incluyen el crecimiento del trofoblasto, la angiogénesis, y la inmunomodulación [55].

La leptina está hoy considerada como un importante regulador durante las primeras fases del embarazo, modulando el crecimiento, la invasión, la apoptosis y la síntesis de proteínas en la placenta [41,56–61]

El control de la proliferación celular es crítico para el correcto desarrollo de la placentación, y está regulada con precisión [62]. Una velocidad alterada en la proliferación del citotrofoblasto se ha asociado con diferentes patologías del embarazo. En este sentido, un incremento se asocia a macrosomía fetal, y una disminución a retraso del crecimiento intrauterino [63]

Otros factores en la circulación materna puede estimular la proliferación, diferenciación y supervivencia de las células trofoblásticas a través de la activación de múltiples kinasas [64,65] y fosfatasas [66].

Durante la placentación el citotrofoblasto y el sincitiotrofoblasto mantienen un grupo de células en contacto con las membranas basales vellosas. En el compartimento extravelloso la proliferación celular favorece la invasión del estroma uterino. Al mismo tiempo, en el compartimento velloso, las células llevan a cabo la fusión sincitial dirigida por factores de transcripción específicos [67].

La leptina es un factor trófico autocrino, producido por las células del trofoblasto, fundamental para el desarrollo normal de la placenta (Fig. 4)

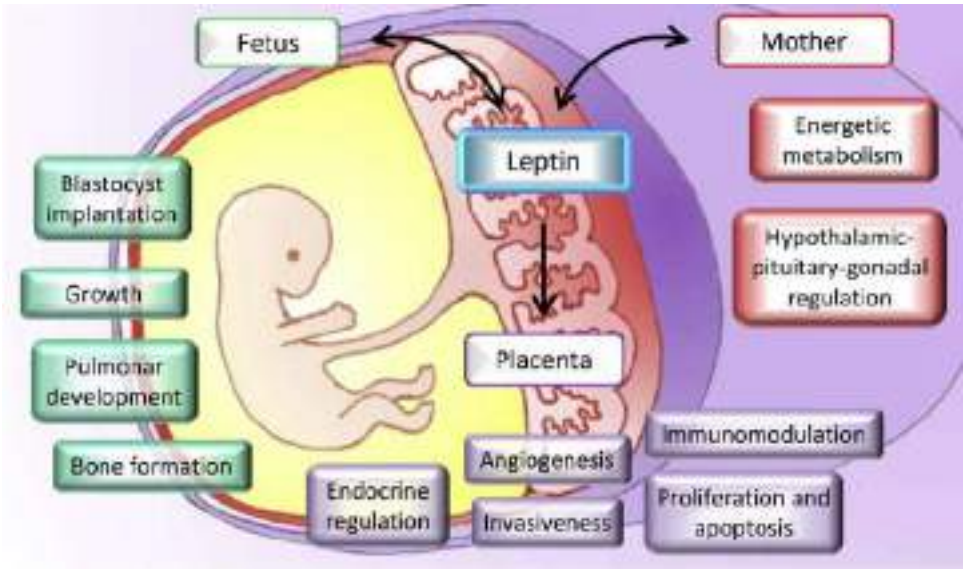

Figura 4. Papel de la leptina en el desarrollo placentario [24]

La leptina actúa durante todo el embarazo facilitando el crecimiento de la placenta y previniendo la apoptosis de las células trofoblásticas, lo que explica el aumento de su expresión en situaciones de estrés durante embarazo, como en la preeclampsia o el retraso de crecimiento intrauterino para intentar evitar la apoptosis de las células trofoblásticas [68,69].

El papel de MAPK en la regulación del trofoblasto está bien documentada tanto en humanos como en sistemas animales [70]. Además, la leptina se ha visto que induce la proliferación en muchas líneas celulares de trofoblasto [71], principalmente en la vía MAPK, como ha demostrado nuestro grupo [72,73]. Además, nuestro grupo también ha encontrado que la leptina estimula la síntesis de proteínas activando la maquinaria de traducción [61]. En este contexto, es interesante mencionar el papel que nuestro grupo ha encontrado de la proteína Sam68 [6,74], una proteína de unión a ARN, un miembro de la familia STAR (signal transduction and activation of RNA metabolism) [75,76]. Así, se ha descrito que la leptina estimula la tirosín fosforilaciín de Sam68 en el trofoblasto, mediando su disociación del ARN, sugiriendo que la señal de la leptina podría modular el metabolismo del ARN [77].

Sam68 puede participar en la señalización de otros receptores, como el de insulina, donde nuestro grupo tiene larga experiencia [78–81]. Además, hemos estudiado el papel de Sam68 en la señal del receptor de la leptina en otros sistemas, como el sistema inmune, donde también media la activación de las vías MAPK, PI3K y JAK/STAT [82,83]. Entonces, el papel de Sam68 en la fisiología placentaria puede ser relevante, dada su participación en la señal de la leptina. Así, en las vellosidades placentarias, el recambio celular está fuertemente regulado por la cascada de apoptosis [84,85], y la leptina previene la apoptosis mediante la activación de MAPK [56,57,60].

3.2. LEPTINA Y DIABETES MELLITUS GESTACIONAL

La diabetes mellitus gestacional (DMG) se caracteriza por una intolerancia a la glucosa diagnosticada durante el embarazo. Es una de las complicaciones más

frecuentes del embarazo y afecta al 3-8% de los embarazos, con una prevalencia creciente debido al incremento de la edad de las embarazadas y el aumento en la prevalencia de la obesidad [86].

La DMG se asocia a numerosas complicaciones incluidas la macrosomía neonatal, alteraciones metabólicas, distrés respiratorio, muerte neonatal y predisposición para el desarrollo de síndrome metabólico y diabetes tipo 2 [87].

La placenta parece jugar un papel clave en la patogénesis de la DMG, ya que las complicaciones metabólicas se resuelven tras el parto. Por tanto, alteraciones en el desarrollo y funciones de la placenta, incluido el sobrecrecimiento han sido implicadas en el desarrollo de la DMG [88].

La DMG se asocia con resistencia a la insulina, hiperinsulinemia e hiperleptinemia, y estas condiciones asociadas pueden afectar el transporte de nutrientes y su aporte al feto [89,90].

Se ha visto que la expresión de leptina y su receptor están aumentadas en la placenta de la mujer con DMG [91]. De hecho, la leptina se ha propuesto como un predictor bioquímico de DMG en el primer trimestre [92,93]. Además, se ha sugerido que la leptina placentaria actuaría como una señal circulante para controlar la homeostasis del feto [94]. Se piensa que la hiperglucemia regula los niveles de leptina en sangre de cordón, y podría explicar el riesgo aumentado de obesidad de los niños expuestos a DMG [95]. Comparando la expresión génica de placentas de embarazadas normales con DMG hay una correlación con una mayor producción de citocinas proinflamatorias, como IL-6 y TNF-alfa, causando un ambiente inflamatorio incrementa la producción de leptina. [96].

Nuestro grupo ha encontrado que la insulina induce la expresión de leptina en células trofoblásticas, incrementando la actividad promotora [97]. Sabemos que la leptina y la insulina comparten vías de señalización a través de sus receptores, como JAK/STAT, MAPK y PI3K. Así, nuestro grupo pudo demostrar que el nivel basal de activación de estas vías está aumentado en la placenta de mujeres con DMG [98], sugiriendo una acción sinérgica en el trofoblasto placentario (Fig. 5).

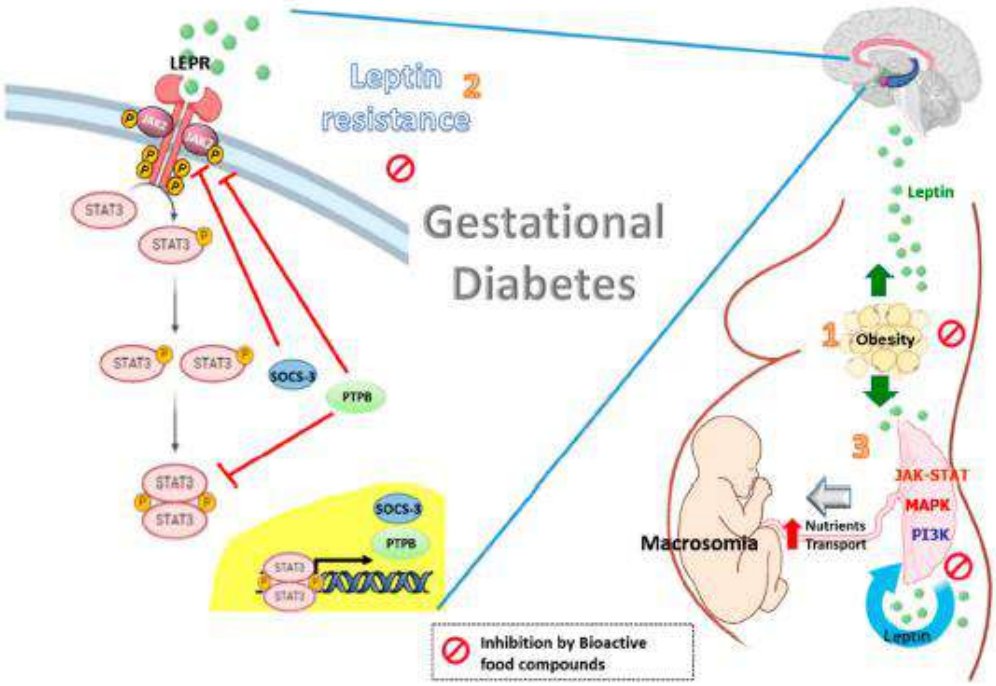

Fig. 5. Papel de la leptina en la diabetes gestacional [99]

Todos estos conceptos están incluidos en una revisión publicada como capítulo de libro que se incluye en esta Tesis doctoral (**cuarto artículo**).

3.3. PLACENTA Y AQUAPORINAS

Varias aquaporinas (AQPs) se expresan en el trofoblasto y en las membranas fetales, con un importante papel facilitando el movimiento de agua a través de las membranas biológicas, los tejidos feto-placentarios entre la madre y el feto [6]. De hecho, el sistema circulatorio materno y de la placenta son interdependientes, y las alteraciones placentarias van a influenciar la circulación materna y viceversa [6] (Fig. 6).

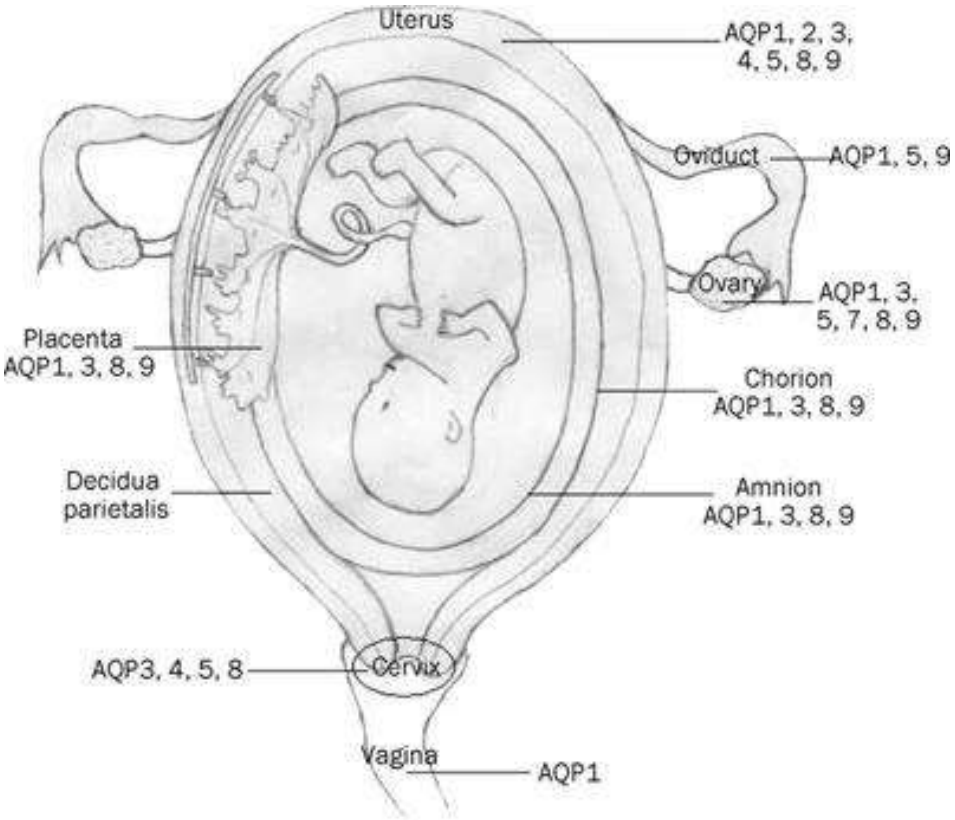

Figura 6. Expresión de AQPs en las estructuras materno-fetales [100]

Todos los requerimientos del feto van a satisfacerse por la transferencia transplacentaria desde la circulación materna, y la necesidad de flujo de agua va a incrementarse a lo largo de la gestación. En este sentido, AQP1, AQP3, AQP8, AQP9 y AQP11 (Fig. 3) podrían participar en la regulación del volumen de líquido amniótico [101–105]. Más aún, se especula que el ambiente metabólico materno puede generar estímulos en la placenta, resultando en la producción de AQPs y citocinas inflamatorias.

Todos estos conceptos están incluidos en una revisión publicada que incluimos en la presente Tesis Doctoral (**quinto artículo**)

Por tanto, aunque el papel funcional de las AQPs en la placenta tenga todavía que estudiarse más a fondo, la expresión de las AQPs en patología del embarazo podría apuntar a nuevas dianas terapéuticas.

En este sentido, la DMG se asocia con un incremento en la incidencia de polihidramnios, debido al incremento del volumen de líquido amniótico, sugiriendo que las aquaporinas (AQP) y su expresión podrían estar alteradas en

la DMG. Además, algunas AQPs como la AQP-9 es un transportador de glicerol, por lo que podría además contribuir al mayor aporte de nutrientes al feto macrosómico. La DMG podría tener aumentada la expresión de alguna AQP, como la AQP-9, y este aumento podría estar mediado por el incremento de leptina y de activación de su receptor. Por eso nos planteamos esta pregunta de investigación en la presente Tesis Doctoral.

ARTÍCULOS DE REVISIÓN

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MDPI

Review

Role of Leptin in Inflammation and Vice Versa

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Abstract: Inflammation is an essential immune response for the maintenance of tissue homeostasis. In a general sense, acute and chronic inflammation are different types of adaptive response that are called into action when other homeostatic mechanisms are insufficient. Although considerable progress has been made in understanding the cellular and molecular events that are involved in the acute inflammatory response to infection and tissue injury, the causes and mechanisms of systemic chronic inflammation are much less known. The pathogenic capacity of this type of inflammation is puzzling and represents a common link of the multifactorial diseases, such as cardiovascular diseases and type 2 diabetes. In recent years, interest has been raised by the discovery of novel mediators of inflammation, such as microRNAs and adipokines, with different effects on target tissues. In the present review, we discuss the data emerged from research of leptin in obesity as an inflammatory mediator sustaining multifactorial diseases and how this knowledge could be instrumental in the design of leptin-based manipulation strategies to help restoration of abnormal immune responses. On the other direction, chronic inflammation, either from autoimmune or infectious diseases, or impaired microbiota (dysbiosis) may impair the leptin response inducing resistance to the weight control, and therefore it may be a cause of obesity. Thus, we are reviewing the published data regarding the role of leptin in inflammation, and the other way around, the role of inflammation on the development of leptin resistance and obesity

Keywords: leptin; inflammation; obesity; leptin resistance; microbiota

1. Introduction

Acute inflammation is a protective response that is engaged to defend and restore physiological functions and homeostasis. Acute inflammation is actually an essential part of the healing process. It starts rapidly, and symptoms may last for a short time, a few days at most. The inflammatory response can only achieve this goal by overriding or suppressing incompatible homeostatic controls. However, in its attempts to restore homeostasis, inflammation may enforce and propagate homeostatic changes, which results in chronic inflammation, a slow condition caused by a misactivation of the immune system that keeps the organism in a long-term state of high alert, which is detrimental and can result in chronic pathological states, even in the case of low-grade chronic inflammation [1,2]. In this context, pathways of systemic inflammation have been recognized as an essential component in the pathogenesis of different multifactorial diseases (type 2 diabetes and gestational diabetes, cardiovascular diseases, cancer, obesity, etc.) encompassing chronic inflammatory diseases [3,4]. Moreover, the inflammatory response observed in these pathophysiological conditions does not seem to be triggered by the classical signals of acute inflammation, infection and injury, but it appears to be supported by tissue malfunction or homeostatic imbalance. In recent years, interest has been captured by the discovery of novel mediators of inflammation, such as adipokines. Several inflammatory stimuli,

such as cytokines and Toll-like receptor (TLR) ligands, induce or inhibit their expression, although this system is not fully understood. Adipokines are soluble proteins secreted by the white adipose tissue, a highly dynamic organ with a huge number of functions in physiological and metabolic processes. In fact, apart from its known roles regulating energy balance and metabolism, the white adipose tissue also modulates inflammatory and immune responses, through the secretion of adipokines. Adipokines comprise of a very heterogeneous group of mediators, some of which proinflammatory proteins, such as leptin.

Leptin, the product of the *LEP* gene, is a 16 kDa peptide hormone secreted mainly from adipose tissue, which plays an integral role in the regulation of body weight and energy expenditure [5]. Circulating leptin levels (physiological range approximately 16 ng/mL) reflect the amount of energy stored in the adipose tissue and are correlated with the degree of obesity. Thus, obese individuals typically produce higher leptin than leaner individuals [6–9]. Initially, the effects of leptin were thought to be only centrally mediated. However, leptin plays a role in a quite diverse range of physiological functions both in the central nervous system and at the periphery. The past 20 years of research on leptin have provided important insights into the intricate network that links nutrition, metabolism, reproduction as well as immune functions [7–10] and inflammation. These actions of leptin are consistent with its production by various tissues and organs, such as the stomach, skeletal muscle, pituitary cells and the placenta [6,11].

This pleiotropic nature of leptin is supported by the universal distribution of leptin receptor (LEPR), which shows structural similarity to the class I cytokine receptor family [12–16]. At least six alternatively spliced forms have been identified, differing in the lengths of their cytoplasmic regions, known as LEPRa, LEPRb, LEPRc, LEPRd, LEPRe and LEPRf [12,16]. The short isoform is distributed in almost all peripheral tissues and seems to mediate the transport and degradation of leptin and besides, it show distinct signaling capabilities that include the activation of mitogen-activated protein kinase (MAPK) pathway [17]. The long form isoform of LEPR (LEPRb) predominates in the hypothalamus in areas that are responsible for the secretion of neuropeptides and neurotransmitters that regulate appetite, body weight [14,15,18] and bone mass [19]. Finally, the product of the cleavage process, the so-called soluble leptin receptor, is the main binding protein for circulating leptin and modulates its bioavailability.

Leptin resistance (impaired signaling) is present in obesity, producing hyperleptinemia. Since leptin acts as a proinflammatory adipokine, the hyperleptinemia may contribute to the chronic inflammatory state of obesity. On the other hand, chronic inflammation may impair leptin action producing leptin resistance by interfering in leptin receptor signaling. The leptin resistance in the hypothalamus impairs the weight control that may lead to obesity.

In the present review, we focus on the role of leptin as a mediator of inflammation in the pathogenesis of several chronic disorders and how this knowledge could be instrumental in the design of leptin-based manipulation strategies to help restoration of abnormal responses. In addition, the role of chronic inflammation in the development of leptin resistance, which may lead to obesity is also reviewed in the present work.

2. Leptin Signaling in Immune Cells

The LEPR is ubiquitously expressed on the surface of immune cells both peripheral (such as monocytes/macrophages, and T and B cells) and CD34+ hematopoietic bone-marrow precursors [20,21]. Similar to other receptors of the family, LEPR lacks intrinsic tyrosine kinase activity and requires the activation of receptor associated kinases of the Janus family (JAKs). While the short-form contains only the JAK2 intracellular signaling site, the LEPRb contains an extracellular domain and an intracellular domain that bears a JAK2 signaling site, as well as three tyrosines (Tyr) that can be phosphorylated. This suggests that the binding of JAK2 is particularly important downstream of leptin.

The JAK (Janus kinases)/STAT (signal transducers and activators of transcription) pathway is one of the main signaling cascades activated by leptin in LEPRb promoting the complete activation [22–24].

After ligand binding, JAKs autophosphorylate and tyrosine phosphorylate various STATs. Activated STATs then dimerize and translocate to the nucleus, where specific gene responses are elicited [15,25]. Different pathways in addition to STATs are known to be involved in LEPR signaling. Thus, the mitogen-activated protein kinase (MAPK) family and the phosphatidylinositol 3-kinase (PI3K) signaling cascade become also activated by leptin, as we have previously found in peripheral blood mononuclear cells [26,27]. Therefore, the JAK2–PI3K, JAK2-Tyr 985–ERK1/2 (extracellular signal-regulated kinases or also called mitogen-activated protein kinases) and JAK2-Tyr 1138–STAT3 pathways have emerged as examples of pathways by which leptin can induce immune cell activation.

2.1. Leptin and Innate Immunity

The innate immune system is affected by leptin, and recent research has uncovered important mechanisms of functional regulation. Innate immune cells respond to infection and also influence the adaptive response. Leptin receptors have been found in monocytes, polymorphonuclear and natural killer (NK) cells.

2.1.1. In Monocytes and Macrophages

Both the long (LEPRb) and short isoforms have been found to be expressed, if in fact, constitutive association of JAK2 and JAK3 with LEPRb has been reported, with the subsequent activation by tyrosine phosphorylation of STAT3 [28], the MAPK family and the PI3K signaling cascade [26,27,29]. It is well-established the role of leptin as a growth factor for the monocytes, promoting phagocytic function and proliferation of circulating monocytes, inducing the production of proinflammatory cytokines (TNF-α, IL-6 and IL-12) and stimulating the oxidative burst as well as the chemotactic responses mediating the inflammatory infiltrate [30,31]. On the other hand, the ROS production in HIV infected patients is an indicator of programmed cell-death in monocytes [32]. In this sense, leptin stimulation of these monocytes partially inhibited the production of ROS [33], suggesting that the antiapoptotic role of leptin may be partly mediated by the inhibition of an oxidative burst, in addition to other signaling pathways, such as MAPK in HIV-positive monocytes [27].

2.1.2. Polymorphonuclear Cells

Polymorphonuclear cells have been found to express the leptin receptor in vitro and in vivo [34,35]. Particularly, in neutrophils, it has been found only in the short form of LEPR [36], which is enough to signal inside the cell through MAPK signaling pathways. In these cells, leptin seems to behave as a survival cytokine, similar to G-CSF and promotes chemotaxis [37,38] and the secretion of oxygen radicals, through direct and indirect mechanisms [34]. In eosinophils and basophils leptin also seems to be a potent activator through its positive role in chemotaxis, cytokines release and cell survival. For instance, in eosinophils, human leptin plays a key role in the host defense system against parasitic infections [39] and, thus, the level of eosinophilia might indicate the relative severity of the infection due to the invasion by the parasites [40].

2.1.3. Human NK Cells

Human NK cells constitutively express both long and short forms of LEPR. In fact, leptin signaling is necessary for normal NK cell immune function. Leptin actions in NK cells include cell maturation, differentiation, activation and cytotoxicity [41,42], as well as increased secretion of IL-12 [43]. Therefore, the main role of leptin in this context is the ability to increase immune activity and cell proliferation and to decrease the apoptotic rate of NK cells.

2.1.4. Other Immune Cells

The expression of leptin and leptin receptors has also been demonstrated on mast cells, suggesting paracrine and/or autocrine immunomodulatory effects of leptin on mast cells [44]. Finally, although

leptin acts as an activator, chemoattractant and survival factor (via NF-κB and PI3K-AKTsignalling), it may also be implicated in maturation and migration of dendritic cells (DCs) [45]. In this context, some studies have shown that immature DCs primed with leptin were licensed to skew the immune response toward the Th1-type and, moreover, it was also able to induce the activity of autologous CD8+ T cells in terms of perforin and IFN-γ production [46].

2.2. Leptin and Adaptive Immunity

Although the mechanisms of leptin regulation of the T cell function are not fully understood, leptin has also been demonstrated to modulate the adaptive immune response, which is classically divided into T helper 1 and 2 immune responses on the basis of the produced cytokine pattern. T helper 1 lymphocytes produce mainly proinflammatory cytokines that are necessary for macrophage activation and the cell-mediated response, whereas T helper 2 lymphocytes secrete modulatory and anti-inflammatory peptides that are important factors for the activation of B cells and basophils. Evidence indicates the role of the leptin in the maintenance of thymic maturation of double positive CD4+/CD8+ cells, reducing thymic apoptosis [47] as well as preventing glucocorticoids-induced apoptosis in thymocytes. In fact, chronic leptin replacement in mutant mice lacking leptin expression (ob/ob mice) restores the T-cell function, increasing the secretion of the proinflammatory cytokines. Thus, the effect of leptin polarizing T cells towards a Th1 response seems to be mediated by stimulating the synthesis of IL-2, IL-12 and IFN-γ and the inhibition of the production of IL-10 and IL-4 [29,30].

Besides, leptin receptor signaling in T cells is required for Th17 differentiation [48], which has a paramount role in the promotion and maintenance of inflammation and autoimmunity [49,50]. Leptin is also able to modulate the regulatory T cells (Treg) function. In this sense, leptin can act as a negative signal for the proliferation of human Treg via the mTOR pathway [51]. This supports the possibility of new antileptin-based approaches for the immunotherapy of conditions characterized by low numbers of Tregs, such as obesity, type 2 diabetes mellitus (T2D) and metabolic syndrome.

Therefore, leptin actions in T cell populations involve different processes leading to increase the immune activity by enhancing the polarization of naive T helper cells to a Th1 phenotype. Moreover, leptin increases Th17 cell proliferation while decreases Treg cell proliferation through mTOR activation.

B cells have emerged as crucial players in regulating inflammation in murine visceral adipose tissue, by presenting antigens to T cells, secreting proinflammatory cytokines, and secreting pathogenic antibodies [52], contributing to local and systemic inflammation [53]. In contrast to macrophages and T cells, little is known about the role of B cells in response to leptin. However, leptin seems to play a central role also in the modulation of B cell compartment. In fact, B cells express the long form of LEPR on the cell surface and leptin induces the secretion of proinflammatory cytokines (such as TNF and IL-6) and the anti-inflammatory and immunoregulatory cytokine IL-10 via JAK–STAT and p38MAPK–ERK1/2 signaling in B lymphocytes [54]. Moreover, leptin is necessary for B cell development and can augment the B cell population by increasing proliferation and decreasing apoptotic rate. Therefore, a role of leptin in B-cells generation and activation has been reported [55].

3. Leptin as a Mediator of Inflammation

3.1. Leptin Deficiency and Infection Diseases

Malnutrition affects around 800 million people of the world population [56]. Malnutrition and fasting are associated with nutrients insufficiency and affects both innate and acquired immunity [57,58]. That is why, people with nutrients insufficiency are vulnerable to infections because of immunosuppression [59] and defective cytokine production [60]. For example, malnutrition induces anti-inflammatory cytokines IL-4 and IL-10 and impairs proinflammatory cytokines IL-2 and IFN-γ production from CD4+ and CD8+ T cells in children. Intriguingly, the systemic leptin levels are reduced in malnutrition and in starvation, suggesting that leptin bridges a link between the nutritional status and immune system of individuals. In fact, leptin-deficiency is associated

with increased susceptibility to several infections, but moreover, certain infections also caused the downregulation of systemic leptin levels and mimic a malnutrition like situation. In this context, it has been reported that a drastic fall in leptin levels during starvation increases susceptibility to lipopolysaccharide (LPS) and tumor-necrosis factor alpha (TNF-α) induced toxicity in mice. However, leptin replacement therapy markedly reverses these deleterious effects and protects the mice from fasting-induced lymphopenia [59].

Phagocytosis is a key event executed by certain immune cells to internalize the foreign pathogen inside the cell and subsequent killing. As mentioned-above, leptin induces phagocytic activity of macrophages and prevents the apoptosis of a variety of immune cells involved in both innate and adaptive immunity. In this sense, a large body of evidence has demonstrated that leptin supplementation reduced the infections of some pathogens; such as bacteria (*Listeria monocytogenes, Klebsiella pneumonia, Escherichia coli, Mycobacterium tuberculosis*, etc. . . .) [61–64] virus, fungus and parasite infections as well as their pathogenicity by increasing the phagocytic activity of macrophages. Even more important is the sepsis, which is a systemic inflammatory response responsible for multiple organ failure and high rate of mortality [65]. In this sense, it has also been reported that leptin replacement and leptin signaling is necessary to induce an adequate antiseptic immune response [66]. In leptin-deficient mice exogenous leptin modulated the immune response against sepsis and tremendously improved the survival rates by reducing IL-6 levels in serum and thereby controlled systemic inflammation [66]. In humans, the patients' recovered from sepsis had higher leptin levels compared to that of non-survivors [67]. Thus, these observations reveal the neuroendocrine regulation of systemic immunity and therapeutic potential of leptin in an infectious disease [68].

The low systemic leptin levels in HIV patients [69] due to reduced adiposity might contribute to immunodeficiency [70].

As mentioned above both low systemic leptin or leptin-deficiency and impaired leptin signaling conditions are associated with increased susceptibility to infections. The impaired leptin signaling could be a cause of defective immunity due to the loss of interdisciplinary regulation among immunologic, metabolic and neuro endocrinological aspects. In this respect, LEPR mutation (Q223R) or polymorphism (rs1137101), which is a homozygous allelic mutation that results in impaired STAT3 signaling is likely to increase the susceptibility for dissemination of infection. Leptin was shown to be protective against *C. difficile* colitis by inducing STAT3 inflammatory pathway, which is impaired in the LEPR Q223R mutation [71].

Suppressor of cytokine signaling 3 (SOCS3) is a protein involved in the negative regulation of cytokines that signal through the JAK/STAT pathway including leptin receptors. SOCS3 typically inhibits T cells proliferation and activation by directly targeting CD28. This is the mechanism of viruses such as hepatitis-B, influenza, HIV, and Epstein Barr virus [72,73], which induce SOCS3 expression to ensure their survival and evade the host immunity by inhibiting IFN-α/β JAK/STAT signaling [74–77]. It has been described that a mutation (Tyr 1138 Ser) in tyrosine 1138 residue located in the intracellular domain of LEP-Rb isoform mediates STAT3/SOCS3 signaling, which results in decreased chemokine production and immune cells recruitment at the site of infection in mucosal gut tissue.

Parasite infections are reported to cause damage to intestinal mucosal epithelial cells by inducing the activation of mesenteric lymph nodes and perturbations in the adjacent adipose tissue to secrete leptin [78]. Thus, parasites may induce the malnutrition state, which is the hall mark of low systemic leptin levels [79] and disturb the host immunity. However, high serum leptin levels were reported in several parasitic infections [80], possibly due to acute inflammation and production of IL-1β, TNF-α and IL-6 caused by the gut infections [81]. This is important as leptin functions as an eosinophil survival factor in humans [39], which plays a key role in the host defense system. In addition, it promotes regeneration and intestinal integrity as well as inhibition of apoptosis in intestinal epithelium [82,83]. In fact, an integral leptin signaling via MAPK, STAT3 and AKT pathways was found to be protective against parasites in intestinal epithelial cells in response to leptin [84]. For instance, leptin was able to

maintain the defense against the *L. donovani* infection through the classical activation of macrophages by inducing the phosphorylation of Erk1/2 and Akt kinase [63].

It has also been suggested that leptin might be a potential adjuvant tool in vaccination strategies as the lack of appropriate immuno-adjuvant could be one of the potential reasons for a lack of efficacy of some vaccines in preclinical studies. In this sense, leptin could restore an inflammatory response without eliciting adverse side-effects since it is produced endogenously [85,86]. However, the immunostimulatory potential of leptin cannot be neglected in vaccines development, as an adjuvant alone [87]. The co-immunization of leptin in conjugation with a vector expressing virulence have shown to be able to produce protective immunity, indicating the importance of leptin and its signaling in the generation of a host protective immune response [88].

Therefore, leptin could be a novel approach for protection against the infections in human population susceptible under certain pathological conditions such as malnutrition, diabetes mellitus or HIV infection [89–92].

3.2. Leptin as an Inflammatory Mediator in the Obesity-Associated Immuno-Metabolic Disorders: Diabetes, Cardiovascular Disease, Autoimmune Diseases and Cancer

The incidence of obesity and its associated disorders is increasing worldwide. It is known that obesity predisposes individuals to an increased risk of developing many diseases, including atherosclerosis, diabetes, certain cancers and some immune-mediated disorders [93–95]. This is because obesity is associated with a chronic inflammatory response, which is characterized by abnormal cytokine production, increased synthesis of acute-phase reactants, such as C-reactive protein (CRP), and the activation of proinflammatory signaling pathways [93]. Figure 1 summarizes these pathophysiological conditions associated with obesity and the possible role of leptin.

Figure 1. Leptin action contributes to chronic inflammation in obesity. Obesity is associated with increased leptin levels, which at the local or systemic level activate the cells of the innate and adaptive immune system. In this context, leptin can directly promote environmental conditions that in turn promote the loss of immune self-tolerance and priming immune cells for Th1 phenotype (proinflammatory). The elevated circulating leptin levels in obesity contribute to the low-grade inflammatory background, which makes obese individuals more susceptible to an increased risk of developing metabolic diseases such as cardiovascular diseases, T2D, as well as, degenerative disease including autoimmunity diseases (multiple sclerosis, thyroiditis, rheumatoid arthritis, intestinal inflammatory disease and knee arthrosis among others) and cancer.

Research in the past few years has identified important pathways that link metabolism with the immune system and vice versa. Many of these interactions between the metabolic and immune

systems seem to be orchestrated by a complex network of soluble mediators derived from immune cells and adipocytes. In this sense, in addition to adipocytes, which are the most abundant cell type in white adipose tissue, adipose tissue also contains preadipocytes (which are adipocytes that have not yet been loaded with lipids), endothelial cells, fibroblasts, leukocytes and, most importantly, macrophages. These macrophages are bone-marrow derived and the number of these cells present in white adipose tissue correlates directly with obesity. In fact, the adipose tissue of obese individuals also contains a large number of macrophages compared to lean individuals [96,97]. Certain cytokines such as, CC-chemokine ligand 2 (CCL2) produced by adipocytes, has recently been identified as a potential factor contributing to macrophage infiltration into adipose tissue. Once macrophages are present and active in the adipose tissue, they, together with adipocytes and other cell types present in the adipose tissue, might perpetuate a vicious cycle of macrophage recruitment and production of proinflammatory cytokines. In fact, macrophages in adipose tissue seem to be the main source of TNFα, however, adipocytes contribute almost one third of the IL-6 concentration in the circulation of patients who are obese. In addition, other products of adipose tissue such as, leptin, are thought to provide an important link between obesity, insulin resistance and related inflammatory disorders. Therefore, in obesity-related high plasma leptin conditions, inflammation would occur when signal transduction pathways was activated, such as the activation of NFκβ, by the binding of leptin to its receptor and subsequent release of the inflammation factors, for instance TNFα [98]. In this sense, the elevated ERK1/2 phosphorylation by leptin is followed by increased NFκB activation and TNFα secretion, which is in agreement with a previous report that indicated leptin has proinflammatory action, involving proinflammatory cytokines TNFα through NFκB regulation [99].

3.2.1. Type 2 Diabetes Mellitus

Type 2 diabetes mellitus or non-insulin dependent diabetes mellitus is a disease of chronic hyperglycemia that leads to severe and sometimes fatal complications such as kidney failure, heart disease and death [100]. The natural history of T2D in humans leads from insulin resistance to compensatory hyperinsulinemia, and pancreatic cell dysfunction [101]. It has been reported that subclinical, low-grade inflammation might have an important role in the pathogenesis of obesity related insulin resistance and T2D [102]. Biomarkers of inflammation, such as TNF, IL-6 and CRP, are present at higher concentrations in individuals who are insulin resistant and obese, and decreased expression are observed after weight loss [103]. Therefore, the presence of these proinflammatory mediators may be biomarkers to predict the development of T2D. They might also lead to a state of vascular endothelial dysfunction and vascular inflammation, all of which promote the development of atherosclerotic cardiovascular disease. In addition, insulin resistance might be partly accelerated by an acute-phase reaction as part of the innate immune response, in which large amounts of proinflammatory mediators are released from adipose tissue. Moreover, since plasma leptin levels are positively correlated with body mass index (BMI) and obesity is a risk factor for T2D, the relationship between leptin and T2D has being extensively studied.

In searching for the mechanisms involved in inflammation-induced insulin resistance, SOCS proteins [104,105], endoplasmic-reticulum (ER) stress [106], the inhibitor of nuclear factor-κB (NF-κB) kinase-β (IKKβ) of NF-κB activation and the JUN N-terminal kinase (JNK) signaling pathways have been all associated with the development of insulin resistance. Intriguingly, activation of these pathways is regulated by leptin, the proinflammatory mediator released mainly by adipocytes that link the immune system with obesity-related insulin and leptin resistance. For instance, leptin signaling is inhibited by the overexpression of SOCS3 [107], which affects JAK/STAT pathway by binding to the phosphorylated Tyrosine-985 (pTyr985) of LEPR and induces dephosphorylation of JAK2 [108]. Protein tyrosine phosphatases (PTPs), the phosphatase and tensin homolog (PTEN), receptor-type PTPe (RPTPe) and PTP1B also induce dephosphorylation of JAK2 and inhibit leptin signaling. The expression of PTP1B and T cell PTP (TCPTP) is upregulated in a high-fat diet and

obesity, and inhibits leptin-mediated STAT3 phosphorylation [109]. This is important as the PTP1B mediated ER stress induces leptin resistance [110,111], possibly by inhibiting STAT3 phosphorylation.

An activated JNK pathway by ligation of TLRs is also an important regulator of insulin resistance in mouse models of obesity. In this sense, TLRs are a family of receptors in the innate immune system that mediate signal transduction pathways through the activation of transcription factors that regulate the expression of proinflammatory cytokines in several cell types and tissues [112]. More specifically, it has been reported that TLR4, involved in modulating the innate immunity (proinflammatory macrophages) [113,114], is an important mediator of insulin resistance and inflammation through its activation both by elevated exogenous ligands (e.g., dietary fatty acids) and endogenous ligands (e.g., free fatty acids), which are elevated in obesity. Moreover, TLR4 activation also leads to increased transcription of proinflammatory genes, resulting in the elevation of cytokine, chemokine as well as reactive oxygen species and eicosanoid levels that promote further insulin-desensitization.

Taken together, several proinflammatory cytokines, SOCS proteins, ER stress, the IKKβ pathway of NF-κB activation and JNK signaling pathways are all associated with the development of insulin resistance, indicating that various proinflammatory mediators released by adipocytes, in addition to the initially described proinflammatory cytokine TNF, link the immune system with obesity-related insulin resistance.

Therefore, increased understanding of theses signaling pathways-mediated effects on insulin action present the opportunity and challenge of developing related therapeutic approaches for improving insulin sensitivity.

3.2.2. Cardiovascular Diseases

It has been suggested that leptin is one of the mediators of atherosclerosis by favoring an inflammatory state that promotes the recruiting of monocytes to the arterial intima, and inducing proinflammatory cytokines [115,116]. Moreover, LEPR is present in atherosclerotic lesions, and ob/ob mice, which are leptin deficient, are protected from atherosclerosis in spite of obesity [117]. On the other hand, it is not clear whether increased leptin or leptin resistance is the mediator of atherosclerosis [118], and clinical prospective studies are needed to further clarify the role of leptin in cardiovascular disease.

3.2.3. Autoimmune Diseases

The prevalence of autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), multiple sclerosis (MS) and type 1 diabetes mellitus (T1D), is increasing in affluent countries and associates with serum leptin levels [119]. Consistently, it has been demonstrated that leptin-deficient mice showed resistance or less susceptibility to the development autoimmune diseases [120]. Serum leptin levels are higher in RA patients with high disease activity, correlate well with disease activity and decrease significantly when disease is well controlled [121]. In fact, the leptin concentrations are significantly higher in patients with active erosive RA [122]. Even though an inverse correlation between leptin concentrations and inflammation exists in patients with active RA, plasma leptin concentrations did not significantly differ from those in healthy controls. This suggests that active chronic inflammation may lower plasma leptin concentrations.

High leptin levels are also related with a higher prevalence of other immune diseases, such as SLE [123], and also with increased susceptibility to the development of osteoarthritis (OA) [124]. In fact, it has been hypothesized that the increased predisposition of females to develop OA could be due to the higher circulating leptin levels observed in females [124] in comparison with males. Recently, leptin has been found to promote SLE by increasing autoantibody production and inhibiting immune regulation [125,126].

Obesity is also associated with other inflammatory autoimmune diseases, such as ulcerative colitis, Crohn's disease and psoriasis [127,128], and increased leptin expression has also been reported in Behcet´s disease, psoriasis, thyroiditis and during the acute phase of ulcerative colitis [129–132]. Besides, in inflammatory bowel disease patients, systemic leptin levels are increased compared to

normal healthy donors [133]. Concerning experimental autoimmune encephalomyelitis (EAE), it has been shown that ob/ob mice are resistant to the development of this model of multiple sclerosis. This resistance is abolished by the administration of leptin, which is accompanied by a switch from a Th2 to Th1 pattern of cytokine release [134]. In addition, and in concordance with these reports, it has been noticed that the onset of the disease is preceded by an increase of circulating leptin [135]. Furthermore, it has been demonstrated that acute starvation, which is accompanied by a decrease in circulating leptin levels, delays the onset of the disease and attenuates the symptoms. Recently, it has been shown that leptin levels are negatively correlated with $CD4^+$ $CD25^+$ regulatory T-cells during multiple sclerosis [134], suggesting that this negative association may have major implications in the pathogenesis of multiple sclerosis, as well as in the development of different autoimmune diseases characterized by Th1 auto-reactivity [134]. This interesting report indicates that leptin is produced by immune cells during acute EAE, and suggests that this hormone could be participating in the development of CNS-inflammatory diseases not only in an endocrine fashion but also by an autocrine or paracrine mechanism. In summary, regulation of leptinemia is complex and additional studies are necessary to clarify whether leptin is a real actor or a simple mediator in the inflammatory process of these autoimmune diseases.

3.2.4. Cancer

Finally, increasing evidence also indicates that obesity is associated with tumor development and progression. Thus, in the context of obesity, the convergence of chronic inflammation, insulin signaling dysregulation, altered availability of lipids and other macromolecules as well as changes in adipokine signaling appear to be involved in the pathogenesis of cancer [136].

Leptin associated to the excess of adiposity influences the risk, prognosis and progression of cancer. Although the underlying mechanisms are still unclear, both leptin and its receptor expression and function have been positively correlated with cancer progression in some endocrine-related cancers [137] and this effect seems to be mainly mediated by LEPR activation of PI3K, ERK1/2 and Jak2/Stat3 signaling pathways [138–141]. These pathways regulate the expression of several cancers related genes such as cyclin D1, COX-2, VEGF and potentiates several procarcinogenic processes including angiogenesis, antiapoptosis, cell proliferation, migration and mesenchymal transformation [142–144]. This contributes to various steps of tumor progression, from cancer stem cell activity, survival, growth and proliferation to metastatic invasion in different types of cancer cells [145–150]. In the inflammatory context, leptin may promote molecular changes capable of modulating the behavior of tumor cells and the surrounding microenvironment, which include cancer and adipose-derived stem cells, cancer-associated adipocytes, epithelial cancer cells, fibroblasts and also immune cells. Leptin modulates both innate and adaptive immunity through its action in different cell types [151]. In this sense, leptin may contribute to the local proinflammatory mechanisms. As an example, it was shown that leptin increases IL-18 expression and secretion in TAMs, leading to increased migration and invasion of breast cancer cells [152].

In addition, leptin has a key role in the antitumor immune defense. This immunomodulatory action of leptin has been demonstrated on NK function, which is crucial for an effective antitumor response [153]. However, the exact role of leptin as a negative or positive modulator could be dependent on the dose or time effect [154,155]. Obesity has recently been found to be favorable for the response to immune checkpoint inhibitors in different tumors [156] so cytokine homeostasis, and more specifically, leptin homeostasis, could also be an important factor considered as both the modulated and modulator of the future efficacy of therapies in cancer.

A causative link between inflammation and carcinogenesis has been demonstrated. Chronic inflammation is a well-established risk factor for cancers, where genetic instability and epigenetic modification could be induced through cytokine signaling or through the generation of reactive nitrogen and oxygen species [157,158]. However, there exists a more complex crosstalk among inflammation, immune cells and cancer cells throughout the phases of elimination, equilibrium and escape in cancer

immunoediting. While cancer-related inflammation confers at first the immunosuppressive activity to the tumor microenvironment (TME), it is also responsible for the epithelial-to-mesenchymal transition (EMT), tumor invasion and also the generation of a premetastatic environment in the context of immunological tolerance [159].

Adipose expansion and inflammation associated to obesity promote the cells from adipose tissue to become part of this cancer microenvironment, thus enhancing protumoral effects. Increased levels of growth factors and cytokines like leptin, decrease proinflammatory TH1 cells and increase TH2 cells and Tregs. Under these conditions, the recruitment of monocytes from the circulation leads to increased tumor-associated macrophages (TAMs) in the tumor microenvironment [157].

There is clear evidence on the association of various adipokines and obesity-related cancers [160]. In this sense, either as an independent factor or by mediating estrogens action, leptin has been proposed as a key link between obesity and different types of cancer. Thus, several data strongly support the involvement of leptin in common endocrine related cancer in women [161], especially, breast cancer [145,162,163]. Additionally, leptin have been suggested as part of the mechanisms involved in the development of obesity-related carcinogenesis in pancreatic [164], prostate [165] and colorectal cancer [166].

3.2.5. Leptin as a Therapeutic Target

Even though leptin was cloned from the obesity animal model ob/ob, which has a mutated leptin gene [167] and therefore obesity may be treated with leptin administration [168], very soon obese humans were found to have increased expression of leptin in adipose tissue [169] and leptin defects are actually rare in human obesity [170]. Thus, only a few families have been identified with leptin deficiency, where leptin replacement restores the normal weight [171,172]. Another pathophysiological leptin deficient state that can benefit from leptin replacement is lipodystrophy [173,174] with good results improving glycemic control and decreasing triglyceride levels. Leptin treatment has also been found to be effective for hypothalamic amenorrhea [175].

4. Inflammation as a Mediator of Leptin Resistance and Obesity

Inflammation is an adaptive response that is triggered by a wide variety of physiological and pathological processes, such as infection and tissue injury, "the classic instigators" [176]. However, these are at one end of a large range of adverse conditions that induce inflammation, and they trigger the recruitment of leukocytes and plasma proteins to the affected tissue site. Once recruited, these cells can initiate many different activities, such as increasing vascularization, recruiting additional immune cells via proinflammatory signaling and initiating the phagocytosis of debris and pathogens. The mediators involved in the onset of systemic immune responses are proinflammatory and include cytokines (IL-1β, IL-6, IL-18, TNF-α and IFN-γ), transcriptional factors (e.g., NF-κB), peptides, chemokines, enzymes, lipids and coagulation factors. When the trigger of the response is successfully neutralized, immune cells shift their activity towards a pro-resolution phenotype via anti-inflammatory signaling, including lipoxins and cytokines (e.g., IL-10, IL-37 and TGF-β).

Tissue stress or malfunction similarly induces an adaptive response, which relies mainly on tissue-resident macrophages and is intermediate between the basal homeostatic state and a classic inflammatory response [176]. Therefore, although the pathological aspects of many types of inflammation are well appreciated, their physiological functions are mostly unknown.

One of the most intriguing aspects of studying inflammation is that the pathways of systemic inflammation have been recognized as an essential component in the pathogenesis of different multifactorial diseases encompassing chronic inflammatory rheumatic disorders, as well as a wide variety of conditions including obesity, T2D, atherosclerosis, autoimmunity and allergy [177,178]. However, these last diseases (obesity, T2D, atherosclerosis and autoimmunity allergy), different to rheumatic disorders, seem to have in common that they involve the disruption of homeostasis of one of several physiological systems that are not directly related to the host defense or tissue repair.

Moreover, in these, the types of inflammatory response are likely more common but of lower magnitud than the classic inflammatory responses induced by infection or injury. Regardless the cause of th inflammatory response, its 'purpose' is to remove the source of the disturbance, to allow the hos to adapt to the abnormal conditions and, ultimately, to restore [179,180]. In this sense, the adaptiv change often provides short-term benefits; however, in a chronic phase, it can become maladaptiv as exemplified by a sustained increase in leptin levels. More specifically, a transient increase in th leptin level during acute inflammation can have a short-term benefit by helping leukocytes and othe cell types during infection and tissue repair. However, sustained leptin resistance could lead t obesity [181,182], cancer and autoimmune diseases. Indeed, many chronic inflammatory disease that are not caused by infection or injury seem to be associated with conditions that were not presen during the early evolution of humans, including the continuous availability of high-calorie nutrient More specifically, hypothalamic inflammation seems to mediate leptin resistance in these chroni inflammatory conditions [183,184] or as a consequence of a fat rich diet [185]. The role of leptin in th development, pathophysiology, acceleration or complications of many diseases as a consequence o obesity seems clear [186–188]. Actually, leptin has been considered a therapeutic target in autoimmun diseases using leptin antagonists [189]. We propose that the chronic inflammation in autoimmun diseases may also contribute to leptin resistance in a vicious circle, as previously hypothesized i animal models, were depletion of perforin-positive dendritic cells, which control inflammatory T cell leads to weight gain and metabolic syndrome [190]. Figure 2 summarizes the role of inflammation i leptin resistance and obesity.

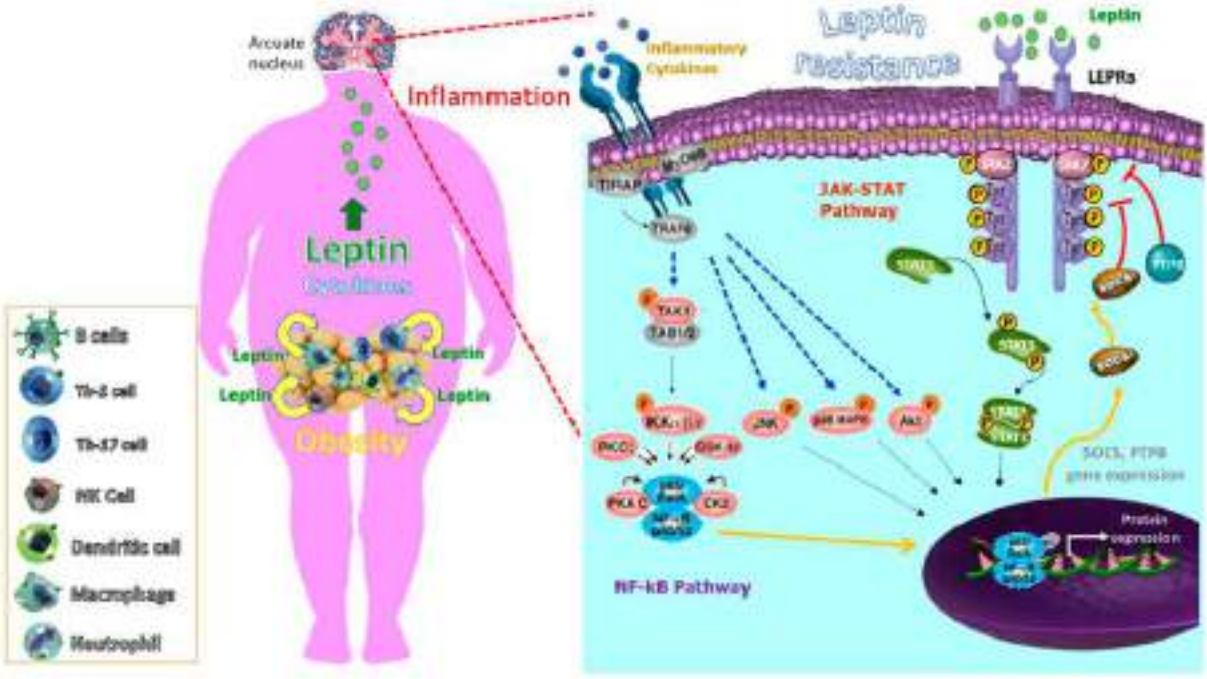

Figure 2. Inflammation contributes to leptin resistance in the brain at the hypothalamic arcuate nucleus, and as a result, alters food intake and energy expenditure leading to obesity. Adipocytes and central nervous system (CNS) cells interact via several secreted factors. In obesity, the adipose tissue is characterized by hypertrophy and increased infiltration of macrophages and other immune cells. The metabolic consequences of adipose tissue dysfunction are an increased synthesis of proinflammatory cytokines and adipokines, such as leptin, which impair adipocyte function. This adipose tissue dysfunction leading to chronic inflammation, not only at the local level but also at the brain level. Moreover, inflammation produced by chronic infection and autoimmune diseases contribute to leptin resistance. Recruitment and activation of NF-KB signaling molecules by proinflammatory cytokines induce SOCS3 and protein tyrosine phosphatases-1B (PTP1B), which are involved in a negative feed-back loop to block LEPR signaling via the JAK/STAT pathway and promoting leptin resistance.

4.1. Infectious Diseases

4.1.1. Viral Infection

In addition to inflammatory diseases some infectious diseases have been related with the development of obesity, coining the new term "Infectobesity" or the obesity of infectious origin [191]. Different viral infections have been associated with obesity, including members of Adenoviridae, Herpesviridae, phages, transmissible spongiform encephalopathies (slow virus) and hepatitides [192]. The mechanisms may include the reprogramming of host metabolism, the exchange of microbiota components, and the adaptation of host immune and metabolic system in the presence of chronic viral infection, which produces changes in cytokine and interferons that may play a role in the development of obesity [193,194]. In the other way around obesity has been found to be an important risk factor for the severity of some viral infections such as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [195] and leptin has also been proposed as the possible link [196].

4.1.2. Bacterial Infection

Mediation of the host defense mechanisms against bacterial infection occurs by an innate immune response as the primary defense and by the adaptive immune response as the secondary defense [197,198]. One of the mechanisms of bacterial escape from host defenses is the upregulation of inhibitory molecules of cytokine signaling, especially the JAK-STAT pathway [199] such as SOCS proteins [200]. The bacterial endotoxin alone can induce adipose tissue expansion [201]. The relationship of microbes and obesity has previously been reviewed [202], and the pathways involved in microbe-induced obesity have also been summarized [203]. Therefore the infectobesity hypothesis seems to be supported by much evidence [204]. The relationship also takes place in the opposite direction. Thus, obesity by excess adiposity can increase the susceptibility to infections [202].

4.2. Microbiota

Interest in the role that the gut microbiota plays in disease has increased in recent years, as evidence of its importance in maintaining normal physiology. It is widely accepted that this consortium of cells provides important biological and metabolic functions that cannot be performed by our human metabolism [205]. A growing body of evidence suggests the gut microbiota participates in whole-body metabolism by affecting energy balance [206–208], glucose metabolism [208–210] and low-grade inflammation [208,210–212] associated with obesity and related metabolic disorders. Therefore, changes in the composition of this complex ecosystem "gut microbiota" have been associated with the development of inflammatory disorders, such as obesity. For example, it has been reported that a high-fat diet profoundly affects gut microbiota composition by reducing *Bifidobacterium* spp. and Bacteroides-related bacteria, *Eubacterium rectale–Blautia coccoides* group content [208,213], as well as, *Lactobacillus* spp. and *Roseburia* spp. [214]. In this context, TLRs could play a critical role in innate immunity by integrating signals from microbiota–host interactions (e.g., proinflammatory signals). The innate immune system detects LPS via its interaction with specific proteins that complex with TLR4 (CD14/TLR4 complex) [215]. Therefore, it can be proposed that fatty acids stimulate the innate immune system, but probably in conjunction with initial stimulation by LPS of the TLR-4/CD14 complex and subsequent TLR-2 stimulation. Moreover, both TLR5 [216] and TLR2 [217] knock out mice exhibited altered gut microbiota composition and these receptors could play a central role in the development of obesity and associated disorders.

Among the putative mechanisms linking the gut microbiota with the development of obesity, growing evidence suggests that the gut microbiota contributes to host metabolism through communication with adipose tissue, which influences the development of metabolic alterations associated with obesity. However, the exact molecular mechanisms underlying this regulation are still under investigation.

Leptin resistance is a hallmark of obesity [9] and it has been demonstrated that gut microbiota control leptin action [211]. More precisely, the altered gut microbiota composition by prebiotics improves leptin sensitivity in diet-induced obese and type 2 diabetic mice [211], suggesting the gut microbiota modulations could be a novel therapeutic target to reset leptin sensitivity during obesity.

4.3. The Paradox of Leptin Sensitization by Inflammatory Cytokines

Similarly to leptin or leptin receptor deficiency, leptin resistance leads to morbid obesity and interleukin-1 receptor 1 (IL1R1) deficiency and the major receptor mediating the biological function of the IL-1 cytokine family (activates inflammatory signaling pathways) also leads to a higher degree of obesity and metabolic disturbance [218–220]. Moreover, it has been demonstrated that LEPR and IL1R1 might physically interact [221]. In fact, IL1R1 has been identified as a mediator that increases leptin sensitization secondary to the action of celastrol, an effective drug treatment of obesity [222]. This effect of the IL1R1 in increasing leptin sensitivity is against the general dogma that cytokine/inflammatory signaling pathways have a key role in aggravation of obesity and associated metabolic diseases [1,223] and support the idea that cytokine signaling could be useful for beneficial metabolic purposes. Thus, the development of cytokine resistance could be one of the mechanisms underlying development of endoplasmic reticulum stress and obesity [222,224].

5. Conclusions

Inflammation is classically recognized as an essential step for the control of microbial invasion. However, now it especially represents an important process for maintenance of biological homeostasis. An aberration of these mechanisms may favor the development of various diseases, in which a relevant role is mediated by the molecular and cellular components of the innate immune system. Moreover, it is well known how the host nutritional status and metabolism can affect also the immune response. In this context, leptin, the adipose tissue-derived cytokine, has been shown to participate in a wide range of biological functions that include the activation of the immune system in the innate-adaptive frontier, underlining the link among immune function/homeostasis, metabolism and nutritional state. Thus, leptin may be one of the mediators of inflammation responsible not only in autoimmune diseases but also in other inflammatory disorders. In the opposite direction, chronic inflammatory states due to metabolic, autoimmune or infectious diseases may lead to leptin resistance at the central level, which is a known cause of obesity, therefore increasing leptin levels and further fueling the inflammation state. However, many aspects concerning leptin's interactions with the inflammation and immune system remain unclear. Novel elements belonging to the innate immunity are continuously discovered (microRNAs, inflammasomes and the danger signals, NK cells), which synergistically enhance inflammatory responses through the integration of a multiplicity of pathways [225]. All of them have allowed us to establish unexpected links among seemingly different chronic diseases, which seems to have inflammation as the "common soil" [225]. That is why the investigation of the role of leptin in the regulation of the immune response remains a challenge for the future. The discovery of common biochemical pathways, which link metabolism and immune tolerance, could be possibly exploited to harness beneficial potential in the modulation of these pathologies.

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Mini review

Role of leptin as a link between metabolism and the immune system

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ABSTRACT

Leptin is an adipocyte-derived hormone not only with an important role in the central control of energy metabolism, but also with many pleiotropic effects in different physiological systems. One of these peripheral functions of leptin is a regulatory role in the interplay between energy metabolism and the immune system, being a cornerstone of the new field of immunometabolism. Leptin receptor is expressed throughout the immune system and the regulatory effects of leptin include cells from both the innate and adaptive immune system. Leptin is one of the adipokines responsible for the inflammatory state found in obesity that predisposes not only to type 2 diabetes, metabolic syndrome and cardiovascular disease, but also to autoimmune and allergic diseases. Leptin is an important mediator of the immunosuppressive state in undernutrition status. Placenta is the second source of leptin and it may play a role in the immunomodulation during pregnancy. Finally, recent work has pointed to the participation of leptin and leptin receptor in the pathophysiology of inflammation in oral biology.

Therefore, leptin and leptin receptor should be considered for investigation as a marker of inflammation and immune activation in the frontier of innate-adaptive system, and as possible targets for intervention in the immunometabolic mediated pathophysiology.

Contents

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1. Introduction

A link between body weight, adipose tissue, and immunity has been hypothesized for a long time, but the precise molecular mediators were unknown until the discovery of leptin in 1994, an adipocyte-derived hormone. Leptin is a non-glycosylated hormone of 146 amino acids [1] with a tertiary structure resembling that of members of the long-chain helical cytokine family (that includes IL-6, IL-11, IL-12, LIF, G-CSF, CNTF, and oncostatin M) [2]. Leptin is synthesized mainly in adipose cells [3] to regulate weight control in a central manner [2]. Circulating leptin levels (normal range 1–15 ng/mL) directly reflect the amount of energy stored in the adipose tissue and are proportional to the body adipose mass both in mice and in humans. Thus, obese individuals typically produce higher leptin than leaner individuals [4–7]. During fasting period and after reduction of body fat mass, there is a decrease in leptin levels that leads to a reduction in total energy expenditure to provide enough energy for the function of vital organs, that is, the brain, the heart, and the liver [8]. However, there is now increasing evidence that leptin has systemic effects apart from those related to energy homeostasis, including regulation of neuroendocrine, reproductive, hematopoietic and immune functions [9]. Even though these effects of leptin decrease are aimed to improve the survival chances under starving conditions, the fall in leptin levels may lead to immune suppression [10], in addition to other neuroendocrine alterations affecting adrenal, thyroid, and sexual/reproductive function [11]. At least, these alterations observed during fasting parallel the decrease in circulating leptin levels. In fact, both ob/ob mice (lacking leptin secretion) and db/db mice (lacking leptin receptor) are not only obese but they also show the immune/endocrine deficiencies observed during starvation [11,12]. In this context, low plasma leptin levels are found in patients with impaired immune response, such as pulmonary tuberculosis patients, and effective treatment restores leptin levels [13]. On the other hand, obesity is now regarded as a pro-inflammatory state, and leptin participates with other adipokines in this pathophysiological state in obese subjects [14].

The pleiotropic nature of leptin is supported by the universal distribution of leptin receptor (LEPR), which may have several isoforms, differing in the length of their cytoplasmic regions [15]. LEPR also shows structural similarity to the class I cytokine receptor family [15–17] and similar to other receptors of this class, LEPR lacks intrinsic tyrosine kinase activity, but requires the activation of receptor-associated kinases of the Janus family (JAKs)

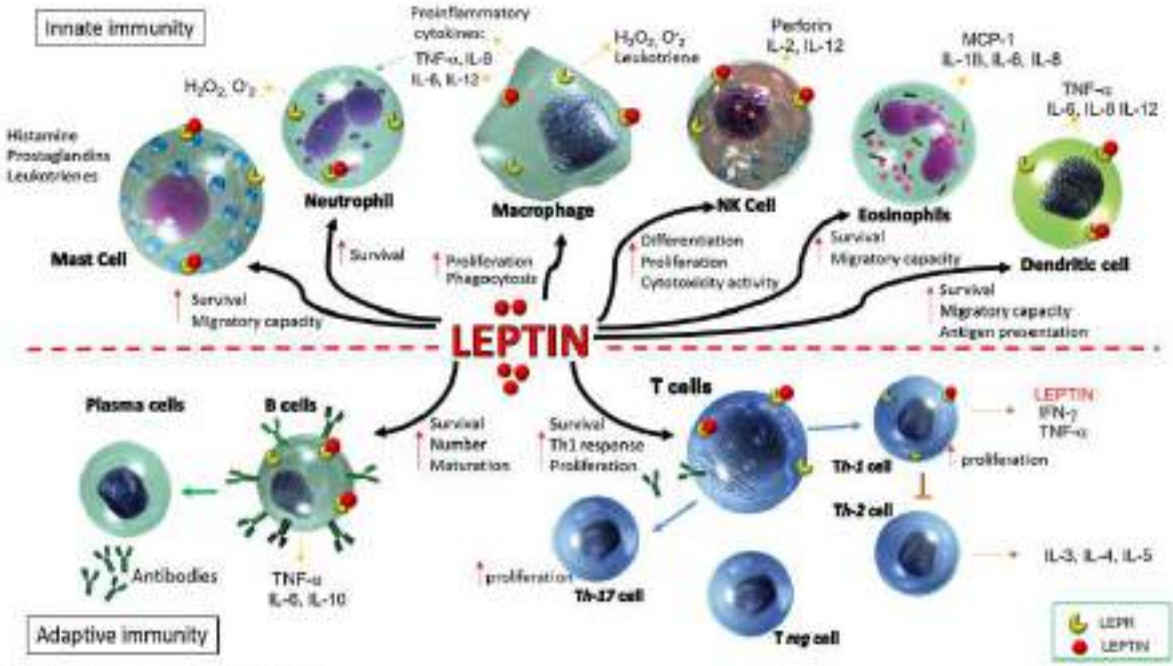

Fig. 1. Effects of leptin on innate and adaptive immune responses.
Leptin affects both innate and adaptive immunity. In innate immunity, leptin modulates the activity and function of Mast cells by enhancing migratory capacity and their survival rate. Leptin also modulates the activity and function of Neutrophils by increasing chemotaxis and the secretion of oxygen radicals (such as hydrogen peroxide, H_2O_2, and superoxide, O^{-2}) through direct and indirect mechanisms. The leptin action seems to be mediated by tumour necrosis factor (TNF) secreted by monocytes/macrophages. Leptin increases phagocytosis by monocytes (macrophages and enhances the secretion of pro-inflammatory mediators. On natural killer (NK) cells, leptin increases cytotoxic capacity and the secretion of perforin and interleukin 2 (IL-2). Leptin can also modulate migratory capacity of human eosinophils, as well as their survival and migratory capacity. Leptin stimulates the release of inflammatory cytokines (including IL-1β, IL-6, IL-8) and chemokines (monocyte chemotactic protein-1). Finally, leptin induces functional and morphological changes in human DCs, licensing them towards Th1 priming and promoting DC survival. Moreover, leptin also increases immature human DC migratory capacity and antigen presentation capacity. In adaptive immunity, leptin affects the generation, maturation and survival of T cells by reducing their rate of apoptosis. On memory T cells, leptin promotes the switch towards T helper-1 (Th1) cell immune responses by increasing interferon γ (IFN-γ) secretion, and facilitates Th17 responses. Conversely, leptin can act as a negative signal for the expansion of human regulatory T cells (Treg). This process is then sustained by an autocrine loop of leptin secretion by Treg cells. Finally, leptin activates B cells to secrete cytokines (i.e., TNF-α, IL-6, IL-10, and TNF-α) as well as the production of IgG2a and delayed type hypersensitivity responses.

[16] which initiate downstream signaling, including members of the STAT (signal transducers and activators of transcription) family of transcription factors [17]. After ligand binding, JAKs autophosphorylate and tyrosine phosphorylate various STATs. Activated STATs then dimerize and translocate to the nucleus, where specific gene responses are elicited [17,18]. Different pathways in addition to STATs are known to be involved in LEPR signaling, including the mitogen-activated protein kinase (MAPK) family and the phosphatidylinositol 3-kinase (PI3K) signaling cascade, as we have previously found in peripheral blood mononuclear cells [19,20] and trophoblastic cells [21–23] (Fig. 2).

Therefore, it has become evident that the control of orexigenic and anorexigenic circuits not only affects the regulation of body weight but also dramatically influences other important physiological and dominant functions, including immune homeostasis [24]. In particular, leptin plays relevant roles in both metabolism and immunity. In this paper we are reviewing the role of leptin as a key element in the frontier of innate and adaptive immune system to mediate an inflammatory response.

2. Leptin and immunometabolism

The survival of the multicellular organisms depends on the capacity to store energy, which is regulated by the communication between the periphery (adipose tissue) and the brain, to inform the body whether there is sufficient amount of energy to mount certain responses or rather, whether limited availability of energy should constrain certain responses. One of these important responses is the ability to fight infection by activating the immune system. The endocrine and immune system are linked by network of cytokines and neuropeptides that modulate the response to infection. This connection is also regulated by the brain in a major way. This is achieved via regulation of peripheral nervous system functions and endocrine responses [25]. This interaction between the immune system and whole-body metabolism has created the new field of immunometabolism, where leptin plays an important role.

Leptin is a key signal from the periphery to provide information to the brain regarding the energy store to control feeding and metabolism. The central effect of leptin in the hypothalamus is mediated, at least in part, by the activation of both the sympathoadrenal axis [26] and the inhibition of hypothalmic-pituitary-adrenal axis [27,28]. The sympathetic nervous system has a major

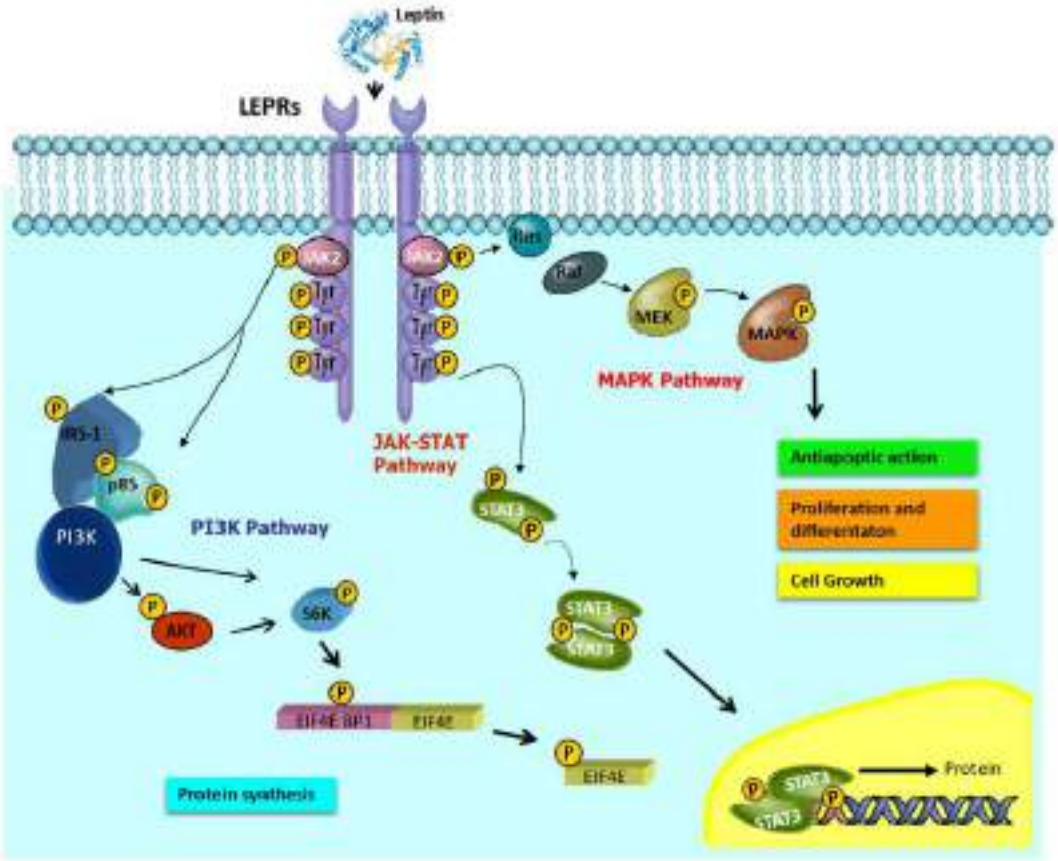

Fig. 2. Leptin signaling via LEPRs.
Leptin binds to LEPR and Jak2 autophosphorylates and phosphorylates Tyr^{985} (Y985 P) and Tyr^{1138} (Y1138 P) on the cytoplasmic domain of LEPR. LEPR phosphorylated Tyr^{1138} mediates the interaction with STATs via its SH2 domain. The STATs translocation allows the Tyr phosporylation by JAKs. STATs then dimerize and translocate to the nucleus to activate gene transcription of target genes. The ERK members of the MAPK family are components of the well defined Ras/Raf/MAPK signaling cascade, which can also be activated by leptin. This pathway has been demonstrated to prevent apoptosis, and to promote proliferation and differentiation as well as cell growth in the trophoblast. Stimulation of the PI3K pathway by leptin also represents a key cascade to exert several different effects of the hormone such as protein synthesis in the placenta.

role in the brain-immune cross-talk, mainly playing an antiinflammatory action [29,30]. Therefore, leptin resistance at central level may prevent a negative feed-back on this antiinflammatory action. On the other hand, the leptin inhibition of the corticotropic axis may contribute to the proinflammatory state found in obesity. In fact animal suggest that decreased hypothalamic-pituitary-adrenal function may play a role in the increased susceptibility and severity of some autoimmune diseases such as multiple sclerosis [31].

3. Leptin and innate immunity

Dissection of the interface connecting the metabolic and immune systems has recently gained wide interest. It is well-known that immune responses require an optimal balance between energy intake and consumption [32]. Early studies, focused on leptin's anorexigenic action, showed that this hormone is able to maintain an adequate energy homeostasis by integrating different orexigenic and anorexigenic signals. There are several connections indeed between adipose tissue and the immune system. First, macrophages and lymphocytes can be normally found in the non-adipose fraction of the adipose tissue [33]. Moreover, white adipocytes have been suggested to share embryonic origin with immune cells, while characterization of adipose tissue-resident lymphocytes led to the notion that this tissue was an ancestral immune organ [33,34]. And more recently, immature haematopoietic cells have been found in adipose tissue, hence it has been proposed as a site for formation and maturation of immune cell precursors [35]. The first evidence of a possible involvement of leptin in the modulation of the immune system derived from the study of its structure and of its receptor which belong the class I cytokine superfamily [16,17,36]. In this sense, LEPR has been shown to have the signaling capabilities of IL-6-type cytokine receptors [16], activating JAK-STAT, PI3K, and MAPK signaling pathways [37,38]. In fact, leptin receptors have been found in monocytes, granulocytes and Natural Killer (NK) Cells (Fig. 1).

3.1. Leptin activation of monocytes

In monocytes, leptin up-regulates phagocytic function [39] via phospholipase activation [40] as well as the expression of adhesion molecules and proinflammatory cytokine secretion, such as TNF-α (early), IL-6 (late), and IL-12 [41,42]). In fact, studies carried out in rodents with genetic abnormalities in leptin or leptin receptors revealed obesity-related deficits in macrophage phagocytosis and the expression of proinflammatory cytokines both in vivo and in vitro, whereas exogenous leptin upregulated both phagocytosis and the production of cytokines [43].

Besides, phenotypic abnormalities in macrophages from leptin-deficient, obese mice have been found [44]. More importantly, leptin deficiency increases susceptibility to infectious and inflammatory stimuli and is associated with dysregulation of cytokine production [45]. More specifically, murine leptin deficiency alters Kupffer cell production of cytokines that regulate the innate immune system. Moreover, leptin stimulates the proliferation of human circulating monocytes in vitro and up-regulates expression of activation markers, such as CD25, CD38, CD69 and CD71 (transferring receptor), and, while it further increases the expression of other activation markers already present at high levels on the surface of resting monocytes, such as HLA-DR, CD11b, and CD11c [46]. Leptin potentiates the stimulatory effect of LPS or PMA on the proliferation and activation of human monocytes and enhances CC-chemokine ligand expression in cultured murine macrophage, through activation of a JAK2-STAT3 pathway [47]. The presence of both isoforms of the leptin receptor was also assessed. Later, it was found that leptin directly induces the secretion of interleukin 1 receptor antagonist in human monocytes [48] and upregulates IP-10 (interferon-gamma-inducible protein) in monocytic cells [49]. It has been reported that leptin augments the synthesis of leukotriene [50] as well as the cholesterol acyltransferases-1, and cyclooxygenase 2 [20,40,46].

A possible role of leptin as a trophic factor to prevent apoptosis has also been found in serum-depleted human monocytes [50], further supporting the role of leptin as a growth factor for the monocyte. Moreover, leptin regulates monocyte function as assessed by in vitro experiments measuring free radical production. Thus, leptin was shown to stimulate the oxidative burst in control monocytes [51], and binding of leptin to the macrophage cell surface increases lipoprotein lipase expression through oxidative stress- and PKC-dependent pathways. In this line, leptin has been found to increase oxidative stress in macrophages [52]. Finally, leptin could act as a monocyte/macrophage chemoattractant inducing in vitro maximal chemotactic responses at 1 ng/mL [53], mediating the inflammatory infiltrate [54], and inducing tissue factor expression in human peripheral blood mononuclear cells [55].

Dendritic cells (DCs) also present leptin receptor on the cell surface [56] (Fig. 1). Thus, leptin has also been found to increase the production of IL-8, IL-12, IL-6, and TNF-α, whereas it decreases MIP-1-α production by dendritic cells. Similar to leptin effect on monocytes, it may increase the survival of DCs, and it may also increase the expression of surface molecules, such as CD1a, CD80, CD83, or CD86. Leptin induces functional and morphological changes in human DCs, directing them towards Th1 priming and promoting DC survival via the PI3K-Akt signaling pathway [57]. The involvement of leptin signaling in DCs survival and maturation has been observed in leptin receptor-deficient db/db mice. Db/db mice displayed markedly reduced expression of costimulatory molecules and a Th2-type cytokine profile, with poor capacity to stimulate allogenic T cell proliferation. Consistent with their impaired DCs phenotype and function, DCs from db/db showed significantly downregulated activities of the PI3K/Akt pathway as well as STAT-3 and IkappaB-alpha. Moreover, the reduced DCs yielded in db/db bone marrow culture was attributed to significantly increased apoptosis, which was associated with dysregulated expression of Bcl-2 family genes [58].

The expression of leptin and leptin receptors has been demonstrated on mast cells (Fig. 1), suggesting paracrine and/or autocrine immunomodulatory effects of leptin on mast cells [59].

3.2. Leptin activation of granulocytes

Human polymorphonuclear neutrophils (PMN) have been found to express leptin receptor in vitro and in vivo [60,61]. However, Zarkesh-Esfahani et al. [61] demonstrated that neutrophils only express the short form of the leptin receptor, which is enough to signal inside the cell, enhancing the expression of CD11b and preventing apoptosis [61,62]. Leptin delayed the cleavage of Bid and Bax, the mitochondrial release of cytochrome c and second mitochondria-derived activator of caspase, as well as the activation of both caspase-8 and caspase-3 in these cells [61]. Therefore, leptin seems to behave as a survival cytokine for PMN, similar to G-CSF. Leptin promotes neutrophils chemotaxis [45,63] and the secretion of oxygen radicals, through direct and indirect mechanisms [61]. Otherwise, when leptin acts as a uremic toxin it interferes with neutrophil chemotaxis [64] and inhibits neutrophil migration in response to classical neutrophilic chemoattractants. The two activities, inhibition of the cell response to chemokines and stimulation of neutrophil migration, could be detected at similar concentrations. On the contrary, neutrophils exposed to leptin did not display detectable $[Ca2^{+}]_i$ mobilization, oxidant

production, or beta2-integrin upregulation [65]. Moreover, leptin also has a stimulating effect on intracellular hydrogen peroxide production in PMN although this effect seems to be mediated by the activation of monocytes [43]. More specifically, leptin modulates neutrophil phagocytosis of *Klebsiella pneumoniae* [66] and in diabetic patients' neutrophils, an increase in leptin serum levels has been correlated with the degree of CD11b expression [67]. On eosinophils, leptin could upregulate cell surface expression of adhesion molecules ICAM-1 and CD18 but suppress ICAM-3 and L-selectin. Moreover, leptin could also stimulate the chemokinesis of eosinophils and induce the release of inflammatory cytokines IL-1beta and IL-6 and chemokines IL-8, growth-related oncogene-alpha, and MCP-1 [33] (Fig. 1).

Leptin has also been found to activate and to inhibit apoptosis of eosinophils. Thus, leptin receptor in eosinophils seems to be a survival factor [68]. Besides, leptin also stimulates chemotaxis, cytokine release and migration of eosinophils, as well as the expression of adhesion molecules, such as ICAM-1 and CD18 [67,69]

Finally, leptin may also enhance survival and induce migration, degranulation and cytokine synthesis of human basophils upregulating CD63, which may have an exacerbating effect on allergic inflammation [70]. Moreover, leptin deficiency has been found to shift mast cells toward an anti-inflammatory actions and protects mice from obesity and diabetes by polarizing M2 macrophages [71].

3.3. Natural killer (NK) cells

In Natural Killer (NK) Cells, leptin increases cytotoxic ability and the secretion of perforin and IL-2 (Fig. 1). Human NK cells constitutively express both long and short forms of LEPR. Besides, the leptin receptors can signal in NK cells, since leptin activates STAT3 phosphorylation in NK cells. Moreover, leptin increases IL-2 and perforin gene expression at the transcription levels in NK cells. Consistent with this role of leptin regulating NK cells, db/db mice have been found to have impaired NK cell function [72,73]. Leptin actions in NK cells include cell maturation, differentiation, activation, and cytotoxicity [42]. Leptin enhances both the development and the activation of NK cells [73], increasing IL-12 and reducing the expression of IL-15 [72]. Finally, it has also been shown that leptin stimulates the production of growth hormone by peripheral-blood mononuclear cells through protein kinase C (PKC) and nitric oxide-dependent pathways [74]. This effect of leptin on the production of growth hormone might be important in immune homeostasis, given the fact that this cytokine-like hormone has marked influences on immune responses by controlling the survival and proliferation of immune cells.

4. Leptin and adaptative immunity

4.1. T cells

Leptin has also been demonstrated to modulate adaptive immune response, which is classically divided into T helper 1 and 2 immune responses on the basis of the cytokine pattern produced. T helper 1 lymphocytes produce mainly pro-inflammatory cytokines that are necessary for macrophage activation and cell-mediated response, whereas T helper 2 lymphocytes secrete modulatory and anti-inflammatory peptides that are important factors for the activation of B cells and basophils. The role of leptin in cell-mediated immunity has been obtained working with ob/ob (leptin-deficient) and db/db (LepR-deficient) mice [75], which, show impaired in vitro T cell mediated immunity, with low IL-2 and IFN-gamma (IFN-γ) production and decreased delayed type hypersensitivity responses in vivo, as compared to normal, age-matched littermate controls [10]. Chronic leptin replacement in ob/ob mice restores the T-cell function, increasing the secretion of the pro-inflammatory cytokines as well as the DTH response in vivo [12]. Besides, these animals show atrophy of lymphoid organs [12], induced by acute nutritional deprivation. Indeed, 48 h acute starvation in normal mice causes a dramatic fall in the total thymocytes count, particularly affecting the $CD4^+CD8^+$ (double positive) and the $CD4^+$ or $CD8^+$ (single positive) compartments when compared to ad libitum fed mice. Leptin replacement during starvation completely protected against these starvation-induced changes in thymocyte number and subpopulation proportion [12]. Thus, evidences indicates that leptin participates in the maintenance of thymic maturation of double positive $CD4^+/CD8^+$ cells, reducing thymic apoptosis [12] as well as preventing glucocorticoids-induced apoptosis in thymocytes.

The activation of T cells induces the expression of the long isoform of the LEPR which is markedly higher in peripheral $CD4^+$ T cells than in $CD8^+$ T cells [19]. Leptin also promotes the expression of adhesion molecules in $CD4^+$ T cells, such as VLA-2 (CD49b) or ICAM-1 (CD54) [10,75] as well as the expression of early activation markers such as CD69 and the expression of late activation markers, such as CD25, or CD71 in both $CD4^+$ and $CD8^+$ T lymphocytes in the presence of suboptimal concentrations of activators such as PHA (2 μg/mL).

Human leptin not only modulates the activation and proliferation of human T lymphocytes but also enhances cytokine production [76]. In fact, dramatic reduction in the adipocyte mass and consequently in circulating leptin levels determines the release of a negative signal that impairs T-cell priming and the production of pro-inflammatory mediators necessary for the induction of a Th1 response. On naive T-cell responses, leptin increases proliferation and IL-2 secretion, through the activation of mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways [34]. On memory T cells, leptin promotes the switch toward T-helper 1 (Th1)/Th17-cell immune responses by increasing IFN-γ and TNF-α secretion, the production of IgG2α by B cells, and DTH responses. Thus, leptin receptor signaling in T cells is required for Th17 differentiation [77]. This process is then sustained by an autocrine loop of leptin secretion by Th1/Th17 lymphocytes [10]. Thus, human leptin enhances the production of IL-2 and IFN-γ in stimulated T lymphocytes. These data are in agreement with the observation of the leptin effect on anti-CD3 stimulation of T cells, which increases the production of the proinflammatory cytokine IFN-γ [78]. The effect of leptin polarizing T cells towards a Th1 response seems to be mediated by stimulating the synthesis of IL-2, IL-12, and IFN-γ and the inhibition of the production of IL-10 and IL-4 [54,76] (Fig. 1). These data regarding leptin modulation of Th1-type cytokine production are in line with the observed effects of leptin stimulating TNF-α and IL-6 production by monocytes [20], further suggesting the possible role of human leptin in the regulation of the immune system inducing a proinflammatory response.

Leptin is also able to modulate regulatory T (Treg) function. In this sense, it has been reported that freshly isolated Tregs produce and secrete leptin and express high amounts of the LEPR [79]; Leptin can act as a negative signal for the proliferation of human Foxp3+ CD4+ CD25+ T(reg); in vitro leptin neutralization, during anti-CD3 and anti-CD28 stimulation, results in marked Treg proliferation, via mTOR pathway [80], thus confirming the negative control that leptin exerts on this cellular subset cells. Moreover, genetic deficiency of leptin (ob/ob mice) is associated with an increased percentage of peripheral Tregs as compared to wildtype mice [79]. The evidence that leptin can act as a negative signal for the proliferation of Treg supports the possibility of new anti-leptin-based approaches for the immunotherapy of

conditions characterized by low numbers of Tregs, such as obesity, T2D, and metabolic syndrome.

4.2. B cells

In contrast to macrophages and T cells, little is known about the role of B cells in response to leptin. However, leptin seems to play a central role also in the modulation of B cell compartment (Fig. 1). In this sense, it has been proposed that leptin may be responsible for most of B cell intrinsic inflammation in obese subjects. In fact, It have been reported that leptin not only increases STAT3 phosphorylation, crucial for TNF-α production, but also decreases AMPK phosphorylation, crucial for E47 activation through p38 MAPK phosphorylation [81].

B cells have recently emerged as crucial players in regulating inflammation in murine visceral adipose tissue (VAT), by presenting antigens to T cells, secreting pro-inflammatory cytokines, and secreting pathogenic antibodies [82], contributing to local and systemic inflammation [83]. In this sense, leptin seems to play a central role also in the modulation of B cell compartment. It has been proposed that leptin may be responsible for most of B cell intrinsic inflammation in obese subjects. Therefore, B cells critically regulate proinflammatory T-cell function [84], and can now be added to the list of immune cells participating in this process, where they activate $CD8^+$ and Th1 cells and release pathogenic antibodies [82].

5. Leptin as a mediator of inflammation

In addition to the immune regulatory actions reviewed earlier, recent evidence shows that leptin acts as a pro-inflammatory cytokine. In fact, leptin expression is not only regulated by food intake but also by various hormones, as well as by several inflammatory mediators [85]. In general, serum leptin levels inversely correlate with glucocorticoid levels, and increase during acute infection, inflammation and sepsis, favored particularly by LPS and cytokines such as TNF-α, IL-6, and IL-1β [86,87]. Such pro-inflammatory mediators, which upregulate leptin expression, contribute in turn to create a loop of acute phase reactants that influence each other in promoting the development of chronic inflammation [88,89]. Furthermore, leptin is produced by inflammatory regulatory cells, suggesting that leptin expression could trigger or participate in the inflammatory process through direct paracrine or autocrine actions [90]. Indeed, circulating leptin levels are highly and promptly increased in experimental models of acute inflammation [91]. Even leptin from adipose tissue is stimulated by pro-inflammatory cytokines such as TNF-α and IL-1β, suggesting that these cytokines stimulate short-term release of stored leptin. In fact, in models of adaptive immune mediated inflammation, leptin deficiency implies an imbalance between Th1 and Th2 lymphocytes [35], causing an altered cytokine secretion which could lead to the aforementioned resistance to inflammation.

The link between leptin and inflammation has been studied in several inflammatory diseases, in which, direct and indirect effects of leptin have been shown.

5.1. Leptin as an inflammatory mediator in the obesity-associated metabolic disorders

Obesity is associated with metabolic disorders, such as insulin resistance, and non- alcoholic [92–95] hepatic steatosis and steatohepatitis [96]. It is thought that the increased leptin levels associated with leptin resistance may contribute to fat accumulation in the liver, through a reduced hepatic oxidation and an increased synthesis of free fatty acids that cause liver inflammation and fibrosis through lipid accumulation and peroxidation that trigger the production of reactive oxygen species (ROS). However, type 2 diabetes is the most significant metabolic disorder associated with obesity and their prevalence is increasing in parallel worldwide [97]. Leptin could be a pro-inflammatory factor contributing to insulin resistance [98]. On the other hand, leptin may also exert insulin-sensitizing effects, and may also regulate beta cell mass and survival [99]. In any case, leptin administration has not any effect on body weight reduction, probably due to the development of central leptin resistance or tolerance in obese patients with already very high circulating leptin levels [100].

5.2. Leptin as an inflammatory mediator in the obesity-associated cardiovascular disease

Obesity is associated with an increased cardiovascular mortality and morbidity [101]. The pathophysiology of obesity is in part mediated by a state of chronic, low-grade inflammation [102,103]. In fact, adipose-tissue-mediated inflammation seems to be the link between obesity and cardiovascular disease [104]. Obesity is almost always characterized by elevated plasma leptin levels, reflecting the increase in fat mass as well as the leptin resistance at central level [105]. In this line, leptin has been shown to be one of the adipokines mediating the pro-inflammatory state of obesity [106]. Moreover, even though more than 30 adipokines may have a role in the adipo-vascular axis, leptin is one of the more studied links between obesity and cardiovascular disease [107]. In this sense, leptin augments the inflammatory milieu that promotes the recruitment of monocytes to the intima, eliciting foam cell formation in macrophages, and inducing secretion of pro inflammatory and atherogenic cytokines [108]. Leptin can also alter cardiomyocyte structure and function [109], and LEPR has been detected in lesions of patients with atherosclerosis, while ob/ob mice are resistant to atherosclerosis. Thus, leptin has also been proposed as an important mediator of atherosclerosis [110,111].

On the other hand, the cardiovascular effects of leptin are controversial [112], and even though there are clinical studies supporting the role of leptin in cardiovascular risk [113], other studies have indicated no clinically relevant association with risk of cardiovascular disease [114,115]. The effects of leptin increasing NO production may have both protective and deleterious effect on endothelial cells, since leptin activates the endothelial NO synthase (eNOS) [116], but also increases the expression and activity of the inducible NO synthase (iNOS) [117]. In any case, leptin may contribute to cardiovascular disease in obesity, since leptin-deficient mice, which are extremely obese, are protected from atherosclerosis [118]. Further studies are therefore needed to address the role of leptin as a biomarker or a target for treatment.

5.3. Leptin as a mediator in autoimmune diseases

The prevalence of autoimmune diseases, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), and T1D, is increased in affluent countries and associates with serum leptin levels [119,120]. Consistently, it has been demonstrated that leptin-deficient mice showed resistance or less susceptibility to the development autoimmune diseases [91]. For example, recent studies have shown that leptin is increased in patients with RA [121]. Serum leptin levels are higher in RA patients with high disease activity, correlate well with disease activity, and decrease significantly when disease is well controlled [88]. In fact, the leptin concentrations are significantly higher in patients with active erosive RA [89]. Moreover, fasting, which is associated with a dramatic decrease in circulating leptin, leads to a decreased CD4 lymphocyte activation and increased IL-4 secretion in patients with RA [122]. Even though a significant inverse correlation between inflammation and leptin

concentrations exists in patients with active RA, plasma leptin concentrations did not significantly differ from those in healthy controls. This suggests that active chronic inflammation may lower plasma leptin concentrations. However, treatment with anti-TNF-α did not change plasma leptin concentrations [123] and in longer therapy, the beneficial effect of on cardiovascular mortality in RA does not seem to be mediated by reduction in serum levels of leptin [124].

High leptin levels are also related with a higher prevalence of other immune diseases, such as systemic lupus erythematosus (SLE) [125], and also with increased susceptibility to the development of osteoarthritis (OA) [126]. In fact, it has been hypothesized that the increased pre-disposition of females to develop OA could be due to the higher circulating leptin levels observed in females [126] in comparison with males. However, whether the increase of plasmatic leptin is a cause or a consequence of the development of those pathologies needs to be elucidated. Recently, leptin has been found to promote SLE by increasing autoantibody production and inhibiting immune regulation [127,128].

Obesity is also associated with other inflammatory autoimmune diseases, such as Crohn's disease, ulcerative colitis and psoriasis [129,130], and increased leptin expression has also been reported in Behcet's disease, psoriasis, thyroiditis and during the acute phase of ulcerative colitis [131–134]. Besides, In inflammatory bowel disease patients, systemic leptin levels are increased compared to normal healthy donors [135]. Nevertheless, clinical studies are contradictory, and further investigation is necessary to clarify leptin involvement in the pathogenesis of intestinal bowel disease [136]. Regarding psoriasis, serum leptin levels, tissue leptin and leptin receptor expression showed a positive correlation with disease duration [137].

Concerning experimental autoimmune encephalomyelitis (EAE), it has been shown that ob/ob mice are resistant to the development of this model of multiple sclerosis. This resistance is abolished by the administration of leptin, which is accompanied by a switch from a Th2 to Th1 pattern of cytokine release [138]. In addition, and in concordance with these reports, it has been noticed that the onset of the disease is preceded by an increase of circulating leptin [139]. Furthermore, it has been demonstrated that acute starvation, which is accompanied by a decrease in circulating leptin levels, delays the onset of the disease and attenuates the symptoms. Recently, it has been shown that leptin levels are negatively correlated with $CD4^+CD25^+$ regulatory T-cells during multiple sclerosis [138], suggesting that this negative association may have major implications in the pathogenesis of multiple sclerosis, as well as in the development of different autoimmune diseases characterized by Th1 auto-reactivity [138]. Noteworthy, it has been shown that leptin is expressed by both macrophages and T-cells infiltrated into the central nervous system during EAE [90]. This interesting report indicates that leptin is produced by immune cells during acute EAE, and suggests that this hormone could be participating in the development of CNS-inflammatory diseases not only in an endocrine fashion but also by an autocrine or paracrine mechanism.

In addition to experimentally induced autoimmune diseases, leptin is also involved in spontaneous autoimmune disease such as type 1 diabetes (T1D) in the non-obese diabetic (NOD) mice model. Leptin accelerates the disease onset and progression, by stimulating autoimmune destruction of β-cells and significantly increased IFN-γ production in peripheral T cells [140]. Moreover, it has been reported that a spontaneous mutation of the LEPR in normally T1D-prone NOD mice suppresses T1D development in the NOD mice by inhibiting activation of effector T cells, demonstrating the important role of leptin signaling in the disease pathogenesis [141]. Therefore, given that scientific evidence has demonstrated the importance of leptin in the activation of autoreactive T cells, responsible for the autoimmune attack during diabetes, it is reasonable to speculate that alterations of leptin/leptin signaling in diabetes-susceptible NOD mice might determine a condition of resistance to the disease or at least a delay in the onset of clinical symptoms associated with it.

In summary, regulation of leptinemia is complex and additional studies are necessary to clarify whether leptin is a real actor or a simple mediator in the inflammatory process of these autoimmune diseases. Moreover, the elevated circulating leptin levels in autoimmune diseases may contribute to the low-grade inflammation that increases the risk of cardiovascular disease in these patients [136].

5.4. Leptin as a mediator in allergic diseases

There are data from different epidemiological studies showing a strong association between obesity and allergy- related diseases [142]. Thus, obesity and weight gain have been associated with an increased risk of asthma and allergic rhinitis, probably due to the common inflammatory condition that pushes the immune system toward a Th2 pattern. Moreover, leptin serum levels seem to depend on allergen exposure in seasonal allergic rhinitis [143]. Besides, Increasing epidemiological data identify a link between obesity and asthma incidence and severity [144]. Importantly, obesity antedates asthma [145], although body mass index does not appear to be a significant modifier of asthma severity [146]. In this line, obesity has also been involved in the exacerbation of asthma, and leptin effect on eosinophilic inflammation has been proposed as a possible mechanism [147]. In fact leptin and other adipokines seem to be more important in obesity- related asthma than other factors such as oxidative stress [148]. Therefore, elevated production of leptin in patients with allergic diseases such as atopic asthma and atopic dermatitis, has led to the concept that leptin could play a crucial immunopathophysiological role in allergic inflammation by activation of eosinophils. In this regard increased eosinophilic activity (chemotaxis and adhesion) associated with high serum leptin and TNF-α levels have been found in atopic asthmatic obese children and adolescents [149].

5.5. Leptin in placenta immunomodulatory signals

The well-established link between leptin, fat stores, and immunocompetence [10], makes leptin a likely candidate for mediating physiological trade-offs between the immune and other systems, especially reproduction. Leptin concentrations are higher in pregnancy (which is a hypermetabolic state and an alteration in neuroendocrine milieu) than in the non-pregnant state [150]. Moreover, it has been found striking homologies between the expression and regulation of cytokines and inflammation- related genes in the placenta at term and in the white adipose tissue. The high level of similarities between the two tissues points to the placenta as a very attractive candidate to promote molecular interplays between immune function and metabolism. Intriguingly, it has become apparent that the placenta is a source as well as a target for the action of leptin [151,152]. More exactly, the human leptin mRNA and proteins are localized to the syncytiotrophoblast at the maternal interface as well as in the villous vascular endothelial cells in direct contact with the fetal blood (Lea et al.) and their expressions are comparable to those of the adipose tissues [151,153]. Moreover, human placental leptin is identical to that derived from adipose tissue in terms of size, charge and immunoreactivity [154]. Although it has the same promoter, the human placental gene has an upstream enchancer with three protein binding sites, known as leptin enchancer region (PLE-3) [155], implying that placental leptin gene is perhaps differently

regulated compared to that in the adipose tissue. In this regard, it has been reported that hypoxia, insulin, glucocorticoids, IL-1α, IL-1β, IL-6, IFN-γ, progesterone, hPL, hCG and cAMP regulate placental leptin expression [156–162] . Leptin receptors (short and long leptin receptor isoforms) also are co-localized with leptin on the syncytiotrophoblast at the maternal interface and multiple signal transduction pathways, such JAK-STAT [21], which has been correlated with trophoblast invasiveness [163], MAPK [21], which mediates a proliferative response, and PI3K [21] which regulates the invasive differentiation of human trophoblasts [164] are activated in response to leptin in human term placenta. Taken together, these data reinforce the view of leptin acting in autocrine as well as paracrine pathways in the human placenta similar to that of the white adipose tissue, suggesting a role for leptin in immunosuppression during pregnancy.

The placenta is a complex organ with a wide diversity of functions that ranges from anchoring the embryo and preventing its rejection by the maternal immune system to enabling the transport of nutrients and waste between mother and the embryo [165]. Human placenta has been reported to produce and secrete various proinflammatory factors including cytokine such as IL-1β, IL-6, TNFα, and prostaglandins (PGs) such as PGE2 and PGF2α [166] (Fig. 3). These inflammatory mediators have been demonstrated to play an important role in a number of normal and abnormal inflammatory processes, including the initiation and progression of human labor [167,168]. In this sense, leptin, which is released by human placenta, is a cytokine-like peptide hormone that acts as an immune regulator. In placenta, leptin is a key modulator of the inflammatory and immune responses, regulating generation of matrix metalloproteinases, arachidonic acid products, nitric oxide induction, and T cell cytokines [166].

Leptin stimulates IL-6 secretion by human term trophoblast cells [169] and human placenta [170]. Moreover, leptin- stimulated proinflammatory cytokine release from human placenta could be abrogated by treatment with the antiinflammatory ERK 1/2 MAPK inhibitor UO126, the PPARγ ligand troglitazone, and the NF-κB inhibitor BAY 11-7082, demonstrating the importance of NF-κB and PPARγ in the regulation of TNFα and IL-6 release from human placenta [171]. Prostaglandins and proinflammatory cytokines that are produced within the intrauterine environment participate in the regulation of myometrial contractility, cervical ripening and rupture of membranes [167,168]. Therefore, leptin, which is produced within the intrauterine environment, may play a role in the processes of human labor and delivery. Nevertheless, that inflammation is a labor related mechanism in both term and preterm deliveries [172–174]. Furthermore, these proinflammatory actions of leptin may also have significant implications in the pathogenesis of various disorders during pregnancy, such as gestational diabetes and preeclampsia, which are characterized by increased placental leptin expression [175–177]. The production of leptin within placenta may contribute to the raised circulating levels of proinflammatory mediators that are evident in these pregnancy diseases, whereas successful pregnancy is associated with downregulation of intra-uterine pro-inflammatory such as TNF-α and interleukin 1 (IL-1). Moreover, leptin can also stimulate myelopoiesis, erythropoiesis, and lymphopoiesis and may promote maturation of the fetal immune system [41]. For example, leptin, might modulates the activation of natural killer (NK) cells (70% of the decidual leukocyte population), which produce an array of angiogenic growth factors including angiopoietin-1 (Ang-1), Ang-2, and VEGF-C and have been implicated in decidual vascular remodeling [178,179] (Fig. 3). Thus, placental leptin may have a local autocrine immunomodulatory or anti-inflammatory role [180].

Finally, a great body of work indicates that leptin secretion is regulated by the steroid hormones [159] implicating leptin as a modulator of placental endocrine function [181]. Moreover, leptin increases matrix metalloproteinase expression in cytotrophoblasts

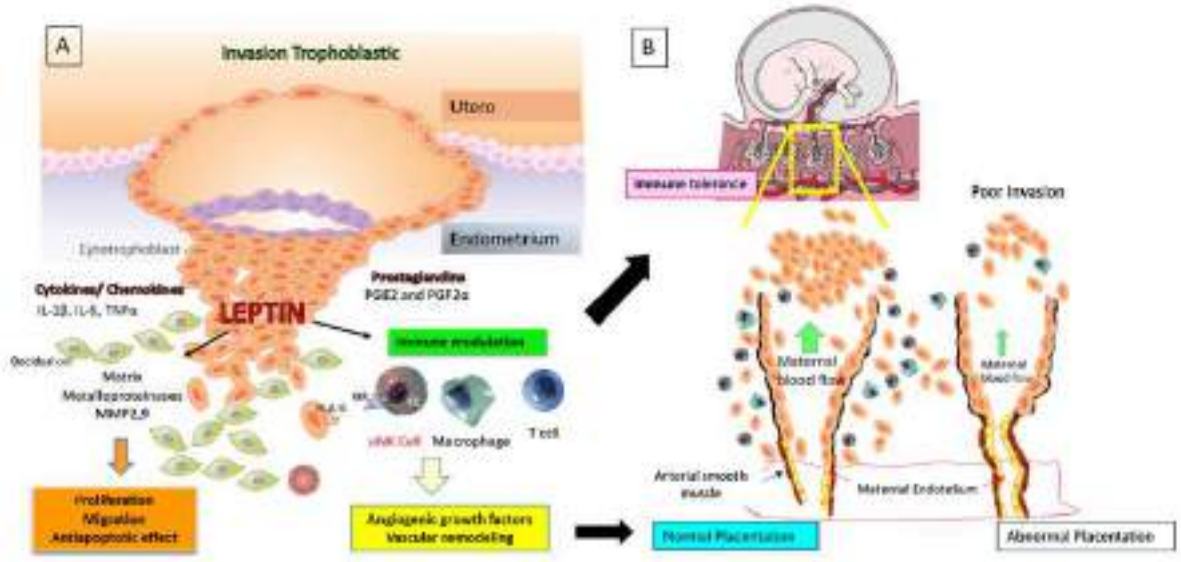

Fig. 3. Scheme illustrating the role of leptin on placenta development and function.
(A) Leptin, secreted by the syncytiotrophoblast, is a pivotal hormone for pregnancy establishment and maintenance, exerting autocrine and paracrine effects that regulate decidualization by stimulating metalloproteinases (MMPs) expression and secretion, promoting proliferation, migration as well as decreasing the apoptotic effects. Leptin seems to intervene in the angiogenesis and immunotolerance by regulating immunomodulation, likely cross talking to diverse uterine cell types, including cytotrophoblast, uterine natural killer (uNK) cells and macrophages. In particular, interaction of paternal human leukocyte antigen (HLA) expressed on cytotrophoblasts with maternal killer cell immunoglobulin like receptors (KIR) are thought to play a pivotal role in placentation and reproductive success. (B) Proliferative cytotrophoblasts migrate into spiral arteries and contribute to uNK cell initiated remodeling within the decidua and the upper part of the myometrium, leading to the widening of artery lumen, which decreases the resistance against blood flow that irrigates the fetus. Failure in this process is associated with pregnancy complications such as abortions or preeclampsia likely due to damage of placental villi provoke by oxidative stress.

[182], stimulates the process of proliferation and protein synthesis, and inhibits apoptosis in human trophoblastic cells [21–23,183].

In summary, the localization of leptin and its receptor in human placental indicates that leptin may have both autocrine and paracrine activities as a local immunomodulatory signal [184] (Fig. 3).

5.6. Leptin and inflammation in oral biology

Compelling evidence has implicated leptin in oral biology functions, thus, it has also been reported the presence of leptin and soluble LEPR in gingival tissue extract both in healthy and inflamed gingival tissues [185,186], in gingival crevicular fluid [187–189] as well as in human chronic periapical lesions [190] and saliva [191,192]. Elevated serum leptin concentration has been associated with increased chronic periodontitis [193]. Moreover, the first evidence has emerged that leptin has effects on dental pulp stem cells, acting as an important modulator of pulpal mesenchymal stem cell differentiation [194], being expressed in ameloblasts, odontoblasts, dental papilla cells and stratum intermedium cells in rat and human tooth germs at the late bell stage[195]. In this way, a recent study has also demonstrated the expression of leptin in rat dental pulp [195]. In addition, we have found that both leptin and LEPR are expressed by healthy and inflamed human dental pulp cells [196,197], as well as periapical granulomas [198,199]. Intriguingly, it has been reported that leptin is synthesized and secreted in vitro by pulp fibroblasts derived from extracted healthy molar teeth [200]. However, other dental pulps cells might also be a source of leptin and LEPR. Thus, which pulp cell type may expresses leptin and LEPR, remain to be elucidated. Therefore, leptin could act by autocrine as well as paracrine pathways and therefore it may play a role in pulpal inflammatory and immune responses similar to that of the white adipose tissues. In this sense, although, adipocytes are not a normal cellular component in dental pulp, human dental pulp stem cells (DPSCs) are capable of differentiating into oil red-O-positive lipid-containing adipocytes [201], expressing in vitro the adipogenic master genes PPARγ2 (peroxisome proliferator- activated receptor gamma two) and lipoprotein lipase (LPL), two adipocyte-specific transcripts [202]. So, pulpal leptin could be secreted by DPSCs suffering adipogenic differentiation. On the other hand, leptin may also have a functional role in dentin mineralization process in dental pulp reparative and immune responses [203].

The dental pulp is a highly innervated tissue with good healing potential after injury, inflammation and angiogenesis is crucial for tooth development and a prerequisite for successful repair after injury and inflammation. Angiogenic growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF-2), and transforming growth factor (TGF-β) have been identified in human dental pulp and dentin matrix [204–206]. In fact, these factors are up-regulated in dental pulp from carious teeth. Therefore, leptin might play an important role mediating angiogenesis by regulating angiogenic growth factor expression by pulpal, similar to that of the others inflamed tissues and cancer [207].

Although bacteria were suggested as the main etiologic agent in periodontal disease, its presence is not enough to initiate the disease, whereas the initiation and progression of periodontal disease depends on the quality of the host immune response. The infiltration of inflamed tissues by natural immune cells such as neutrophils, eosinophils, and macrophages is an important feature of inflammation. Macrophage infiltration of the adipose tissue is present in obesity, where it could expand adipocytes or neighboring pre-adipocytes (in turn responsible for the production of chemotactic signals leading to macrophage recruitment) [208,209]. Similarly to what occurs in obesity inflamed pulp is characterized by the infiltration of inflammatory cells such as lymphocytes, macrophages, dendritic cells and neutrophils, and consequently, pulpitis, is formed [210]. Moreover, it is well known that chemokines regulate the trafficking of lymphocytes, and CC-chemokine ligand 20 (CCL20) shows to play a crucial role in the recruitment of memory T cells [211] and immature dendritic cells into inflammatory lesions [212]. All together and taking into account that leptin is associated with an increased expression of CCL20 [213] as well as the high relative amount of leptin in inflamed pulp, it has been suggested that leptin could regulate the production of chemotactic signals and the trafficking of lymphocytes during pulpal inflammatory response. It has been demonstrated that CCL20 expression is induced by stimulation with caries-related bacteria invading deeply into the dentinal tubules as well as by proinflammatory cytokines in the inflamed pulpal lesions [214]. When the number of macrophages increases during the innate response of the dentin/pulp complex to caries [215], leptin can regulate monocyte function as assessed by in vitro experiments measuring free radical production via PKC-dependent pathways [51,216]. Moreover, as previously demonstrated in monocytes in immune system, leptin could stimulates the production of pro-inflammatory cytokines such as TNF-α and IL-6, and enhances CC-chemokine ligand expression in human dental pulp macrophage, through phosphorylation-activation of the JAK-STAT pathway [217,218].

In summary, even though the role of leptin and its receptors has not been fully clarified in oral biology, the leptin expression in human dental pulp cells, in gingival tissue and in gingival crevicular fluidic as well as in serum and saliva, point to a role for leptin mediating the response of the host to the infectious and inflammatory stimuli with the host's production of pro-inflammatory cytokines.

6. Conclusions

In conclusion, during the last decade, there has been a growing understanding of how host nutritional status and metabolism can affect the immune response. In this context, leptin, the adipose tissue-derived cytokine, is able to participate in a wide range of biological functions that include the activation of the immune system in the innate- adaptive frontier, underlining the link among immune function/homeostasis, metabolism, and nutritional state. Thus, leptin might broadly influence vital functions not only by tuning caloric balance but also by affecting immune responses. In this context, leptin may be one of the mediators of inflammation responsible not only in autoimmune diseases but also in other inflammatory disorders such as periodontitis, metabolic syndrome and gestational diabetes. However, many aspects concerning leptin's interactions with inflammation and immune system remain unclear. That is why the investigation of the role of leptin in the regulation of the immune response remains a challenge for the future. The discovery of common biochemical pathways, which link metabolism and immune tolerance, could be possibly exploited to harness beneficial potential in the modulation of these pathologies. For example, taking into consideration the detrimental effect of the increased circulating leptin on inflammation, it could be suggested that the control of the amount of bioavailable leptin by using a specific soluble receptor or the blockade of leptin receptor, by using monoclonal humanized antibodies might be a good way to avoid undesired leptin actions in autoimmune- inflammatory diseases. In any case, plasma leptin concentration may be a biological marker of the inflammation status as well as the onset and evolution of autoimmunity, and a maker of undernutrition that may mediate impaired immune response.

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Leptin action in normal and pathological pregnancies

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Abstract

Leptin is now considered an important signalling molecule of the reproductive system, as it regulates the production of gonadotrophins, the blastocyst formation and implantation, the normal placentation, as well as the foeto-placental communication. Leptin is a peptide hormone secreted mainly by adipose tissue, and the placenta is the second leptin-producing tissue in humans. Placental leptin is an important cytokine which regulates placental functions in an autocrine or paracrine manner. Leptin seems to play a crucial role during the first stages of pregnancy as it modulates critical processes such as proliferation, protein synthesis, invasion and apoptosis in placental cells. Furthermore, deregulation of leptin levels has been correlated with the pathogenesis of various disorders associated with reproduction and gestation, including polycystic ovary syndrome, recurrent miscarriage, gestational diabetes mellitus, pre-eclampsia and intrauterine growth restriction. Due to the relevant incidence of the mentioned diseases and the importance of leptin, we decided to review the latest information available about leptin action in normal and pathological pregnancies to support the idea of leptin as an important factor and/or predictor of diverse disorders associated with reproduction and pregnancy.

Keywords: leptin • reproduction • placenta • polycystic ovary syndrome • recurrent miscarriage • pre-eclampsia • gestational diabetes • growth restriction

Introduction

Adipose tissue acts as an endocrine organ, secreting different molecules or adipokines [1]. Leptin is produced and secreted predominantly from adipose tissue into the circulation. Circulating leptin levels reflect adipose tissue size and also change with nutritional state [2]. Furthermore, leptin is considered as a pleiotropic hormone that regulates not only bodyweight but many other functions, including vascular function, bone and cartilage growth, immune system and systemic inflammatory response as well as the normal physiology of the reproductive system [3, 4].

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A link between bodyweight, adipokines and success of pregnancy has been proposed, although it is not fully understood [5–7]. The observations that human and rodents with congenital leptin deficiencies are sterile and that anorexia and obesity modify the onset of puberty in opposite ways, led to the idea that leptin is an important player in reproduction [8]. In this way, leptin was the first adipokine claimed to be the 'missing link' between fat and reproduction [9].

Leptin mediates its effects by binding to leptin receptors (LepRs) expressed in the brain and peripheral tissues [2]. Different variants of LepR have been described, but the long isoform of LepR (LepRb) is primarily responsible for leptin signalling. LepRb is strongly expressed in specific nuclei of the hypothalamus, a region of the brain that is involved in the control of appetite, and there it regulates energy homoeostasis and neuroendocrine function, among other functions [10]. In addition, leptin has direct effects on many cell types on the periphery. LepRb is expressed in lung, kidney, adipocytes, endothelial cells, blood cells, stomach, muscle, liver, pancreatic islets, osteoblast, endometrium, placenta and umbilical cord [2, 11].

Leptin or LepR deficiencies not only cause severe obesity but also abnormalities in haematopoiesis, immunity, angiogenesis, bone formation, blood pressure and reproduction. Mutations in the leptin gene, in human and/or mouse models, result in infertility or significant reproductive dysfunction [8, 12]. Leptin is required for the release of gonadotrophin-releasing hormone (GnRH) from the pituitary, and as a consequence, female *ob/ob* mice (deficient in leptin) have reduced oestrogen levels and exhibit low uterine weight [13, 14]. Male *ob/ob* mice also show reduced GnRH levels and diminished production of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) as well as testosterone, an essential hormone for the maintenance of male fertility and testicular function [15].

Therefore, leptin can act as metabolic switch connecting the nutritional status of the body to high energy consuming processes. The energy requirements of pregnancy are those desired for correct maternal gain to ensure the growth of the foetus, placenta and associated maternal tissues [12]. Another key observation that built on the link between leptin and reproduction is the secretion of leptin from human placenta, further establishing an association between leptin and pregnancy [8, 16]. Placental formation during human gestation is crucial for embryonic progress and successful pregnancy outcome, allowing metabolic exchange and production of steroids, hormones, growth factors and cytokines that are critical for the maintenance of pregnancy [17, 18]. Trophoblast cells play an essential role in the development of placenta. These cells differentiate into two distinct types: extravillous and villous trophoblast. In the extravillous pathway, cytotrophoblasts (CT) proliferate, differentiate into an invasive phenotype and penetrate into the maternal decidua and myometrium. Meanwhile, in the villous pathway, mononuclear CT fuse to form a specialized multinuclear syncytium called syncytiotrophoblast (ST) [19].

In normal pregnancy, trophoblast invasion is a critical step in remodelling the maternal spiral arteries to adequately perfuse the developing placenta and foetus [20]. Failure of invasion processes may lead to miscarriage or pregnancy disorders such as pre-eclampsia (PE) or intrauterine growth restriction (IUGR) [21, 22]. In this sense, deregulation of leptin levels has been implicated in the pathogenesis of various disorders of reproduction and gestation, such as polycystic ovary syndrome (PCOS), recurrent miscarriage, gestational diabetes mellitus (GDM), PE and IUGR [23].

Leptin mediates the crosstalk between adipose tissue and reproduction

Reproductive function depends on the energy reserves stored in adipose tissue and the reproductive system. The large energy needs for pregnancy was the original rationale to explain the disruption of reproductive function by low fat reserves [24]. This led to the hypothesis of an endocrine signal that conveys information to the brain about the size of fat stores [25]. Thus, leptin was the first adipokine claimed to be the 'missing link' between fat and reproduction [9]. Leptin modulates satiety and energy homoeostasis [26, 27], but is also produced by placenta. Thus, it was suggested that the effects of placental leptin on the mother may contribute to endocrine-mediated alterations in energy balance, such as the mobilization of maternal fat, which occurs during the second half of pregnancy [28, 29]. In addition, leptin has been found to influence several reproductive functions, including embryo development and implantation [30]. Moreover, animal models have demonstrated that leptin-deficient mice are infertile, and fertility can be restored by exogenous leptin [31]. This adipokine may therefore play a critical role in regulating both energy homoeostasis and the reproductive system [32].

Leptin increases the secretion of gonadotrophin hormones, by acting centrally at the hypothalamus [33]. In addition, because leptin has been shown to be influenced by steroid hormones and can stimulate LH release, leptin may act as a permissive factor in the development of puberty [34].

Leptin can also regulate ovary functions [35–38]. Thus, leptin resistance and/or hyperleptinaemia in obesity lead to altered follicle function, whereas conditions in which nutritional status is suboptimal, leptin deficiency results in hypothalamic–pituitary gonadal axis dysfunction [39, 40].

In addition, a significant role of leptin in embryo implantation was proposed. Leptin and leptin receptor are specifically expressed at the blastocyst stage [41], and it was also reported that leptin is present in conditioned media from human blastocysts, promoting embryo development, suggesting a function in the blastocyst–endometrial dialog [42].

Role of leptin in placenta development

The implantation process involves complex and synchronized molecular and cellular interplay between the uterus and the implanting embryo, and these events are regulated by paracrine and autocrine factors [18]. In 1997, leptin was described as a new placental hormone in humans [29]. Circulating leptin levels are significantly increased during pregnancy and decreased after birth, revealing an

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important role of leptin during gestation [43, 44]. Placental production of leptin is one of major source of higher levels in maternal circulating leptin other than maternal gain of fat mass [45]. Leptin is now considered an important regulator during the first stages of pregnancy which has physiological effects on the placenta, including angiogenesis, growth and immunomodulation [28, 46–51]. Figure 1 highlights the main actions of leptin in the maternal–foetal interface.

The control of cell proliferation is critical for a correct placental development, and it is finely regulated [52]. During placentation, CT and ST keep a subset of cells in direct contact to the villous basement membranes. In the extravillous compartment, cell proliferation favours the invasion of the uterine stroma. Similarly, in the villous compartment cells undergo syncytial fusion directed by specific transcription factors [53]. It was shown that leptin induces proliferative

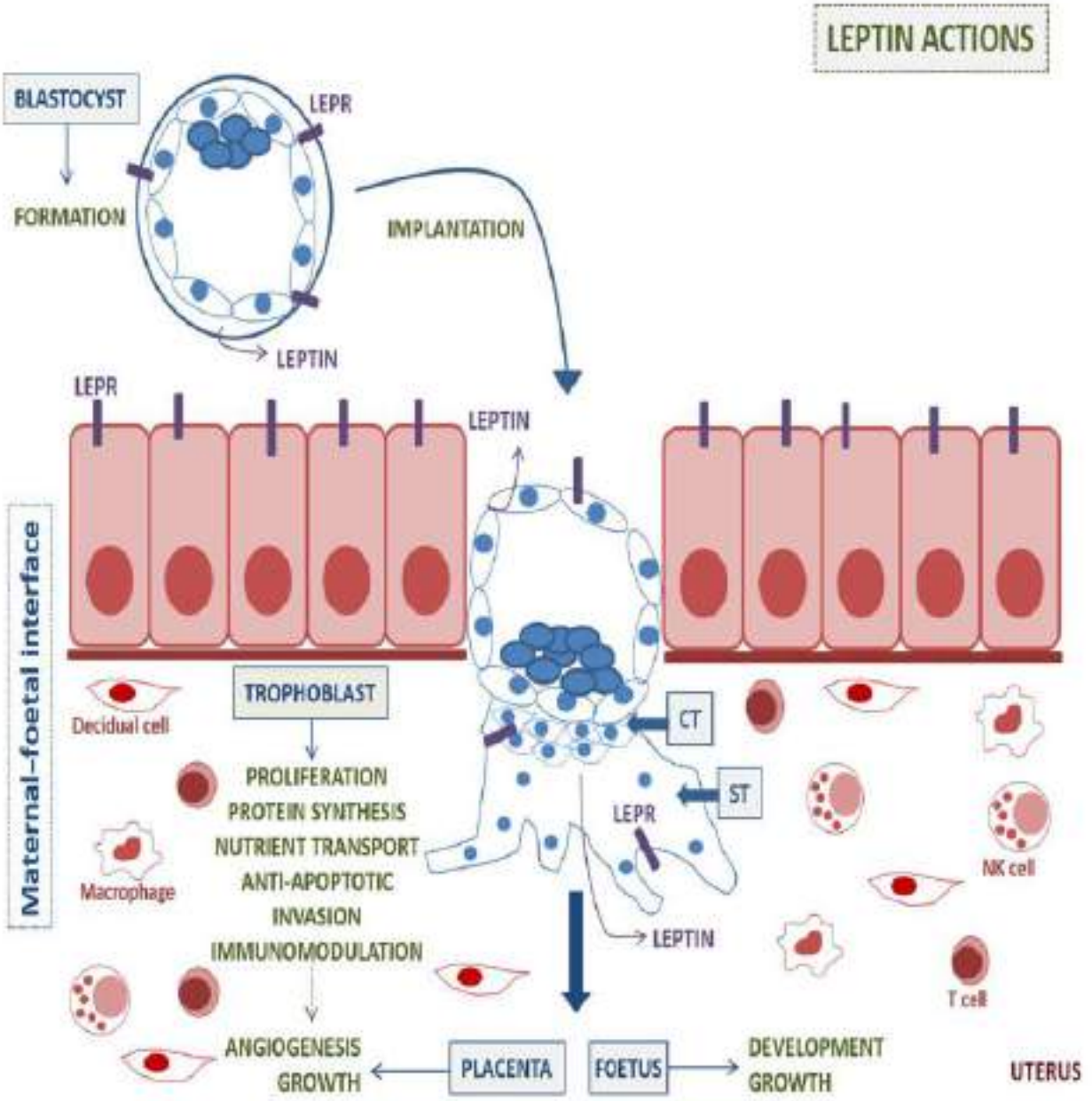

Fig. 1 Leptin action during the first stages of pregnancy. Leptin seems to play a crucial role during the first stages of pregnancy as it modulates critical processes such as implantation and placentation, as well as foetus development. This figure summarizes the most important actions of leptin during early gestation (green), highlighting its role in the maternal–foetal interface. It is also shown the different type of cell which expresses leptin receptor (LEPR) and produce leptin (violet). CT: cytotrophoblast, ST: syncytiotrophoblasts.

activity in many human cell types [54–56], *via* mitogen-activated protein kinase (MAPK) activation [57]. We have demonstrated that leptin promotes proliferation of trophoblast cells by this MAPK pathway [46, 51] and stimulates protein synthesis by the activation of translation machinery in trophoblastic JEG-3 cells, a human placenta choriocarcinoma cell line [47, 58].

In this line, multiple signal transduction pathways are activated in response to leptin both in trophoblastic JEG-3 cell culture and in human term placenta [46]. Leptin receptor requires activation of receptor-associated kinases of the Janus family (JAK) [59]. After ligand binding, JAKs autophosphorylate and tyrosine phosphorylate various signal transducers and activators of transcription (STATs). In this context, leptin is able to stimulate Janus kinase (JAK)–STAT pathway by mainly promoting JAK-2, the most important JAK isoform to mediate physiological effects of leptin [60], and STAT-3 tyrosine phosphorylation in the human placenta choriocarcinoma JEG-3 cell line, as well as in trophoblast cells from human term placenta. STAT-3 activity has been correlated with trophoblast invasiveness [61]. In this context, it is interesting to mention the role of Sam68, an RNA-binding protein originally identified as the substrate of Src kinase during mitosis and a member of the signal transduction and activation of RNA metabolism (STAR) family [62, 63]. Leptin stimulates Tyr-phosphorylation of Sam68 in the trophoblast, mediating the dissociation from RNA, suggesting that leptin signalling could modulate RNA metabolism [64, 65]. Moreover, phosphorylated Sam68 interacts with STAT-3 in response to leptin in trophoblastic JEG-3 cells [66], suggesting that Sam68 seems to play an important role mediating biological function of leptin [66].

In human trophoblastic cells, it has also been demonstrated that leptin induces the phosphorylation of the extracellular signal-regulated MEK and the extracellular signal-regulated ERK 1/2 [46]. Moreover, it is well-established that the ERK pathway is essential for reproduction in general, and for the control of trophoblast penetration and invasion [67], as well as placental development [68].

Besides, leptin activation of phosphatidylinositol 3-kinase (PI3K) pathway has been described in many systems, including placenta [47, 58], leading to phosphorylation of Protein kinase B (PKB), also known as Akt and inactivation of glycogen synthase kinase 3 (GSK-3), as well as the activation of the translation machinery.

In placental villi, cell turnover is tightly regulated, *via* apoptotic cascade [69]. In normal pregnancy, apoptosis is an essential feature of placental development and it is well-established that trophoblast apoptosis increases with placental growth and advancing gestation [70]. Leptin prevents early and late events of apoptosis *via* MAPK pathway [46, 51]. The role of leptin was also studied under different stress conditions such as serum deprivation and hyperthermia. Under serum deprivation, leptin increased the anti-apoptotic B-cell lymphoma 2 (BCL-2) protein expression, while down-regulated the pro-apoptotic BAX and BH3 interacting domain death agonist (BID) proteins expression as well as caspase-3 active form and cleaved poly [ADP-ribose] polymerase 1 (PARP-1) fragment in Swan-71 cells, a first-trimester trophoblast cells isolated from a 7-week normal placenta [71] and placental explants. In addition, it was demonstrated that p53 and its phosphorylation of serine 46 (Ser-46), phosphorylation involved in the selectivity of apoptotic target genes, are down-regulated by leptin suggesting that leptin plays a pivotal role for apoptotic signalling by inhibiting p53 [48]. Recent studies have demonstrated that MAPK and PI3K pathways are necessaries for this anti-apoptotic leptin action and it was also demonstrated that murine double-minute type 2 also known as E3 ubiquitin–protein ligase (MDM-2) expression is regulated by leptin [49]. In placental explants cultured at high temperatures (40°C and 42°C), the extent of Ser-46 phosphorylation of p53 and the expressions of p53-regulated apoptosis-inducing protein 1 (p53AIP1), a potential mediator of apoptosis depending on p53, p21 and Caspase-3 are increased and these effects are significantly attenuated by leptin, indicating that leptin is a pro-survival placental cytokine [50]. Figure 1 highlights the main actions of leptin in the maternal–foetal interface.

Leptin as an immunomodulator during pregnancy

One of the most important placental functions is to prevent embryo rejection by the maternal immune system to enable its correct development [72]. To ensure normal pregnancy, trophoblast differentiation requires potent immunomodulatory mechanisms to prevent rejection of ST and invasive trophoblast by alloreactive lymphocytes and natural killer (NK) cells present in maternal blood and decidua [73]. Inflammatory mediators such as interleukin-1 β (IL-1β), interleukin-6 (IL-6), tumour necrosis factor α (TNFα) and prostaglandins are produced and secreted by the human placenta and these cytokines play an important role in a number of normal and abnormal inflammatory processes, including the initiation and progression of human labour [74–76]. There are several homologies between the expression and regulation of cytokines and inflammation-related genes in the placenta and in the white adipose tissue. In this regard, leptin effects include the promotion of inflammation and the modulation of innate and adaptive immunity [64, 77, 78]. Thus, placental leptin acts as an immune modulator, regulating the generation of matrix metalloproteinases (MMPs), arachidonic acid products, nitric oxide production and T-cell cytokines [76]. Interestingly, leptin expression is also regulated by interleukin-1 α (IL-1α), IL-1β, IL-6 and interferon-ϒ (IFN-ϒ) [44, 79, 80].

It was reported that leptin stimulates IL-6 secretion in human trophoblast cells [81, 82]. In addition, TNFα release from human placenta is also stimulated by leptin, and it was demonstrated that nuclear transcription factor NF-kappa B (NF-κB) and peroxisome proliferator-activated receptor γ (PPAR-γ) are important mediators of this effect [83]. Recently, we have found that leptin induces human leucocyte antigen G (HLA-G) expression in placenta. HLA-G has potent immunosuppressive effects promoting apoptosis of activated CD8+ T lymphocytes, the generation of tolerogenic antigen-presenting cells and the prevention of NK cell-mediated cytotoxicity. These data place leptin as a placental cytokine which confers to trophoblast cells a tolerogenic phenotype to prevent immunological damage during the first steps of pregnancy [84].

Pro-inflammatory leptin actions may also have significant implications in the pathogenesis of various disorders associated with

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pregnancy, such as GDM and PE, which are characterized by increased leptin expression. In this sense, placental leptin may contribute to the incremented circulating levels of pro-inflammatory mediators that are evident in these pregnancy diseases, whereas successful pregnancy is associated with down-regulation of intrauterine pro-inflammatory cytokines [23, 85, 86].

Leptin and pathologies associated with pregnancy

Polycystic ovary syndrome

PCOS, the most common endocrine disorder in females and major cause of anovulatory infertility, affects approximately 15% of women during reproductive ages [32]. It is characterized by hyperandrogenism, chronic oligoanovulation and polycystic ovarian morphology [87]. Peripheral insulin resistance appears to play a crucial role in the pathogenesis of PCOS [88]. However, the aetiology of PCOS is not fully understood yet. The deregulated secretion of adipokines, including leptin, plays a role in the pathogenesis of PCOS [89]. Besides, PCOS could be associated with increased prevalence of gestational disorders such as miscarriage, GDM and PE [90].

Different studies have shown augmented leptin levels in women with PCOS [91–93]. A recent work confirmed that leptin serum concentrations are increased in obese women with PCOS, while adiponectin levels are decreased [94]. Furthermore, the authors suggested the higher leptin levels may be related to the hyperinsulinaemic characteristic of obesity and PCOS [94]. In this sense, as women with PCOS also commonly present overweight and obesity [95], the symptoms mentioned could be a consequence of the hyperleptinaemia due to the gain of fat mass. A recent preliminary investigation proposed leptin as strong biomarker of hyperandrogenic PCOS women, suggesting metabolic and inflammatory biomarkers may be increased in PCOS. Interestingly, offspring from PCOS patients have increased inflammatory markers such as matrix metalloproteinase-9 (MMP-9) and S100 calcium-binding protein A8 or calgranulin A (S100A8), suggesting that these children may exhibit increased chronic low-grade inflammation [96]. In fact, it has been reported that increased leptin concentrations may be correlated with insulin resistance, metabolic disorder, infertility and even cardiovascular disease risk in PCOS, which may contribute to the aetiology and development of PCOS [97]. Elevated leptin levels could be one of the mechanisms underlying insulin-mediated ovarian androgen production, as high leptin levels are associated with elevated testosterone levels [98].

Recurrent miscarriage

Recurrent miscarriage is defined as the loss of three or more consecutives pregnancies before the 20th week of gestation with or without previous live births. Genetic, endocrine, anatomical, immunological, thrombophilic and environmental factors have been implicated in recurrent miscarriage. However, no cause can be found in up to 50% of cases. In patients who have early recurrent miscarriages, some proteins such as human chorionic gonadotrophin (hCG), glycodelin and galectin-1 are down-regulated in the ST. Moreover, animal and human studies indicate that alterations in leptin signalling may increase the risk for pregnancy loss [99].

Serum leptin concentration was found elevated in women with recurrent miscarriage in comparison to control group [100]. However, in women who subsequently miscarried, it was found that at weeks 5–6 and 7–8 plasma leptin concentrations are also lower than women who subsequently had a term birth [101]. In addition, low serum leptin concentrations were observed in women suffering spontaneous miscarriage during the first trimester [102]. However, Tommaselli *et al.* did not find significant differences in maternal serum leptin levels, probably due to the heterogeneity of miscarriage in terms of pathogenesis [103].

Single nucleotide polymorphisms (SNP) of LEPR within domains necessary for receptor activation or the cytoplasmic domains may be associated with impaired signalling capacity. In this line, the A223G polymorphism of LEPR is associated with increased risk of pregnancy disorders like PE [104]. On the other hand, Chin *et al.* [105] did not find a correlation between this polymorphism and recurrent miscarriage. However, other studies have reported that these genetic variants are associated with pregnancy recurrent loss (Table 1) [100, 106].

Gestational diabetes mellitus

GDM is the most common pregnancy metabolic disorder and is defined as the type of glucose intolerance that develops in the second trimester and third trimester of the pregnancy, resulting in hyperglycaemia of variable severity [107]. Aberrant development and functioning of the placenta, including placental overgrowth, have been implicated as important factors that contribute to GDM-associated complications [108, 109]. GDM is associated with a high perinatal morbidity and mortality as well as insulin resistance, hyperinsulinaemia and hyperleptinaemia, and these GDM-associated conditions disturb placental nutrient transport and foetal nutrient supply [110, 111]. It has been found that leptin and leptin receptor expressions are increased in placenta from GDM [23, 85] and, in fact, leptin was proposed as a first-trimester biochemical predictor of GDM [112, 113]. In addition, it was suggested that hyperinsulinaemia may regulate placental leptin production acting as a circulating signal to control foetal homoeostasis [114, 115]. Furthermore, it is though that maternal glucose regulates cord blood leptin levels and this could explain why newborns exposed to GDM have an increased risk of obesity [116]. Comparison of the placental gene expression profile between normal and diabetic pregnancies indicates that increased leptin synthesis in GDM is correlated with higher production of pro-inflammatory cytokines such as IL-6 and TNFα, causing a chronic inflammatory environment that enhances leptin production [117].

Our group has reported that insulin induces leptin expression in trophoblastic cells by increasing leptin promoter activity [118]. It is known that leptin and insulin share several signalling pathways, such

Table 1 LEP and LEPR single nucleotide polymorphisms (SNPs) in pathologies associated with pregnancy

Pathologies associated with pregnancy	SNP	Description
Polycystic ovary syndrome	–	No LEP or LEPR SNPs have been described
Recurrent miscarriage	LEP-2548G/A	GA genotype and G allele are associated with risk of RM
Gestational diabetes mellitus	LEP-2548G/A	A allele is associated with risk of gestational diabetes mellitus (GDM)
Pre-eclampsia	LEP-2548G/A	A allele is associated with PE
	LEPR A223G	G allele is associated with increased risk of severe PE
	LEPRG1019A	GA genotype and G allele are associated with severe PE
	LEPR A668G	A allele is associated with severe PE
Intrauterine growth restriction	–	No LEP or LEPR SNPs have been described

as JAK2/STAT-3, MAPK and PI3K. Moreover, we could demonstrate that in GDM placenta is increased the basal phosphorylation of STAT-3, MAPK 1/3 and PKB, with resistance to a further stimulation with leptin or insulin *in vitro*, suggesting synergistic interaction and a crosstalk between insulin and leptin signalling in human placenta [23].

On the other hand, GDM is associated with increased incidence of polyhydramnios, due to an increase in amniotic fluid volume, suggesting that aquaporins (AQP), such as AQP9 expression could be altered in GDM [119, 120]. Besides, when maternal circulating glucose levels are controlled they have normal amniotic fluid volume. AQP9 is also a transporter for glycerol and may also provide this substrate to the foetus. In this context, we have found that AQP9 messenger RNA (mRNA) and protein expressions are elevated in placentas from women with GDM. These data could suggest that during GDM the overexpression of AQP9, which correlates with higher leptin plasma levels, increments glycerol transport to the foetus and may help to cover the increase in energy needs that occur during this gestational metabolic disorder [121].

Pre-eclampsia

PE is a potentially life-threatening hypertensive disorder affecting ~2–7% of all pregnancies. Approximately 1% of cases are severe, causing stillbirth or the need for extreme preterm delivery. It is characterized by hypertension, systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg after 20 weeks of gestation, and proteinuria. Some of the established risk factors are age younger than 20 years or older than 40 years, primiparity, excess bodyweight of the mother, multifoetal pregnancy and familiar and individual history of PE [122]. Increasing evidence supports that the pathogenesis of PE involves improper placental development, due to dysfunctional proliferation, migration and invasion of CT into the uterus. This leads to inappropriate spiral artery remodelling, decreased placental blood flow and placental hypoxia [123].

Leptin expression is increased in pre-eclamptic placentas, and many studies suggest a positive correlation between elevated serum levels and PE [124–126]. Moreover, it has been shown that leptin concentration is higher in term PE but not in preterm PE [126]. Thus, leptin has been proposed as a link between body mass index and PE, but the role of obesity or leptin in the pathogenesis of PE is not obvious [126]. Leptin up-regulation could be attributed to placental stress, mainly by the hypoxia present in pre-eclamptic placenta. Furthermore, serum leptin levels seem elevated in PE even before the clinical onset of the disease, suggesting a possible prognostic value [127]. In addition, leptin inhibits increased apoptosis of placental cells during PE. Also, as leptin is a potent angiogenic factor, enhanced placental leptin could increase blood supply to the placenta by neovascularization. Furthermore, leptin is involved in the regulation of placental nutrient transporters, suggesting that hyperleptinaemia in PE is a compensatory response to boost nutrient delivery to the underperfused placenta [117].

However, the role of leptin in PE should be evaluated cautiously as it has recently been found no association of leptin levels with PE [128]. SNPs in the LEPR gene have also been investigated in relation to severe PE. In this sense, it was reported that variants of LEPR such as A223G polymorphism may individually modify the risk of severe PE (Table 1) [104].

Intrauterine growth restriction

The failure of arterial remodelling results in malperfusion of the placenta [129]. The incapacity of the placenta to deliver an adequate supply of nutrients to the foetus is termed placental insufficiency and results in IUGR, affecting up to 5–10% of pregnancies in developed countries. IUGR is characterized by a birthweight of <2.5 kg and is associated with a high incidence of perinatal morbidity and mortality and increased risk of cardiovascular and metabolic diseases in adulthood [130]. IUGR represents a period of true foetal malnutrition followed by a period of weight recovery after birth, which leads to changes in adipose tissue with important long-term consequences [131]. Specifically, IUGR is frequently associated with inflammation and infarcts within the villi, implying abnormal villous development [132]. At the same time, several growth factors and signalling molecules have been implicated in IUGR, including vascular endothelial growth factor and leptin [133].

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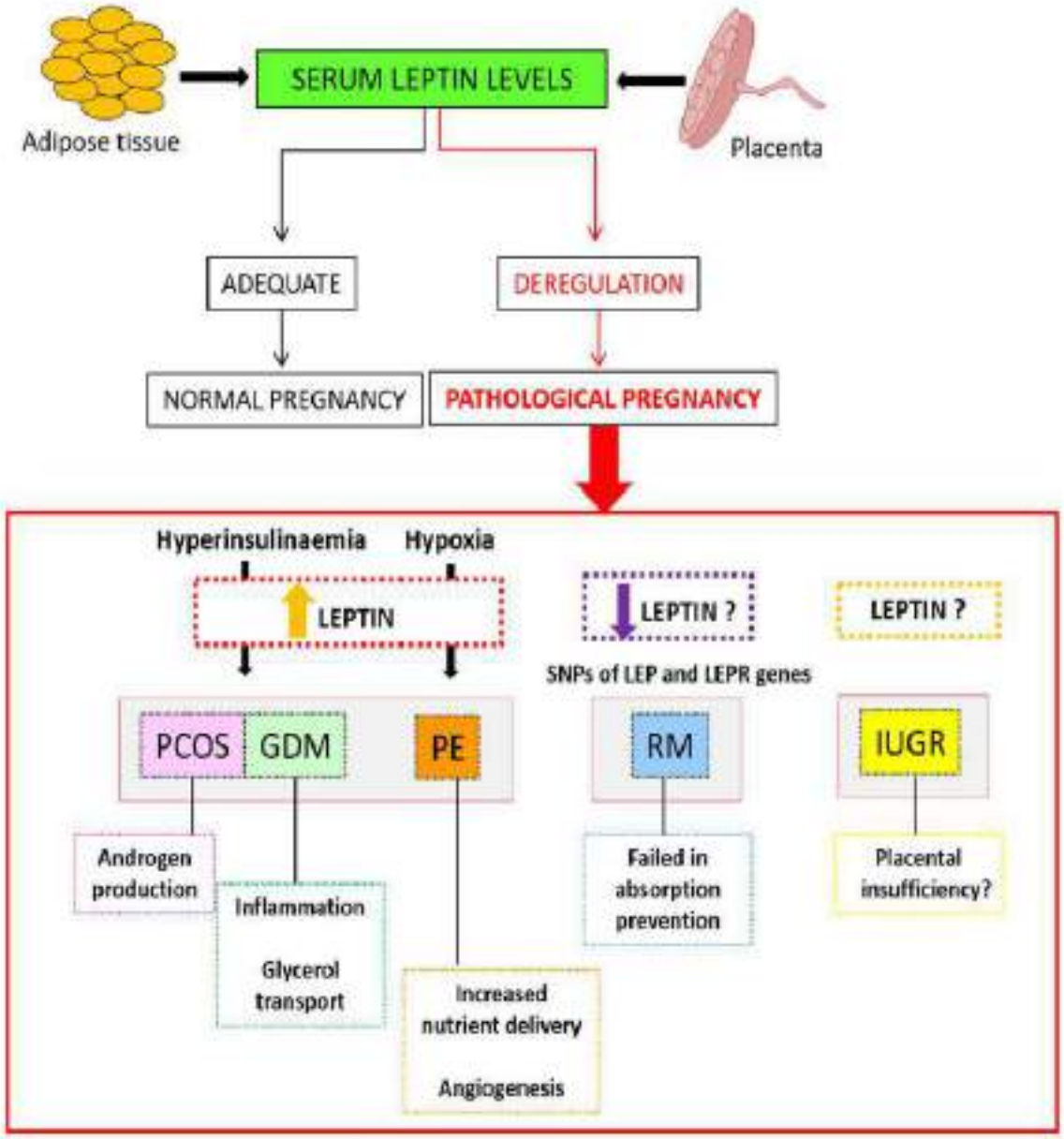

Fig. 2 Leptin association pregnancy disorders. Deregulation of leptin levels has been correlated with the pathogenesis of various disorders associated with reproduction and gestation, including polycystic ovary syndrome (PCOS), recurrent miscarriage (RM), gestational diabetes mellitus (GDM), pre-eclampsia (PE) and intrauterine growth restriction (IUGR). This figure summarizes the link between leptin and the mentioned diseases, including suggested causes and consequences of these pathologies.

Diverse studies demonstrated lower circulating leptin levels in IUGR neonates at birth, due to reduced fat mass and/or lower placental production, suggesting leptin as a growth factor that intervenes during foetal intrauterine development [134, 135]. Compared with normal birthweight controls, leptin levels become higher in IUGR children and adults, suggesting an adaptive leptin resistance beneficial for catch-up growth or an adipocyte dysfunction associated with IUGR [136]. However, other studies have reported that maternal

serum leptin concentrations were significantly higher in pregnancies complicated by foetal growth restriction and growth-restricted foetuses show umbilical cord leptin concentrations lower than those in normal foetuses, suggesting that it could be due to a compensatory mechanism in which small placentas produce more leptin [137]. Simultaneously, it was suggested that placental insufficiency is associated with an increase in placental leptin production [138]. Furthermore, some studies demonstrated that leptin levels are lower in IUGR, but differences were not significant [139, 140], and it was also reported that cord blood leptin levels did not differ significantly in IUGR compared to normal pregnancies [141]. These data suggest that the association between leptin and IUGR is controversial. Finally, it was reported that the mother of foetuses with growth restriction has a body composition pattern characterized by slightly increased fraction of fat mass and increased serum leptin levels [142].

Conclusions

In conclusion, it could be affirmed that leptin plays an integral role in the normal physiology of the reproductive system. Leptin controls reproduction depending on the energy state of the body, and sufficient leptin levels are a prerequisite for the maintenance of reproductive capacity. The present review was focused in placental leptin effects during gestation, when leptin levels are increased due to leptin production by trophoblastic cells. Thus, leptin has a wide range of biological functions on trophoblast cells and a role in successful establishment of pregnancy. In this sense, leptin promotes proliferation, protein synthesis and survival of placental cells. These actions are very important as cell proliferation and apoptotic cascades are critical for the correct placental development and function. Moreover, leptin is involved in the promotion of trophoblast invasion which represent a key event during early pregnancy. Besides, it is suggested an important role of leptin in the regulation of immune mechanisms at the maternal interface.

On the other hand, observational studies have demonstrated that states of leptin overabundance, deficiency or resistance can be associated with abnormal reproductive function. Clinical studies demonstrate an impact of obesity on the risk of infertility, and it is also established that obesity may lead to deregulation in leptin function that results in maternal disease [143]. In this context, leptin deregulation has been implicated in the pathogenesis for at least some disorders associated with reproduction and pregnancy, such as PCOS, recurrent miscarriage, GDM, PE and IUGR. It is well accepted that increased leptin levels are detected in women with PCOS and that may be correlated with insulin resistance, metabolic disorders and infertility. Recurrent miscarriages are associated with altered leptin levels, but the relationship is open for discussion. On the other hand, SNPs of leptin and LEPR genes are risk factors for miscarriage. Leptin and leptin receptor expressions are increased in placentas from GDM, which may be relevant to control foetal homoeostasis. PE is also characterized by enhanced leptin concentrations, even before the clinical onset of the disease, suggesting a possible prognostic significance. Finally, the association between IUGR and leptin levels is controversial. Figure 2 summarizes the link between leptin and the mentioned diseases, including suggested causes and consequences of these pathologies.

Different therapeutic strategies based on leptin administration have been described. Patients with leptin mutations show a marked restoration of fertility as well as weight loss and improvements in immune function after leptin therapy. Furthermore, leptin replacement therapy improves the reproductive abnormalities associated with hypothalamic amenorrhoea (such as failure to menstruate, infertility and premature osteoporosis) [144]. On the other hand, compounds that could reverse leptin resistance and act as leptin sensitizers could be beneficial to treat pathologies associated with hyperleptinaemia [145]. A number of evidence suggested that leptin might have potential as a treatment for diverse pathologies including the malfunctioning of the reproductive system.

Further investigation is needed to fully elucidate the association of leptin with pathological pregnancy and to establish leptin as a biomarker for pathologies associated with pregnancy.

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Conflict of interest

The authors declare no conflict of interest.

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Chapter

Leptin and Gestational Diabetes Mellitus

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Abstract

Emerging research has highlighted the importance of leptin in fetal growth and development, independent of its essential role in the regulation of feeding and energy metabolism. Leptin is now considered an important signaling molecule of the reproductive system, since it regulates the production of gonadotropins, the blastocyst formation and implantation, the normal placentation, as well as the feto-placental communication. Placental leptin is an important cytokine which regulates placental functions in an autocrine or paracrine manner. Leptin seems to play a crucial role during the first stages of pregnancy as it modulates critical processes like proliferation, protein synthesis, invasion, and apoptosis in placental cells. Furthermore, deregulation of leptin levels has been correlated with the pathogenesis of various disorders associated with reproduction and gestation, including gestational diabetes mellitus (GDM). Due to the relevant incidence of the GDM and the importance of leptin, we decided to review the latest information available about leptin action in normal and GDM pregnancies to support the idea of leptin as an important factor and/or predictor of diverse disorders associated with reproduction and pregnancy.

Keywords: leptin, reproduction, placenta, GDM, microRNAs

1. Introduction

Adipose tissue acts as an endocrine organ, secreting different molecules or adipokines. A link between body weight, adipokines, and success of pregnancy has been proposed, although it is not fully understood [1]. Leptin was the first adipokine claimed to be the "missing link" between fat and reproduction [2]. Leptin is considered as a pleiotropic hormone that regulates not only body weight but also many other functions, including the normal physiology of the reproductive system [3]. Importantly, this hormone is also produced by other tissues, especially placenta [4].

Placental formation during human gestation is crucial for embryonic progress and successful pregnancy outcome, allowing metabolic exchange and producing steroids, hormones, growth factors, and cytokines that are critical for the maintenance of pregnancy [5, 6]. Trophoblast cells play an essential role in the development of placenta. These cells differentiate in two distinct types: extravillous and villous trophoblast. In the extravillous pathway, cytotrophoblasts proliferate,

differentiate into an invasive phenotype, and penetrate in the maternal decidua and myometrium. Meanwhile, in the villous pathway, mononuclear cytotrophoblasts fuse to form a specialized multinuclear syncytium called syncytiotrophoblast [7]. In normal pregnancy, trophoblast invasion is a critical step in remodeling the maternal spiral arteries to adequately perfuse the developing placenta and fetus [8]. In this sense, deregulation of leptin levels has been implicated in the pathogenesis of gestational diabetes mellitus (GDM) [9].

2. Leptin and reproduction

Reproductive function depends on the energy reserves stored in the adipose tissue. The large energy needs for a hypothetical pregnancy was the original rationale to explain the disruption of reproductive function by low fat reserves. This led to the hypothesis of an endocrine signal that conveys information to the brain about the size of fat stores [10]. Thus, leptin was the first adipokine claimed to be the "missing link" between fat and reproduction [2]. Leptin modulates satiety and energy homeostasis [11, 12] but is also produced by the placenta. Thus, it was suggested that the effects of placental leptin on the mother may contribute to endocrine-mediated alterations in energy balance, such as the mobilization of maternal fat, which occurs during the second half of pregnancy [13, 14]. In addition, leptin has been found to influence several reproductive functions, including embryo development and implantation [15]. Moreover, animal models have demonstrated that leptin-deficient mice are subfertile and fertility can be restored by exogenous leptin [16]. This adipokine may therefore play a critical role in regulating both energy homeostasis and the reproductive system [17].

Leptin increments the secretion of gonadotropin hormones, by acting centrally at the hypothalamus [18]. In addition, because leptin has been shown to be influenced by steroid hormones and can stimulate LH release, leptin may act as a permissive factor in the development of puberty [19].

Leptin can also regulate ovary functions [20–23]. Thus, leptin resistance and hyperleptinemia in obesity lead to altered follicle function, whereas in conditions in which nutritional status is suboptimal, leptin deficiency results in hypothalamic-pituitary gonadal axis dysfunction [24, 25].

In addition, a significant role of leptin in embryo implantation was proposed. Leptin receptor (LEPR) is specifically expressed at the blastocyst stage [26], and it was also reported that leptin is present in conditioned media from human blastocysts, promoting embryo development, suggesting a function in the blastocyst-endometrial dialog [27].

3. Leptin and placenta

The implantation involves complex and synchronized molecular and cellular events between the implanting embryo and uterus, and these events are regulated by autocrine and paracrine factors [5]. Fetal growth depends on the ability of the placenta to supply nutrients adequate to meet fetal demand, which increases as gestation progresses. Villous cytotrophoblast is a progenitor cell population that produces daughter cells to support the expansion of the syncytium as placental surface area increases as well as the expansion of cytotrophoblast columns, which contain the cells destined to invade maternal decidua [28]. The placenta grows exponentially in the first and early second trimester, but growth has slowed down by term [29]. Therefore, placental growth, especially in early gestation, is a

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prerequisite of a high-capacity transport interface. In 1997, leptin was described as a new placental hormone in humans [14]. In fact, during pregnancy, circulating leptin levels are also increased due to leptin production by trophoblastic cells [30]. After delivery, leptin levels return to normal levels [31].

To alter intracellular signaling and function, leptin must bind to the receptor (LEPR) [32]. There are six different isoforms of LEPR (a–f) that are produced by alternative RNA splicing [33]. The only isoform that has a transmembrane domain that is capable of activating signal transduction pathways is LEPRb, whereas the other five short LEPR isoforms have either a truncated or no transmembrane domain and are unable to activate signaling pathways [33]. Activation of LEPRb results in an upregulation of a number of signal transduction pathways, including the Janus kinase/signal transducers and activators of the transcription pathway (JAK/STAT), as well as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways [34]. Research findings do indicate that there may be fetal-to-maternal leptin exchange across the placenta [35]. However, to date, it is not known which receptor is mediating this transportation.

Leptin has physiological effects on the placenta, including angiogenesis, growth, and immunomodulation [13]. Leptin is now considered an important regulator during the first stages of pregnancy, modulating proliferation, invasion, apoptosis, and protein synthesis, in placenta [36–41].

The control of cell proliferation is critical for a correct placental development, and it is finely regulated [42]. Altered rates of cytotrophoblast proliferation are associated with different pathologies; levels are enhanced with increased fetal growth (macrosomia) and diminished in fetal growth restriction [42]. Others factors in maternal circulation might coordinately stimulate proliferation, differentiation, and survival [43, 44] through the activation of multiple kinases [43–45] and phosphatases [45].

During placentation, cytotrophoblasts and syncytiotrophoblast keep a subset of cells in direct contact to the villous basement membranes. In the extravillous compartment, cell proliferation favors the invasion of the uterine stroma. Similarly, in the villous compartment, cells undergo syncytial fusion directed by specific transcription factors [46].

The role of MAPK in regulating trophoblast turnover is well documented in both human and animal systems [43, 44, 47]. Moreover, it was shown that leptin induces proliferative activity in many human cell types [48–50], mainly via MAPK activation [51]. We have demonstrated that leptin promotes proliferation of trophoblast cells by this MAPK pathway [41, 52]. We have also found that leptin dose-dependently stimulates protein synthesis by the activation of translation machinery [36, 53].

In this context, it is interesting to mention the role of Sam68, an RNA-binding protein originally identified as the substrate of Src during mitosis and a member of the signal transduction and activation of RNA metabolism (STAR) family [54, 55]. Leptin stimulates Tyr-phosphorylation of Sam68 in the trophoblast, mediating the dissociation from RNA, suggesting that leptin signaling could modulate RNA metabolism [48, 56]. Recent data indicate that microRNAs have a fundamental role in a variety of physiological and pathological processes. In this context, studies of microRNA expression have revealed that some microRNAs are abundantly expressed in the placenta [57]. However, the signature of miRNAs in the placenta has yet to be elucidated.

In placental villi, cell turnover is tightly regulated, via apoptotic cascade [49]. In normal pregnancy, apoptosis is an essential feature of placental development, and it is well stablished that trophoblast apoptosis increases with placental growth and advancing gestation [50]. Leptin prevents early and late events of apoptosis via MAPK pathway [41, 52]. The role of leptin was also studied under different stress

conditions like serum deprivation, hyperthermia, and acidic stress [39, 40]. Under serum deprivation, leptin increased the anti-apoptotic BCL-2 protein expression, while it downregulated the pro-apoptotic BAX and BID proteins expression as well as caspase-3 active form and cleaved PARP-1 fragment in Swan-71 cells and placental explants. In addition, it was demonstrated that p53 and its phosphorylation in Ser-46 are downregulated by leptin suggesting that leptin plays a pivotal role for apoptotic signaling by p53 [37]. Recent studies have demonstrated that MAPK and PI3K pathways are necessary for this anti-apoptotic leptin action, and it was also demonstrated that MDM-2 expression is regulated by leptin [38]. In placental explants cultured at high temperatures (40 and 42°C) and a pH acid (<7.3), the expression of Ser-46 p53, p53AIP1, p21, and caspase-3 is increased, and, these effects are significantly attenuated by leptin, indicating that leptin is a pro-survival placental cytokine [39, 40].

4. Leptin and immune system in placenta

One of the most important placental functions is to prevent embryo rejection by the maternal immune system to enable its correct development [51]. To ensure normal pregnancy, trophoblast differentiation requires potent immunomodulatory mechanisms to prevent rejection of syncytiotrophoblast and invasive trophoblast by alloreactive lymphocytes and natural killer (NK) cells present in maternal blood and decidua [58]. Inflammatory mediators such as IL-6, IL-1β, TNFα, and prostaglandins are produced and secreted by the human placenta, and these cytokines play an important role in a number of normal and abnormal inflammatory processes, including the initiation and progression of human labor [59–61]. There are several homologies between the expression and regulation of cytokines and inflammation-related genes in the placenta and in the white adipose tissue. In this regard, leptin effects include the promotion of inflammation and the modulation of adaptive and innate immunity [56, 62, 63]. Thus, placental leptin acts as an immune modulator, regulating the generation of matrix metalloproteinases, arachidonic acid products, nitric oxide production, and T cell cytokines [61]. Interestingly, leptin expression is also regulated by IL-6, IL-1α, IL-1β, and IFN-Ɣ [31, 64, 65].

It was reported that leptin stimulates IL-6 secretion in human trophoblast cells [66, 67]. In addition, TNFα release from human placenta is also stimulated by leptin, and it was demonstrated that NF-κB and PPAR-γ are important mediators of this effect [68]. Recently, we have found that leptin induces HLA-G expression in placenta. HLA-G has potent immunosuppressive effects promoting apoptosis of activated CD8+ T lymphocytes, the generation of tolerogenic antigen-presenting cells, and the prevention of NK cell-mediated cytotoxicity. These data place leptin as a placental cytokine which confers to trophoblast cells a tolerogenic phenotype to prevent immunological damage during the first steps of pregnancy [69].

Pro-inflammatory leptin actions may also have significant implications in the pathogenesis of various disorders during pregnancy, such as GDM, which is characterized by increased leptin expression. In this sense, placental leptin may contribute to the incremented circulating levels of pro-inflammatory mediators that are evident in these pregnancy diseases, whereas successful pregnancy is associated with downregulation of intrauterine pro-inflammatory cytokines [9, 70, 71].

5. Leptin and gestational diabetes mellitus

Gestational diabetes mellitus, characterized by glucose intolerance diagnosed during pregnancy, is one of the most common complications in pregnancy and

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affects 3–8% of all pregnancies [72, 73]. The prevalence of GDM has increased in recent decades due to increased average age of pregnant females and increased risk of obesity [74]. However, GDM is associated with numerous complications including macrosomia, neonatal metabolic disorders, respiratory distress syndrome, and neonatal death as well as a predisposition for the development of metabolic syndromes and typ. 2 diabetes [75, 76].

The placenta is thought to have a critical role in the pathogenesis of gestational diabetes mellitus, as GDM-associated complications resolve following delivery. Therefore, abertrant development and functions of the placenta, including placental overgrowth, have been implicated as important factors that contribute to GDM-associated complications [77, 78]. GDM is associated with insulin resistance, hyperinsulinemia, and hyperleptinemia, and these GDM-associated conditions disturb placental nutrient transport and fetal nutrient supply [79, 80].

It has been found that leptin and LEPR expressions are increased in placenta from GDM [9, 70], and, in fact leptin was proposed as a first-trimester biochemical predictor of GDM [81, 82]. In addition it was suggested that hyperinsulinemia may regulate placental leptin production acting as a circulating signal to control fetal homeostasis [73, 83]. Furthermore, it is thought that maternal glucose regulates cord blood leptin levels, and this could explain why newborns exposed to GDM have an increased risk of obesity [84]. Comparison of the placental gene expression profile between normal and diabetic pregnancies indicates that increased leptin synthesis in GDM is correlated with higher production of pro-inflammatory cytokines such as IL-6 and TNFα, causing a chronic inflammatory environment that enhances leptin production [85].

Our group has reported that insulin induces leptin expression in trophoblastic cells by increasing leptin promoter activity [86]. It is known that leptin and insulin share several signaling pathways, such as JAK2/STAT-3, MAPK, and PI3K. Moreover, we could demonstrate that in GDM, the basal phosphorylation of STAT-3, MAPK 1/3, and Akt is increased in the placenta, with resistance to a further stimulation with leptin or insulin in vitro, suggesting synergistic interaction between insulin and leptin signaling and action in human placenta [9].

On the other hand, GDM is associated with increased incidence of polyhydramnios, due to an increase in amniotic fluid volume, suggesting that aquaporins (AQP), such as AQP9 expression, could be altered in GDM [87, 88]. Besides, when maternal circulating glucose levels are controlled, they have normal amniotic fluid volume. AQP9 is also a transporter for glycerol and may also provide this substrate to the fetus. In this context, we have found that AQP9 mRNA and protein expressions are overexpressed in placentas from women with GDM. These data could suggest that during GDM the overexpression of AQP9, which correlates with higher leptin plasma levels, increments glycerol transport to the fetus which may help to cover the increase in energy needs that may occur during this gestational metabolic disorder [89].

Nevertheless, even though any nutritional or lifestyle intervention aimed to reduce weight produce a decrease in leptin levels, both in gestational diabetes and in general population, no therapeutic intervention, using leptin as a pharmacological target, has so far been used in the management of gestational diabetes.

6. Leptin and microRNAs

Gene expression can be regulated by short (18–22-nucleotide) noncoding RNAs, microRNAs, derived from long primary transcripts (pre-microRNAs) through sequential processing by two enzymes, Drosha and Dicer, and then incorporated

into the RNA silencing complex, where they target homologous mRNAs. In mice, loss or inactivation of Dicer leads to multiple developmental defects [90, 91], and it has been demonstrated that in human placenta, cytotrophoblast proliferation is increased following Dicer [92]; however, the individual microRNAs responsible for these effects are unknown. In silico network analysis identified microRNAs (miR-145 and let-7a) that influence the expression of components of nodal signaling pathways. The large network is bridged by nodal molecules, such as mitogen-activated protein kinase (MAPK1/2), and AKT, which are recognized components of pro-mitogenic signaling pathways [20]. In fact, the role of MAPK1/2 in regulating trophoblast turnover is well documented in both human and animal systems [43, 44, 47]. In this context, we have reported an increased activation of MAPK 1/2 in response to leptin in trophoblastic cells from the human placenta. Thus, it is tempting to speculate that altered microRNAs expression influences the leptin expression and contributes to the pathogenesis of the GDM. However, the signature of microRNAs in the leptin expression in the placenta both in normal pregnancy and GDM remains to be elucidated. Therefore, it will be interesting to determine, in future studies, the combined role of these microRNAs in the leptin expression in normal placenta and in placenta from pregnancy pathology associated with altered placental growth (e.g., GDM) in order to clarify the regulation of placental growth by leptin.

7. Leptin in fetal development

Obesity is associated with significantly elevated plasma leptin concentrations due to an increase in white adipose tissue compared with healthy individuals [93]. As obesity rates are increasing rapidly in the Western world, so is increasing the number of obese women who become pregnant. Importantly, obese pregnant women have significantly elevated plasma leptin concentrations compared with nonobese pregnant women throughout pregnancy [94]. Even though no differences in placental leptin production has been shown, there is a downregulation of LEPRb expression in the placenta of obese mothers, which would cause placental leptin resistance (in addition to the central leptin resistance that occurs during normal pregnancy) that may be attempting to modulate fetal growth under high-energy conditions [95, 96]. Despite the complications associated with pregnancies in obese women, the offspring may be growth restricted, normal weight, or macrosomic. However, after birth, babies born from obese mothers are exposed to elevated leptin concentrations in the maternal milk [97], which suggests that the postnatal environment may increase infant growth and development, increasing the risk of developing a number of diseases in adulthood. Therefore, alterations in maternal-placental-fetal leptin exchange may modify the development of the fetus and contribute to the increased risk of developing disease in adulthood.

8. Conclusions

In conclusion, it could be affirmed that leptin controls reproduction depending on the energy state of the body and sufficient leptin levels are a prerequisite for the maintenance of reproductive capacity. The present review was focused in placental leptin effects during gestation, when leptin levels are increased due to leptin production by trophoblastic cells. Thus, leptin has a wide range of biological functions on trophoblast cells and a role in successful establishment of pregnancy. In this sense, leptin promotes proliferation, protein synthesis, and survival of placental

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cells. These actions are very important since cell proliferation and apoptotic cascades are critical for the correct placental development and function. Moreover, an important role of leptin in the regulation of immune mechanisms at the maternal interface has been suggested.

Observational studies have demonstrated that states of leptin overabundance or resistance can be associated with GDM. Moreover, it is also established that obesity may lead to deregulation in leptin function that results in maternal disease and clinical studies demonstrate an impact of obesity with an increased risk of a number of diseases in adulthood, including metabolic disease. In this context, leptin deregulation has been implicated in the pathogenesis of GDM. It is well accepted that leptin and LEPR expressions are increased in placentas from GDM, which may be relevant to control fetal homeostasis. Moreover, a role for microRNAs in the regulation of placental growth has been suggested, and expression profiling in the studies has shown expression and gestational changes in microRNA levels that demand functional evaluation. Further investigation is needed to fully elucidate the association of leptin with GDM and to stablish leptin as a biomarker for this pathology or the development of microRNA-based approaches to therapeutic targeting for correcting the abnormal placental growth and cell turnover seen in GDM.

Disclosure of interests

The authors declare no conflict of interest.

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CHAPTER FOURTEEN

Aquaporins and placenta

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Contents

Abstract

Water is the major component of cells and tissues. The fetal body consists of about 70 90% water and its fluid balance is dependent on the mother. In fact, abortion, premature birth, amniotic fluid volume abnormality, malformation and fetal growth restrictions might result when the homeostasis of the maternal-fetal fluid exchange is disrupted. Thus, maternal-fetal fluid balance is critical during pregnancy. In this sense, several mechanisms, including aquaporins (AQPs) have been reported to play important roles in maternal-fetal fluid balance. AQPs are small membrane proteins (about 30 kDa), present in different organs, that increase the permeability of water, as well as other small uncharged molecules to be transported across the bilayer cell membranes. Several aquaporins are expressed in placenta, and aquaporins play key roles in the placental function. Even though aquaporins have a proven crucial role in water homeostasis, the physiological and pathological importance of aquaporins as glycerol channels is not fully understood. This review focuses on advances in our knowledge of the roles of aquaporins in placental cells, particularly the roles of AQP3 and AQP9 in placental metabolism and points to the pathophysiological importance of glycerol channels in placenta, as well as the signal transduction pathways activated by them. Moreover, the regulation of aquaporins expression by different placental hormones, such as leptin and the mechanisms involved will be discussed.

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1. Introduction

Aquaporins (AQPs) are a class of membrane water channels, with a wide range of distribution in different organs (Gonen & Walz, 2006) and whose primary function is to facilitate the passive transport of water across the plasma membrane of the cell in response to osmotic gradients (Carbrey & Agre, 2009). It is well known that AQPs not only increase cell membrane water permeability (5- to 50-fold) as compared with membranes in which water moves primarily through the lipid bilayer, but also facilitate the permeability to uncharged small molecules, such as glycerol (Fu et al., 2000), urea and ammonia (Jelen et al., 2012; Musa-Aziz, Chen, Pelletier, & Boron, 2009). In this sense, 13 AQPs isoforms have been identified in humans, many of which have a broad tissue distribution (Gonen & Walz, 2006) and each one has a unique cellular and subcellular distribution with little overlap between homologs. AQPs are subcategorized as "classical aquaporins," the most numerous (AQP0, 1, 2, 4, 5, 6 and 8), that are selective only for water, "aquaglyceroporins" (AQP3, 7, 9 and 10) which enable the transport of water, glycerol and urea, although AQP9 also facilitates the flux of some solutes such as monocarboxylates, purines and pyrimidines (Ishibashi, 2009; Tsukaguchi, Weremowicz, Morton, & Hediger, 1999), and finally, "super-aquaporins" (AQP11 and 12) which are localized in the cytoplasm and whose permeabilities have not yet been fully determined (Ishibashi, 2009). In addition to the transport of water and glycerol, AQPs could potentially transport gasses such as CO_2, ammonia and nitric oxide (NO), as well as larger polar solutes such as sugars, hydrogen peroxide and even some ions (Bienert et al., 2007; Fu et al., 2000; Hara-Chikuma et al., 2012; Herrera, Hong, & Garvin, 2006; Musa-Aziz et al., 2009; Sui, Han, Lee, Walian, & Jap, 2001; Tsukaguchi et al., 1999; Yool & Weinstein, 2002), although, some evidences are conflicting. On the other hand, it is not known whether the transport of molecules other than water and glycerol is biologically important.

AQPs are involved in a variety of physiological and cellular functions, such as peritoneal dialysis (Ni et al., 2006), pleural fluid transport (Song, Yang, Matthay, Ma, & Verkman, 2000), intraocular pressure and aqueous fluid production (Zhang, Vetrivel, & Verkman, 2002), corneal endothelium fluid transport (Kuang et al., 2004), brain edema and neuroexcitation. Moreover, many authors have reported the expression of AQPs (particularly AQP1) in the human placenta and fetal membranes suggesting that AQPs

may contribute to amniotic fluid volume regulation (Beall et al., 2007). In this sense, the developing fetus requires large volumes of water, which are obtained from the maternal circulation through the placenta. Placental function and the maternal circulatory system are intrinsically dependent upon one another, and perturbations in placental function are likely to influence the maternal circulation and vice versa (Damiano, 2011). Of note that human placenta is hemomonochorial, in which the syncytiotrophoblast, a single layer of polarized epithelium separates the maternal and fetal circulation (Stulc, 1997). Formation of this interface relies on coordinated interactions among transcriptional, epigenetic, and environmental factors (Maltepe, Bakardjiev, & Fisher, 2010). The diversity of functions performed by the placenta is very broad, ranging from anchoring the embryo to preventing its rejection by the maternal immune system to enabling the transport of nutrients, gasses, water, ions, and waste products between mother and embryo (Maltepe et al., 2010). Therefore, placenta is a key organ for supporting fetal growth and as the water is transported to the fetus from the maternal circulation across the placenta and amnion, the placental water transport is essential for fetal water acquisition. However, the actual mechanisms of the regulation of placenta water transfer and intramembranous reabsorption are poorly understood. Both phenomena involve the flow of water across biological membranes, which is a function of membrane water channels (Beall et al., 2007). In this sense, it has been demonstrated the expression of AQPs throughout pregnancy in placenta and uterus. More specifically, of the 13 known mammalian AQP genes, (AQP1, AQP3, AQP8, AQP9, and AQP11), 5 of them are known to be expressed in the human amnion (Damiano, 2011; Mann, Ricke, Yang, Verkman, & Taylor, 2002; Mobasheri, Wray, & Marples, 2005; Wang, Chen, Beall, Zhou, & Ross, 2004; Wang, Kallichanda, Song, Ramirez, & Ross, 2001), and each AQP may have a different function with specific roles in transport. For instance, AQP1 and AQP3 likely play a dominant role in passive water movement across the amnion. In this line, gene expression of AQP1 and AQP3 and their protein levels are increased in the human amnion. Other observations suggest that AQP8 and AQP9 water channels are likely fundamental to the regulation of fetal water and solutes flow both in intramembranous absorption and in placental water transfer from mother to fetus (Damiano, 2011; Wang et al., 2004, 2001). However, the role of AQPs in regulating amniotic fluid volume is poorly understood and further studies are needed to compare the relative water conductance of individual AQP water channels in the amnion.

Placenta has the particularity of being a temporary organ in continuous change, both in its macroscopic characteristics and in relation to the expression of different molecules with hormonal or transport functions. In fact, dynamic changes in fetal fluid accumulation and fetal weight, as well as placental weight, take place during gestation (Bazer, Thatcher, Martinat-Botte, & Terqui, 1988; Wu, Bazer, Hu, Johnson, & Spencer, 2005). In this regard, placenta and its associated extraembryonic membranes express also AQP in a spatial and temporal manner with higher mRNA levels for AQP1, 3, 9, and 11, but lower mRNA levels for AQP4, 5, and 8, in chorionic villi in the early stages of human pregnancy (10–14 weeks of gestation) (Escobar et al., 2012).

In humans, with the progression of placentation, two pathways of cytotrophoblast differentiation lead to the formation of two distinct phenotypes. In the villus, cytotrophoblast cells undergo cellular fusion and differentiation to form syncytiotrophoblast, while the extravillous trophoblast proliferates and migrates into the decidua, remodeling the pregnant endometrium (Malassiné & Cronier, 2002). In this sense, the AQP1 mRNA is detected in placental blood vessels and in the syncytiotrophoblast (Stulc, 1997), while AQP3 in trophectoderm (Zhu et al., 2009). Moreover, both AQP1 and AQP3 have been identified in amnion epithelium and chorionic cytotrophoblast cells (Zhu et al., 2009). Wang et al. further demonstrated the presence of AQP3 protein in human placental syncytiotrophoblast and cytotrophoblast cells of the chorionic and amnion epithelium (Wang, Amidi, Beall, Gui, & Ross, 2006). Conversely, AQP4 expression decreased in the syncytiotiophoblast, but increased in endothelial cells and stroma of placental villi between the first and the third trimesters of gestation (De Falco et al., 2007). These data demonstrate a distinct expression profile of AQPs at different stages of gestation in women and suggest that the regional and temporal regulation of AQPs plays important roles in normal pregnancy, fetal growth, and homeostasis of amniotic fluid volume. Therefore, the correct placental morphogenesis underpins pregnancy success and offspring phenotype through tight regulation of transport of nutrients, gasses and waste between the mother and fetus. That is why, the control of cell proliferation and apoptosis are critical for a correct placental development, which is a process finely regulated (Genbacev & Miller, 2000). In this context, it is well known that the role of leptin as a multifunctional hormone that regulates not only body weight homeostasis but also implantation and placentation in pregnancy (Chehab, 2014; Friedman & Halaas, 1998). Leptin promotes growth, proliferation and cell survival of trophoblastic cells

(Pérez-Pérez et al., 2008) by activating MAPK, and PI3K signaling pathways. Intriguingly, these signal transduction pathways as well as pathways that involve cyclic adenosine monophosphate (cAMP), and protein kinase C (PKC), are also known to regulate AQPs expression in animals in a cell-specific manner (Zhang, Ding, Shen, Wu, & Zhu, 2012). Moreover, expression of different AQPs can be regulated by distinct MAPK signal transduction pathways within the same cell. More specifically, the cAMP-PKA-dependent pathway plays a role in regulating expression of AQP1, 3, 8, and 9 in the placenta and fetal membranes (Belkacemi, Beall, Magee, Pourtemour, & Ross, 2008; Zhang et al., 2012). Moreover, it has been reported the regulation of aquaglyceroporins (AQP3, 7, and 9) through the PI3K/Akt/mTOR pathway in placental cells (Rodríguez Catalán, Gómez-Ambrosi, García-Navarro, et al., 2011), suggesting that, even though the physiological and pathological relevance of these aquaglyceroporins (as glycerol channels) is not fully understood, the highly expression in placenta seem to indicate an important role in the homeostasis of metabolism.

There are also several pregnancy molecules and hormones, commonly increased in the course of pregnancy, that involved in leptin up-regulation in the placenta. For example, leptin expression has been shown to be up-regulated by different pregnancy hormones, such as chorionic gonadotrophin, and 17 beta-estradiol, and by second messengers, such as cyclic adenosine 5′-monophosphate, mediated also through MAPK and PI3K signaling pathways (Gambino, Maymo, et al., 2012; Gambino, Perez, et al., 2012; Maymo et al., 2010, 2011, 2012, 2009). Consistent with these observations, high expression levels of AQPs by the same pregnancy molecules and steroid hormones has been also observed in placenta. For example, a concentration-dependent effect of human chorionic gonadotrophin (hCG) is to increase the abundance of AQP9 protein in human placentae (Marino, Castro-Parodi, Dietrich, & Damiano, 2010). Moreover, AQP1 expression in trophoblast-like cells is up-regulated by both arginine vasopressin and cAMP agonists, suggesting that modulation of AQP1 expression by maternal hormones may contribute to fetal-placental-amnion water homeostasis during gestation (Belkacemi, Beall, Magee, Pourtemour, & Ross, 2008). Therefore, all together, suggests that modulation of AQPs expression by maternal hormones contributes to fetal-placental-amnion water homeostasis during gestation. In fact, insulin and leptin regulate expression of AQP3 and AQP9 through the PI3K/Akt/mTOR pathway in placental cells. Moreover, exposure to insulin and leptin might increase the abundance of several AQPs,

such as AQP3, which might facilitate their migration via PI3K-dependent and independent mechanisms (Cao et al., 2013). In fact, recently, unexpected cellular roles of AQPs have been reported, including proliferation, apoptosis, and cell migration (Verkman, 2011). AQP-dependent cell migration, in which the polarization of AQPs to the leading edge of migrating cells facilitates the entry of water into extending lamellipodia in the direction of cell movement, is one mechanism that has been proposed to facilitate AQP1-dependent tumor angiogenesis and metastasis (Papadopoulos, Saadoun, & Verkman, 2008). Moreover, it has been shown that AQP facilitates the proliferation of some types of tumor cells through mechanisms that may involve altered signaling pathways (Di Giusto et al., 2012; Hara-Chikuma & Verkman, 2008; Jung, Park, Jeon, & Kwon, 2011; Zhang et al., 2010). This is important as trophoblastic cells express an invasive phenotype during implantation.

In placental villi, cell turnover is tightly regulated, via apoptotic cascade (Heazell et al., 2008). In normal pregnancy, apoptosis is an essential feature of placental development and it is well-established that trophoblast apoptosis increases with placental growth and advancing gestation (Sharp, Heazell, Crocker, & Mor, 2010). The intracellular K+ depletion generate an osmotic gradient that drives water out of the cell, which, finally, activate the apoptotic caspases (Chen et al., 2008). Regulators of apoptosis are now considered to have a major role in maintaining the integrity of villous trophoblast (Heazell et al., 2008). In fact, it has been suggesting a role central of leptin as a paracrine/autocrine signal, which could be an endogenous component of the proliferation/apoptosis regulatory machinery of trophoblast cells via MAPK pathway (Pérez-Pérez et al., 2008, 2009). Intriguingly, AQPs, stimulated by leptin, might mediate the loss water and subsequent cell shrinkage in the apoptotic cells (Jablonski, Webb, McConnell, Riley, & Hughes, 2004). Particularly, it has been demonstrated that only AQP3 might be important in the regulation of villous trophoblast apoptosis and consequently, an abnormal expression of this protein could alter this tightly regulated process (Szpilbarg et al., 2016). Moreover, as mentioned above, in early pregnancy, a moderately hypoxic environment is crucial for appropriate embryonic development since normal proliferation and differentiation of trophoblastic cells may be driven by low-oxygen concentration in the decidua (Castro-Parodi et al., 2013; James, Stone, & Chamley, 2006). Oxygen enrichment of fetal blood is promoted by partial pressure differences in the feto-maternal circulation and, for example, when PCO2 is incremented or the pH is reduced, hemoglobin affinity for oxygen decreases producing

acidosis. Thus, an adequate exchange of oxygen and carbon dioxide by placenta is crucial to manage acid-base balance within a narrow pH, so, acidic environment could impair the correct development of the fetus as well as the anatomy and function of the placenta (Avagliano et al., 2015; Bobrow & Soothill, 1999). For example, failures in pHi homeostasis could alter various critical cellular functions such as water movements and cell volume regulation. In this respect, aquaporins are also key regulators of cell volume and intracellular ions. Five AQPs are expressed in the human placenta, although their functional significance remains unclear (Damiano, 2011). Possibly, changes in pHi by modifying might alter the selectivity of placental AQPs to water. Therefore, syncytiotrophoblast acid/base balance is necessary to maintain optimal solute transport rates across the placenta. However, the actual mechanisms of the regulation of acid/base balance by AQPs are poorly understood.

The developing fetus requires large volumes of water, which are obtained from the maternal circulation through the placenta. Once in the conceptus, water is partitioned into several compartments, including the amniotic fluid. In this, intramembranous transport pathway, which moves the fluid across the fetal amnion and chorion into the fetal vasculature, is considered a particularly important pathway for regulating amniotic fluid volume homeostasis (Beall et al., 2007). Moreover, changes in the expression of the AQP8 and AQP9 have been shown in pregnancies complicated by idiopathic polyhydramnios (large-for-gestational-age fetuses and macrosomia). In this sense, gestational diabetes mellitus (GDM) is a frequent pregnancy complication associated with increased incidence of polyhydramnios (Sohaey, Nyberg, Sickler, & Williams, 1994). Thus, GDM is a health problem that increases the risk of death during the perinatal period due to fetal macrosomia which is, in addition, accompanied by a larger size and weight of the placenta to support the increased needs of the macrosomic fetus. Therefore, in those subjects that develop polyhydramnios, altered *trans*-amnion water transport due to impared AQP gene expression in GDM could be the cause of the increased amniotic fluid volume (Dashe, Nathan, McIntire, & Leveno, 2000; Santolaya-Forgas, Mehta, & Castracane, 2006). For example, in vitro studies showed an up-regulation in various transport systems, such as amino acids (Vaughan, Rosario, Powell, & Jansson, 2017), fatty acids (Segura et al., 2017), and glucose (Stanirowski, Szukiewicz, Pazura-Turowska, Sawicki, & Cendrowski, 2018) in GDM placenta. Thus, alteration of placenta function may be the reason for abnormal fetal growth (Osmond, Nolan, King, Brennecke, & Gude, 2000)

observed in this pathology of pregnancy (Jansson et al., 2006). In this context, it could be necessary to know the modulation factors, such as fetal or maternal hormones (Oliveira, Carnes, França, Hermo, & Hess, 2005; Pastor-Soler et al., 2002) or other endocrine factors (Vilariño-García et al., 2016), by which AQPs can be regulated. Intriguingly, women with GDM have increased plasma leptin levels (Lepercq et al., 1998) and insulin levels (Sagawa et al., 2002). Our group has recently described that insulin induces leptin expression in trophoblastic cells (Pérez-Pérez et al., 2016) and moreover, increased insulin and leptin levels may modulate the expression of AQPS, such as AQP9, as it was previously described in placenta (in syncytiotrophoblast; Castro Parodi et al., 2011), as well as adipocytes and hepatocytes (Rodríguez, Catalán, Gómez-Ambrosi, & Frühbeck, 2011). Since, AQP9 trophoblast was overexpressed in placentas from women with GDM, the leptin/leptin receptor system could play an important role, since the system is activated in the placenta from GDM by acting on the MAPK and PI3K pathways (Pérez-Pérez et al., 2013, 2015). Therefore, it could be speculate that leptin could mediate the increased expression of AQP9 (or others aquaglyceroporins), which might increase the transport of glycerol to the fetus and thus to contribute to attend the increased energy intake requirements in the macrosomic fetus in GDM. However, the functional role of AQPs in placenta remains to be elucidated and its expression and regulation in abnormal pregnancy may point to new potential therapeutic targets for the pathology of pregnancy.

Moreover, abnormal placentation and altered expression of a several trophoblast transporters are also associated to other pregnancy complication named preeclampsia. This condition is exclusively for human gestation and affects 7–10% of pregnancies worldwide (Giachini et al., 2017). Although its etiology remains unclear, it is well accepted that defects in placentation are also the main predisposing factors for preeclampsia (Hawfield & Freedman, 2009; Huppertz, 2008; Myatt, 2002). Briefly, an insufficient transformation of the maternal spiral arteries resulting in a decrease in blood supply to the fetoplacental unit and consequently, fluctuation in oxygen levels, increase of the oxidative stress and the apoptosis of the trophoblast, concluding in the maternal endothelial dysfunction. In this sense, villous syncytiotrophoblast is involved in the fetal-maternal oxygen and nutrient exchange and an altered expression of a variety of trophoblast transporters and channels are altered in preeclamptic placentas (Castro Parodi et al., 2011; Damiano, Zotta, & Ibarra, 2006; del Monaco et al., 2006; Dietrich, Szpilbarg, & Damiano, 2013; Szpilbarg & Damiano, 2017). More specifically, an abnormal expression of AQP3 and AQP9 were shown in these placentas, suggesting an altered transport of water, urea, and glycerol in placenta form preeclampsia.

The reduced expression of AQP3 in placentas from pregnancies complicated by preeclampsia is consistent with previous findings in explants exposed to oxygen changes (Szpilbarg et al., 2016), in which, AQP3 protein expression decreased after oxygen deprivation, and the subsequent reoxygenation failed to restore AQP3 to basal levels, possibly due to the oxidative damage of the plasma membrane of syncytiotrophoblast. Along with this idea, it has been speculated that the decreased expression of AQP3 might be an adaptive response of the placenta to reduce the trophoblast apoptosis, which is related to the clinical manifestations of preeclampsia. However, further studies are needed to elucidate this issue.

In conclusion, a number of AQPs have been found to be expressed in trophoblast and in fetal membranes with an important roles in facilitating water movement across the biological membranes of maternal- and fetal-placental tissues and organs, as well as between mother and fetus. These requirements are satisfied primarily via transplacental transfer from the maternal circulation, suggesting a need for increased placental water flux with advancing gestation. In this sense, AQP1, AQP3, AQP8 and AQP9 may be involved in regulation of the amniotic fluid volume. Moreover, it could be speculated that the maternal metabolic environment may generate stimuli within the placenta resulting in the altered production of aquaporins and inflammatory cytokines, such as leptin, whose expression is normal under control pregnancy. Thus, deregulation of leptin function in the placenta may be implicated in the pathogenesis of various disorders during pregnancy, such as GDM and preeclampsia (Bajoria, Sooranna, Ward, & Chatterjee, 2002; Sagawa et al., 2002) by alternating AQPs expression. In fact, while AQP3 and AQP9 are normally expressed in the normal trophoblastic cells, AQP9 is up-regulated in abnormal pregnancy. However, this could not be related to an increase in water flux and hypotheses concerning the function of placental AQPs are still speculative. There are many unanswered questions about the roles for AQPs in water transport by placenta, endometrium, and fetal membranes, as well as in relevant processes required for a normal placental development, such as cell migration and apoptosis. Moreover, although GDM and preeclampsia are complex and multisystemic disorders resulting from multiple simultaneous mechanisms, AQPs may be contributing as part of this network of alterations that give rise to the diverse clinical manifestations of these pathologies. Therefore, although the functional role of AQPs in placenta remains to be elucidated, AQPs expression and regulation in abnormal pregnancy might point to new potential therapeutic targets for the pathology of pregnancy.

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OBJETIVOS

Con los antecedentes expuestos en la introducción nos propusimos los siguientes objetivos:

La leptina estimula la secreción de gonadotropinas, pero otros estudios correlacionan la hiperleptinemia con falta de función ovárica, en especial la síntesis de estrógenos en el PCOS. Nuestra **hipótesis** es que la baja expresión de aromatasa para la síntesis de estrógenos en la granulosa de mujeres con PCOS se debería a una resistencia a la acción de la leptina., y que la acción de la leptina sobre la expresión de aromatasa sería positiva y mediada por la proteína de señalización Sam68, como hemos demostrado en otros sistemas. La expresión de Sam68 podría estar disminuida en la granulosa de las mujeres con PCOS y mediar en parte la resistencia a la leptina en la granulosa.

Objetivo 1. **Investigar el papel de Sam68 en la señalización del receptor de leptina en células de la granulosa**

- Comprobar la fosforilación de Sam68 por la activación del receptor de leptina en células de la granulosa
- Estudiar la expresión de Sam68 en respuesta a la leptina
- Evaluar la importancia de Sam68 en las vías de señalización activadas por leptina, inhibiendo su expresión o sobreexpresando Sam68
-

Objetivo 2. **Estudiar el efecto de la leptina en la expresión de la aromatasa en células de la granulosa normal y de mujeres con PCOS**

Objetivo 3. **Estudiar la expresión de Sam68 en granulosa de mujeres con PCOS en comparación con la granulosa de mujeres control**

Nuestro grupo ha demostrado el efecto trófico de la leptina sobre las células trofoblásticas humanas, por lo que planteamos **la hipótesis** que la leptina

podría prevenir la apoptosis de las células trofoblásticas inducidas por agresiones como la alta temperatura o un pH bajo.

Objetivo 4. Estudiar el efecto in vitro de la leptina inhibiendo la apoptosis de las células trofoblásticas promovida por la alta temperatura, así como la participación de la vía de p53 en este efecto antiapoptótico.

Objetivo 5. **Estudiar el efecto in vitro de la leptina inhibiendo la apoptosis de las células trofoblásticas promovida por un pH bajo, así como la participación de la vía de p53 en este efecto antiapoptótico**

La AQP-9, un transportador de agua y glicerol podría contribuir al mayor aporte nutricional del feto macrosómico de la diabética gestacional, por lo que nuestra **hipótesis** es que la expresión de AQP-9 estaría aumentada en el trofoblasto de mujeres con diabetes gestacional y la leptina, cuya expresión está aumentada en esta patología podría mediar esta mayor expresión de AQP-9.

Objetivo 6. Comparar la expresión de AQP-9 en trofoblasto de mujeres con diabetes gestacional y en mujeres control.

Objetivo 7. **Investigar el efecto de la leptina in vitro sobre la expresión de AQP-9 en trofoblasto**

RESULTADOS

Los resultados de los objetivos 1, 2 y 3 están contenidos en la publicación:

Sam68 mediates leptin signaling and action in human granulosa cells: possible role in leptin resistance in PCOS.
Vilariño-García T, Pérez-Pérez A, Santamaría-López E, Prados N, Fernández-Sánchez M, Sánchez-Margalet V.Endocr Connect. 2020 Jun;9(6):479-488. doi: 10.1530/EC-20-0062.

como **sexta publicación** de la Tesis.

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RESEARCH

Sam68 mediates leptin signaling and action in human granulosa cells: possible role in leptin resistance in PCOS

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Abstract

Introduction: Polycystic ovary syndrome (PCOS) is a complex metabolic disorder associated with ovulatory dysfunction, hyperandrogenism, obesity, and insulin resistance, that leads to subfertility. Sam68 is an RNA-binding protein with signaling functions that is ubiquitously expressed, including gonads. Sam68 is recruited to leptin signaling, mediating different leptin actions.
Objective: We aimed to investigate the role of Sam68 in leptin signaling, mediating the effect on aromatase expression in granulosa cells and the posible implication of Sam68 in the leptin resistance in PCOS.
Materials and methods: Granulosa cells were from healthy donors (n = 25) and women with PCOS (n = 25), within the age range of 20 to 40 years, from Valencian Infertility Institute (IVI), Seville, Spain. Sam68 expression was inhibited by siRNA method and overexpressed by expression vector. Expression level was analysed by qPCR and immunoblot. Statistical significance was assessed by ANOVA followed by different post-hoc tests. A P value of <0.05 was considered statistically significant.
Results: We have found that leptin stimulation increases phosphorylation and expression level of Sam68 and aromatase in granulosa cells from normal donors. Downregulation of Sam68 expression resulted in a lower activation of MAPK and PI3K pathways in response to leptin, whereas overexpression of Sam68 increased leptin stimulation of signaling, enhancing aromatase expression. Granulosa cells from women with PCOS presented lower expression of Sam68 and were resistant to the leptin effect on aromatase expression.
Conclusions: These results suggest the participation of Sam68 in leptin receptor signaling, mediating the leptin effect on aromatase expression in granulosa cells, and point to a new target in leptin resistance in PCOS.

Key Words

- Sam68
- leptin
- polycystic ovary syndrome (PCOS)
- aromatase
- signalling pathways

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Introduction

Sam68, the Src-associated substrate during mitosis of 68 kDa, also known as KHDRBS1, is a member of the family of RNA binding proteins (1, 2, 3). This protein participates as a scaffold protein recruited in various signal transduction pathways (4, 5, 6, 7), linking signaling pathways and RNA metabolism regulation (3). Both Sam68 splicing activity as RNA binding ability and other functions are mainly regulated by phosphorylation (8, 9). In this sense, the tyrosine phosphorylation of Sam68 has been previously implicated in cell proliferation, growth as well as in differentiation processes through different mechanisms. In fact, Sam68 phosphorylation has been reported to be stimulated by mitogenic and trophic hormones such as leptin (via PI3K and MAPK pathways activation (10, 11)) (Fig. 1), where it has been linked to cellular growth and differentiation processes. Moreover, numerous physiological roles for this RNA binding protein have been reported using Sam68-deficient mice. For example, Sam68 protein is highly expressed in the gonads, where its ablation causes male infertility, due to defects in spermatogenesis (10, 12). Besides, Sam68$^{-/-}$ females are severely subfertile, and they show a delay in the age of first pregnancy, increased breeding time for successful pregnancy and yielded smaller litters (13). Thus, a function for Sam68 in reproduction and fertility has been suggested (13, 14), and modulation of its activity and/or expression levels probably could mediate various pathological situations.

Polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects 5–10% of women at reproductive age (15). It has unknown origin and various clinical presentations during the life span. PCOS is characterized by the presence of ovulatory dysfunction, clinical and/or biochemical hyperandrogenism and polycystic ovarian morphology (16). Women with PCOS are more likely to require *in vitro* fertilization (17).

A pivotal role in the pathophysiology of this syndrome is played by visceral adiposity (18, 19), and therefore, current research is increasingly focusing on the discovery of novel biomarkers to further elucidate the complex pathophysiology of PCOS. In this sense, alterations of Sam68 metabolic pathways would contribute to reduce the oocyte viability, leading to subfertility or infertility observed in PCOS, which is why we aim to study the role of Sam68 in women with PCOS. More specifically, according to the previously described participation of Sam68 in leptin signaling (6), we aim to investigate the role of this protein in the signal transduction pathways that are activated by leptin in granulosa cells from healthy controls.

Leptin resistance is a common finding in obesity, where it has been shown to cause ovulatory dysfunction and infertility (frequently associated with PCOS) (20). Hyperleptinemia may inhibit the development of the mature oocyte directly and affect ovarian and adrenal steroidogenesis (21).

On the other hand, a number of in vitro studies have reported that leptin may stimulate estrogen expression by increasing the expression of the intracellular

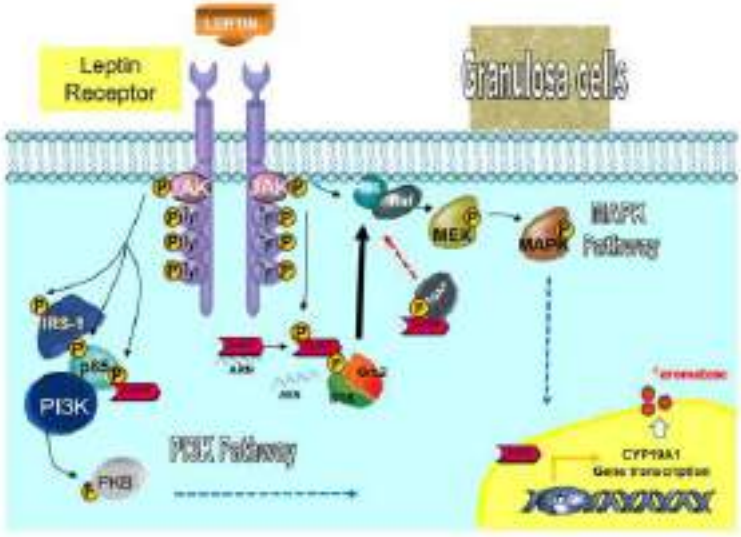

Figure 1
Mechanisms of leptin action in granulosa cells. The cartoon shows the signaling pathways that mediate the leptin effects on granulosa cells. Sam68 contains a domain for the RNA binding activity and it may interact with both RNA targets, as well as other proteins containing Src homology 3 (SH3) and SH2 domains through proline-rich sequences and tyrosine-phosphorylated residues, respectively. Sam68 is a scaffold protein recruited in various signal transduction pathways linking signaling pathways (PI3K and MAPK pathway) and RNA metabolism regulation. Sam68 functions are mainly regulated by phosphorylation. This tyrosine phosphorylation of Sam68 has been implicated in cell proliferation, growth as well as in differentiation processes through different mechanisms. Finally, Sam68 has been related to leptin-dependent PI3K and MAPK pathways activation, where it has been shown to be associated to Grb2, GAP and PI3K regulatory subunit in a different cellular system.

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aromatase enzyme, which catalyzes the rate-limiting step in the conversion of C19 androgens (androstenedione and testosterone) to C18 estrogenic steroids (estrone and estradiol). Thus, conflicting results of high leptin levels correlating with decreased aromatase expression (22, 23) may be due to leptin resistance. In this sense, diminished signaling has been found in GCs from PCOS women even though leptin levels are increased in follicular fluid (24). Low aromatase activity has also been demonstrated in women with PCOS. Therefore, we have hypothesized that the lower expression of aromatase in GC from PCOS could be caused by leptin resistance and that Sam68 could be an underlying factor and thus a novel target in PCOS.

In the present study, we aimed to investigate the role of Sam68 in leptin signaling pathways in GCs, mediating the expression of aromatase, as well as to analyze the relationship between Sam68 and aromatase expression in GCs from patients with PCOS, in comparison with healthy controls.

Materials and methods

The study was approved by the Institutional Ethics Committee for human research of the Virgen Macarena University Hospital and conducted according to the principles expressed in the Declaration of Helsinki. All included adult participants provided written informed consent before the collection of samples

Subjects

We included women with PCOS from Valencian Infertility Institute (IVI), Seville, Spain. All women were evaluated through a standardized screening protocol which has been previously described in detail elsewhere (25). PCOS was diagnosed according to the Rotterdam criteria in the presence of two or more of the following criteria: oligo- and/or anovulation, clinical and/or biochemical signs of hyperandrogenism and polycystic ovarian morphology as assessed by transvaginal ultrasound (26). We also included healthy donor women without PCOS with regular menstrual cycles (21–35 days), as well as women undergoing IVF/ICSI treatment in the IVI clinic. Women were within the age range of 20 to 40 years, and obese subjects, patients with endometriosis and poor ovarian response were excluded from the study. No differences were found in the mean age (33.3 ± 5.2 PCOS subjects, and 25.2 ± 4.8 control subjects) or BMI (25.4 ± 0.4 PCOS, and 24 ± 1.8 control subjects).

Human granulosa cell isolation and culture

Luteinized granulosa cells (GCs) from follicular aspirates were isolated using the protocol described in the literature by Ferrero *et al.* (27). GCs from healthy donor were seeded in six-well dishes and incubated overnight (37°C, 5% CO2) to enable removal of non-adherent cells. Subsequently, GCs from each patient were washed and cultured for 24 h in Mc Coy's medium (BioWhittaker®) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 g/mL streptomycin at 37°C in 5% CO2. Next, GCs were treated with or without leptin (10 nM) for 10 min or 16 h in medium without FCS. The recombinant human leptin was provided by Sigma (Sigma Chemical); 10 nM dose of leptin was used for both the experiments of inhibition and sobrexpression of sam68 corresponding to our previously described results of optimal dose response (28).

The cell lisates were washed with cold PBS and solubilized for 30 min at 4°C in lysis buffer containing 20 mM Tris, pH 8, 1% Nonidet P-40, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 0.4 mM sodium orthovanadate. Total protein levels were determined by the bicinchoninic acid method (29) using BSA as standard.

Immunoprecipitation

Soluble cellular lysates (0.5 mg of protein) from GCs were precleared with 50 µL of protein A-sepharose (Pharmacia, Uppsala, Sweden) for 2 h at 4°C by end-over-end rotation.

The precleared cellular lysates were incubated with anti-Sam68 (C-20) 1:1000 from Santa Cruz Biotechnology for 3 h at 4°C (30). Next, 50 µL of protein A-sepharose was added to immune complexes and incubation continued for 2 h. The immunoprecipitates were washed three times with lysis buffer. We added 30 µL of SDS-stop buffer containing 100 mM dithiothreitol (DTT) to the immunoprecipitates, followed by boiling for 5 min. Soluble supernatants were then resolved by Western blotting by using 7–10% SDS–PAGE and electrophoretically transferred onto nitrocellulose membranes (11).

Western blotting analysis

Cell lysates and immunoprecipitates were denatured with 30 µL of SDS-stop buffer containing 100 mM of DTT followed by boiling for 5 min. The soluble supernatants were then resolved by 7–10% SDS-PAGE and electrophoretically transferred onto nitrocellulose

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membranes. The membranes were blocked with buffered saline – 0.05% Tween 20 (PBST) containing 3% albumin for 1 h at 23°C. The blots were then incubated with primary antibody for 1 h. Anti-Sam68 (C-20) 1:1000 was from Santa Cruz Biotechnology, anti-GAPDH 1:1000 and anti phosphotyrosine (4G10) 1:1000 were from Millipore, anti-phospho-ERK1/2 (pT202-Y204/ pT185-Y187) 1:1500, anti-phospho-AKT (pS473) 1:3000, and anti-phospho-IRS-2 1:2000 were from Cell Signalling Technology.

After the incubation with primary antibody, the membranes were washed in PBST and further incubated with the corresponding secondary antibodies using horse radish peroxidase-linked anti-rabbit/anti-mouse 1:10,000 immunoglobulin (GE Healthcare). Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (SuperSignal from Pierce).

Transfection experiments

GCs from healthy donors were plated onto six-well dishes containing 2 mL of Mc Coy's medium plus 10% FCS and incubated overnight (37°C, 5% CO_2) to enable removal of non-adherent cells. Medium was replaced, and transfection of cells was performed. For experiments involving gene silencing, cells were transfected with 2µg of siRNA oligonucleotides of Sam68 (Integrated DNA Technology) using LipofectAMINE (Life Technologies) transfection reagent according to the manufacturer's instructions.

Duplex sequences: forward, 5′-CGCAGAACA AAGUUACGAAGGCUAC-3′; reverse, 5′-GUAGCC UUCGUAACUUUGUUCUGCGUA-3′. Typically, 40 pmol of the Sam68 siRNA duplexes were transfected using 5µL of LipofectAMINE (Life Technologies). For experiments involving overexpression of Sam68, between 1 and 3µg of DNA was transfected into GCs. The pcDNA3 vector was used to equalize the amount of DNA in each transfection.

Following transfection, the medium was replaced with serum-free medium for another 24 h and the cells were stimulated with or without 10 nM leptin for 10 min. Transfection analyses were performed by duplicate in each of at least three independent experiments.

RNA extraction and quantitative real-time-PCR (qRT-PCR) assay

Relative abundance of aromatase and Sam68 mRNA was determined by qRT-PCR. After treatment with leptin, total RNA from GCs cultures was extracted using TRISURE reagent (Bioline Co) according to the manufacturer's instructions. Total RNA from both healthy donors and PCOS women was extracted. Concentration of the isolated RNA were estimated at 260 and 280 nm with purity in A_{260}/A_{280} ratio around 2.0. For cDNA synthesis, 5µg of total RNA was reverse transcribed at 55°C for 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche). qRT-PCR was performed using the following primers based on the sequences of the National Center for Biotechnology Information GenBank database:

Sam68 (GeneBank accession: NM_006559.3):
forward, 5′-TTTGTGGGGAAGAT TCTTGG-3′;
reverse, 5′ GGGGGTCCAAAGACTTCAAT-3′.
cyclophilin (GeneBank accession: NM_000942):
forward, 5′ CTTCCCCGATACTTCA-3′;
reverse, 5′-TCTTGGTGCTACCTC-3′3′.
Aromatase (GeneBank accession: M18856):
forward, 5′ CCCTTCTGCGTCGTGTCAT-3′;
reverse, 5′ GATTTTAACCACGATAGCACTTTCG -3′.
RPS-7 (GeneBank accession: NC_000002.12):
forward, 5′ ACCAAGAACTTTTTGCCCCT-3′;
reverse, 5′ ATGTCCCCCGAGTAATTTCC-3′.

Quantitative RT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start Universal SYBR Green), and PCRs were performed on a Chromo 4 DNA Engine (Bio-Rad). Reaction contained 10µM of forward and reverse primer, 3µL of cDNA and the final reaction volume was 20µL. The reaction was initiated by preheating at 50°C for 2 min, followed by heating at 95°C for 10 min. Subsequently, 41 amplification cycles were carried out as follows: denaturation 15 *s* at 95°C and 1 min annealing and extension at 58°C. The threshold cycle (CT) from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta ct}$ method. For the treated samples, evaluation of $2^{-\Delta\Delta ct}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin or RPS-7) and relative to the untreated control.

Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as means±s.d. in arbitrary units. Arbitrary units were calculated as normalized band intensity in Western blot analysis. Statistical analysis was performed using the Graph Pad Prism computer program (GraphPad

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Software). Statistical significance was assessed by ANOVA followed by different post hoc tests, as indicated in each figure. A P value of <0.05 was considered statistically significant.

Results

Leptin increased Sam68 phosphorylation and mRNA expression in granulosa cells from healthy donors

We have previously demonstrated the Tyr-phosphorylation of Sam68 in response leptin in different cell systems (11, 28, 30, 31, 32). Now, to assess whether Sam68 is involved in leptin's action on human GCs from healthy donors, we incubated GCs for 10 min in medium with or without different leptin concentrations (0.1–10 nM) (Fig. 2). Leptin (Fig. 2A) increased the Sam68 phosphorylation in a dose-dependent manner, in GCs, as determined by Western blot analysis. This effect was dose dependent, and the maximal effect was achieved at 10 nM leptin. The amount of total protein in every sample was controlled using anti-Sam68 antibodies.

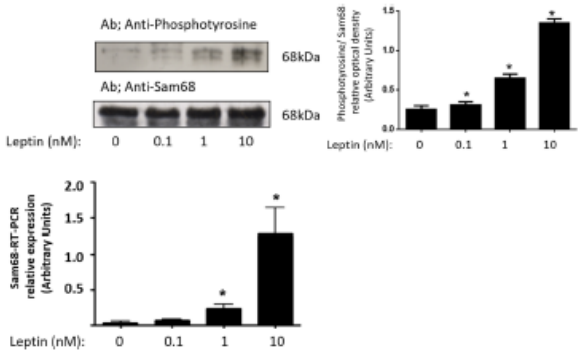

Figure 2

Leptin increases Sam68 phosphorylation in human GCs from healthy donors. (A) GCs were incubated in the absence or presence of leptin (0.1–10 nM) for 10 min, lysed and the soluble clarified cell lysates were subjected to immunoprecipitation with anti-Sam68 antibodies. Immunoprecipitates were resolved by SDS-PAGE and Western blot with anti-phosphotyrosine antibodies. The lysates were analyzed by immunoblot using the anti-Sam68 antibodies to control the amount of protein in every lane. A representative experiment run in duplicates from three different donors is shown. Densitograms with s.e. are shown. *$P < 0.05$ vs control. (B) Total RNA was extracted as described in Materials and Methods and Sam68 mRNA was quantified with qRT-PCR in independent experiments. Cyclophilin was used as internal standard. Results shown are expressed as means ± s.e. from three independent experiments, run in triplicates, *$P < 0.05$ vs control. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison post hoc test.

In order to further study the effects of leptin on Sam68 expression, qRT-PCR analysis was carried out using cyclophilin as an internal control. GCs were independently incubated in the absence of serum with and without leptin (0.1–10 nM) for 16 h. As shown in Fig. 2B, expression level of Sam68 gene was increased in response to leptin, dose-dependently, and maximal effect was achieved at 10 nM leptin.

Sam68 down-regulation prevents leptin activation of signaling pathways in granulosa cells from healthy donors

Leptin receptor is known to activate phosphatidylinositol-3 kinase (PI3K) and mitogen-activated protein kinase (MAPK). To test the effects of Sam68 down-regulation on leptin signaling, human GCs from healthy donors were used and immunoblotting was employed to analyze the phosphorylation of kinases.

Cells were grown to 60–70% confluence in six-well dishes and were first transfected using Sam68 siRNA or NC1 negative control siRNA duplexes and incubated in the absence or presence of leptin for 10 min as previously indicated in Material and Methods. Both anti-Sam68 and anti-GAPDH antibodies were used as control of Sam68 down-regulation and loading control, respectively. This approach achieved a decrease in Sam68 expression ranging between 60 and 70% of the control value (data not shown).

We measured the activation of PI3K pathways by employing antibodies that specifically recognize the phosphorylated forms of the central kinase PKB. Moreover, we also measured the activation of MAPK pathways by employing antibodies that specifically recognize the phosphorylated forms of ERK1/2. As shown in Fig. 3, leptin-mediated PKB and ERK1/2 phosphorylation was significantly reduced in GCs where Sam68 was down-regulated. This effect was almost completely abolished by decreasing the expression of Sam68, suggesting the role of Sam68 in the leptin signaling pathways in GCs.

Finally, to connect Sam68 expression with the mechanistic effect that exerts over the main pathways PI3K and MAPK under leptin stimulation, we next focus on the effect of siRNA Sam68 down-regulation on the phosphorylated forms of insulin receptor substrate-1 (IRS-1) in human GCs. As it was demonstrated using immunoblotting analysis with anti-phospho-IRS-1 antibodies, down-regulation of Sam68 in GCs significantly decreased the phosphorylation of IRS-1 in response to leptin (Fig. 3).

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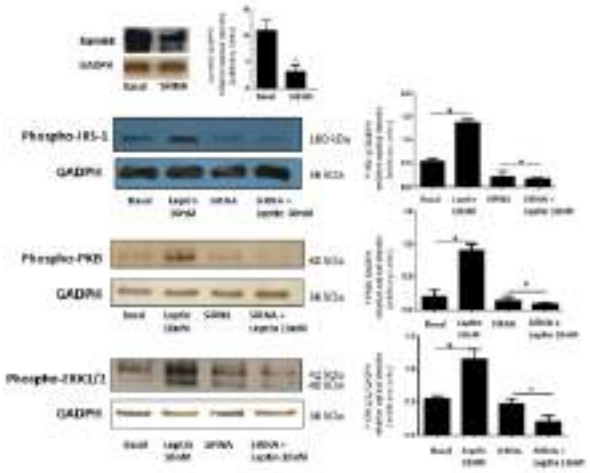

Figure 3
Sam68 siRNA prevents the leptin-dependent activation of PI3K and MAPK pathways in human GCs from healthy donors. GCs were transfected with Sam68 siRNA or NC1-scrambled negative control siRNA (basal) duplexes as described in Materials and Methods and incubated in the absence or presence of 10 nM leptin for 10 min. GCs lysates were separated by SDS–PAGE and Western blot analysis was performed by using anti-P-IRS-1, anti-P-PKB and anti-P-ERK1/2 antibodies to study leptin activation of the PI3K and MAPK signaling pathway. Sample protein loading was controlled by using anti-GAPDH antibodies. We show the control of the Sam68 expression inhibition as well as the corresponding densitometric analysis of three independent experiments as means ± s.e.m. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the controls un-treated and 'ns' no statistically significant difference according to Bonferroni's multiple comparison post hoc test.

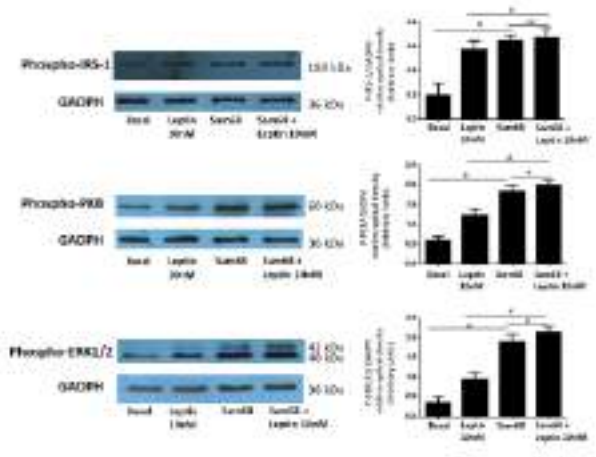

Figure 4
Sam68 overexpression increases the leptin-dependent activation of PI3K and MAPK pathways in human GCs from healthy donors. GCs were transfected with Sam68 plasmid and pcDNA3 (empty vector) as control (basal) as described in Materials and Methods, and incubated in absence or presence of leptin 10 nM for 10 min. GCs lysates were separated by SDS–PAGE and Western blot analysis was performed by using anti-P-IRS-1, anti-P-PKB and anti-P-ERK1/2 antibodies to study leptin activation of the PI3K and MAPK signaling pathway. Sample protein loading was controlled by using anti-GAPDH antibodies. We show the corresponding densitometric analysis of three independent experiments as means ± s.e.m. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control and Sam68 plasmid without leptin. # indicate significant differences from leptin 10 nM and 'ns' no statistically significant difference according to Bonferroni's multiple comparison post hoc test.

Sam68 overexpression increases leptin activation of signaling pathways in human GCs from healthy donors

To further study the effect of Sam68 in leptin signaling pathways, we investigated the up-regulation of Sam68 by transfecting GCs from healthy donors with a pcDNA3 expressing Sam68 plasmid and pcDNA3 vector (empty vector) as control. Following transfection during 24 h, the medium was replaced with serum-free medium for another 24h. Next, GCs were incubated in the absence or presence of 10 nM leptin for 10min. As shown in Fig. 4, up-regulation of Sam68 increased the leptin-dependent activation of PI3K and MAPK pathways in GCs. More specifically, up-regulation of Sam68 increased the phosphorylation of IRS-1, PKB and ERK1/2. Maximal effect was obtained at 10 nM leptin, suggesting again the role of Sam68 in the leptin signaling pathways in GCs.

Effect of leptin on aromatase gene expression

Although leptin was found to significantly increase aromatase gene expression and protein level (33, 34, 35), its effect on aromatase in GCs from PCOS women is contradictory and unknown. Therefore, both GCs from healthy donors and PCOS women were grown for 24 h and treated with 10 nM leptin for 24h. qRT-PCR analysis was carried out as previously indicated in Material and Methods, using RPS-7 as an internal control for reaction efficiency. The results showed that leptin stimulated significantly the expression of aromatase mRNA in GCs from healthy donors (Fig. 5A), possibly via activation of the canonical signal transduction pathways, MAPK and PI3K, as previously shown in other cellular systems (36). However, no significant changes in aromatase mRNA was detected after of 24-h leptin treatment in GCs from PCOS women (Fig. 5B), suggesting that some factors aberrantly expressed in PCOS could be inhibiting the leptin signal transduction pathways.

Decreased Sam68 and and aromatase expression in human GCs from PCOS women compared to GCs from healthy donors

Since Sam68 protein is highly expressed in the gonads, we aimed also to compare the expression of Sam68 in GCs

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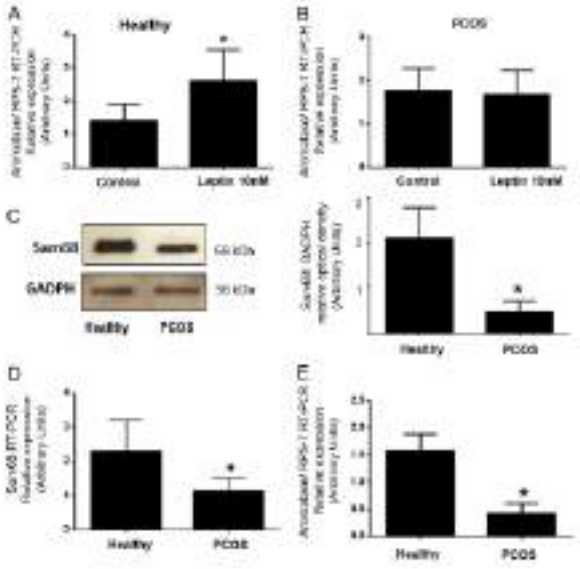

Figure 5
Effect of leptin on aromatase mRNA expression and diminished expression of Sam68 and aromatase in GCs from PCOS women. GCs samples were obtained from 25 healthy donors and 25 PCOS women. GCs were cultured in a medium containing 0% FCS for 24 h. (A) GCs from healthy donors were treated with or without (control) 10 nM leptin for 24 h. (B) GCs from PCOS women were treated with or without (control) 10 nM leptin for 24 h. Aromatase gene expression was analyzed by qRT-PCR, normalized against RPS-7 and compared to the control group. (C) Representative Western blot analysis of Sam68 protein level in GCs from healthy donors and PCOS women. GCs lysates were denatured and resolved by SDS-PAGE with anti-Sam68 antibodies. Loading controls were performed in the same membranes with anti-GAPDH. We show the corresponding densitometric analysis of three independent experiments as means ± s.e.m. Statistical analyses were performed by ANOVA. (D) Relative mRNA level of Sam68 in GCs from healthy donors and PCOS women. Sam68 mRNA was quantified with qRT-PCR. Cyclophylin was used as internal standard. Paired *t*-test was performed to examine the difference in mRNA level of Sam68 and statistical significance was considered when *P* value was < 0.05. (E) Relative mRNA level of aromatase in GCs from healthy donors and PCOS women, quantified by qRT-PCR. RNA was extracted as described in Materials and Methods. RPS-7 was used as internal standard. Paired *t*-test was performed to examine the difference in mRNA level of aromatase, and statistical significance was considered when *P* value was < 0.05. Asterisks indicate significant differences from the control according to Mann–Whitney *U* test.

from patients diagnosed with PCOS vs those obtained from healthy donors, used as controls. We evaluated mRNA expression by means of qRT-PCR, using cyclophilin as an internal control for reaction efficiency. Moreover, immunoblotting analysis of Sam68 was performed as previously described in Materials and methods. As show in Fig. 5C and D, Sam68 expression was lower in GCs from PCOS women than in GCs for healthy donors, suggesting a role played by Sam68 in these cells, which might allow this protein to affect different biological processes, such as, the aromatization step promoted by the aromatase enzyme. That is why we aimed also to compare the expression of aromatase in GCs from patients diagnosed with PCOS vs those obtained from healthy donors, used as controls. As show in Fig. 5E, aromatase expression was lower in GCs from PCOS women than in GCs for healthy donors, suggesting a role played by Sam68 in these cells.

Effect of leptin on aromatase mRNA expression in overexpressed-Sam68 GCs from healthy donors

Since Sam68 was observed to be downregulated in GCs from PCOS women and Sam68 modulates the leptin signaling pathways in GCs, as previously demonstrated, in order to further study the mechanical effect of leptin on aromatase expression, GCs from healthy donors were transfected with Sam68 plasmid and pcDNA3 as control. GCs were treated with or without (control) 10 nM leptin for 48 h. As shown in Fig. 6, transfection with Sam68 plasmid significantly increased the expression levels of aromatase in GCs. Moreover, this effect of the upregulation of Sam68 on the expression of aromatase were more significantly increased when GCs were stimulated with 10 nM leptin for 48 h, suggesting a function of Sam68 in the aromatization step stimulated by leptin in GCs and, therefore, a possible role of Sam68 in the complex pathophysiology of PCOS.

Discussion

Reproductive dysfunction and infertility are common manifestations in PCOS (37). Understanding PCOS

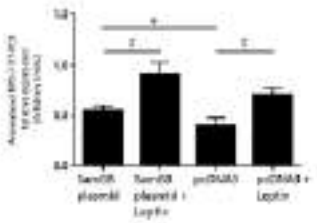

Figure 6
Effect of leptin on aromatase mRNA expression in control and Sam68-overexpressed GCs from healthy donors. GCs were transfected with Sam68 plasmid and pcDNA3 as control, as described in Materials and Methods, and incubated in absence or presence of leptin 10 nM for 48 h. Aromatase gene expression was analyzed by qRT-PCR, normalized against RPS-7. Data represent the mean ± s.e.m. value of triplicate samples in three independent experiments. Paired *t*-test was performed to examine the difference in mRNA level of aromatase, and statistical significance was considered when *P* value was < 0.05. * Indicate significant differences from the control pcDNA3; # indicate significant differences from the control pcDNA3 with leptin and ^ indicate significant differences from the Sam68 plasmid, according to Bonferroni's multiple comparison post hoc test.

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pathophysiology and its association with reproductive and metabolic disturbances is essential for addressing women's health and for expanding knowledge on how to treat this highly multifaceted syndrome.

Recently, a function for Sam68 in reproduction and fertility has been suggested (13, 14). This is a scaffold protein that could be recruited in various signal transduction pathways (6, 38), including leptin signaling. In the present work, we aimed to study the role of Sam68 in leptin signaling in GCs. More specifically, since Sam68 is a protein, whose structural characteristics permit multiple types of post-translational modifications, we aimed to study whether acute leptin administration affects the tyrosine phosphorylation of Sam68 in GCs. Thus, we have confirmed the Tyr-phosphorylation of Sam68 upon leptin stimulation in GCs. Moreover, this effect of leptin is dose dependent, in a similar way to that previously observed in other systems. As a result of this tyrosine phosphorylation of Sam68 in response to leptin, the RNA binding capacity of Sam68 is diminished in GCs (data not shown), being consistent with previous reported data demonstrating that tyrosine phosphorylation of Sam68 by kinases of the Src (39) and Brk (9, 40) family negatively regulates its RNA binding function.

Other receptor systems have been found to positively modulate the expression of kinases substrates and other molecules recruited to the receptor signaling upon stimulation with the specific ligand (41). That is why the possible participation of Sam68 in leptin receptor signaling and the regulation of Sam68 expression by leptin in GCs from healthy donors were also investigated. In this context, we also found that leptin stimulation of GCs increased the expression of Sam68 in a similar way to that previously observed in trophoblastic cells (32). This further supports the possible role of Sam68 in the signaling of leptin in GCs. Therefore, as previously shown in many different cellular systems, leptin activates both MAPK and PI3K pathways in GCs. Now, by using both silencing gene expression strategy as well as gene overexpression, we have demonstrated that Sam68 is mediating leptin action in GCs, by the participation in the leptin-dependent activation of MAPK and PI3K signaling pathways.

Regarding the mechanism whereby Sam68 may mediate the activation of these pathways, it has previously been reported that Sam68 is associated with the SH3 and SH2 domains of proteins (39), suggesting a role of Sam68 in the MAPK pathway. Moreover, association of Tyr-phosphorylated Sam68 with the regulatory subunit of PI3K has been previously demostrated in peripheral blood mononuclear cells in response to leptin. This interaction may enhance the activation of PI3K pathway, which may support a role of Sam68 also in the activation of this pathway by leptin. Particularly, IRS-1 is a key protein linking PI3K and MAPK signaling pathways (42). Therefore, since Sam68 seems to regulate IRS-1 expression, as we observed in the Sam68 down-regulation (no significantly) and up-regulation experiments, the role of Sam68 stimulating PI3K and MAPK signaling pathways may be also mediated by IRS-1 in GCs. However, the mechanism whereby Sam68 may modulate IRS-1 expression is intriguing and remains to be investigated. Thus, Sam68 plays a role in the transduction of the leptin signal from the plasma membrane to RNA metabolism via a rapid mechanism mediated by phosphorylation, and therefore, some of the effects of leptin in GCs may be mediated, at least in part, by modulation of RNA metabolism.

Leptin is a well-characterized obesity-associated factor with biological effects on survival, growth and proliferation in different biological systems (43, 44, 45). Moreover, leptin has also been shown to be a potent stimulator of aromatase expression and activity in isolated adipose stromal cells (36, 46) and luteinized granulosa cells (34). However, some studies have shown contradictory results. In this sense, increased leptin levels in follicular fluid from women with PCOS has been found, and it may be used as a negative predictive marker in *in vitro* fertilization (47). Nevertheless, the increased leptin levels may be due to leptin resistance, as previously suggested (24). Thus, the investigation of the leptin action in GCs from women with or without PCOS is relevant to further understand this problem. Therefore, we also aimed to investigate the expression of aromatase by leptin stimulation and the different expression of both aromatase and Sam68 in GCs from healthy donors and women with PCOS. We found that leptin increased significantly the aromatase gene expression in GCs from healthy donors, possibly, via activation of the canonical signal transduction pathways, MAPK and PI3K, as previously shown in other cell types (34). This finding futher emphasizes the role of leptin in aromatase expression in GCs. Moreover, this study also confirms a decreased aromatase gene expression in response to leptin in GCs from women with PCOS, suggesting a significant resistance to leptin action. Since we have also found that Sam68 is downregulated in GCs from PCOS, the resistance to leptin action on aromatase expression in GCs from PCOS women may be partly mediated by the lower expression of Sam68. Moreover, basal expression of aromatase was also decreased in PCOS. This is important since hyperandrogenism is a critical factor of the pathophysiologic changes and clinical

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features (in both lean and obese women) associated with PCOS (15). Nevertheless, it should be taken into account the limited number of women investigated in the present study. Larger studies which include more subjects should be carried out.

In summary, the current study further supports the regulation of aromatase by leptin in GCs and provides, for the first time, a novel mechanistic insight into the mechanism underlying this effect, via Sam68, as well as the possible role of this protein in leptin resistance in PCOS.

In conclusion, these results suggest the participation of Sam68 in leptin receptor signaling, mediating the leptin effect on aromatase expression in GCs, and point to a new target in leptin resistance obseved in GCs from patients with PCOS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

T V-G, A P-P and E S-L performed the experiments. N P and M F-S analyzed the data. E S-L carried out the sample collection and preparation. T V-G and A P-P participated in the literature search and manuscript revision. V S-M conceived and designed the research and wrote the paper.

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Leptin reduces apoptosis triggered by high temperature in human placental villous explants: The role of the p53 pathway

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ABSTRACT

Maternal fever is common during pregnancy and has for many years been suspected to harm the developing fetus. Whether increased maternal temperature produces exaggerated apoptosis in trophoblast cells remains unclear. Since p53 is a critical regulator of apoptosis we hypothesized that increased temperature in placenta produces abnormal expression of proteins in the p53 pathway and finally caspase-3 activation. Moreover, leptin, produced by placenta, is known to promote the proliferation and survival of trophoblastic cells. Thus, we aimed to study the possible role of leptin preventing apoptosis triggered by high temperature, as well as the molecular mechanisms underlying this effect.

Fresh placental tissue was collected from normal pregnancies. Explants of placental villi were exposed to 37 °C, 40 °C and 42 °C during 3 h in the presence or absence of 10 nM leptin in DMEM-F12 medium. Western blotting and qRT-PCR was performed to analyze the expression of p53 and downstream effector, PS3AIP1, Mdm2, p21, BAX and BCL-2 as well as the activated cleaved form of caspase-3 and the fragment of cytokeratin-18 (CK-18) cleaved at Asp396 (neoepitope M30).

Phosphorylation of the Ser 46 residue of p53, the expression of PS3AIP1, Mdm2, p21, as well as caspase-3 and CK-18 were significantly increased in explants at 40 °C and 42 °C. Conversely, these effects were significantly attenuated by leptin 10 nM at both 40 °C and 42 °C. The BCL2/BAX ratio was also significantly decreased in explants at 40 °C and 42 °C compared with explants incubated at 37 °C, which was prevented by leptin stimulation.

These data illustrate the potential role of leptin for reducing apoptosis in trophoblast explants, including trophoblastic cells, triggered by high temperature, by preventing the activation of p53 signaling.

1. Introduction

Maternal fever is common during pregnancy and has for many years been suspected to harm the developing fetus [1,2]. 1 in 5 women report having experienced fever on at least one occasion while being pregnant [1–5]. Therefore, given the high proportion of pregnant women who are exposed to fever, a small increase in the risk of these outcomes would make maternal fever a public health concern. In animal models it was studied, as a marker of maternal fever, the effect of raising body temperature during pregnancy and it has been reported that prenatal exposure to elevated body temperature leads to increased prevalence of adverse health outcomes in the offspring. It was evidenced that even a short exposure to elevated maternal body temperature is able to lead to cell death, membrane and vascular disruptions, as well as placental infarction. These disturbances may alter the integrity of the offspring [1,6] causing growth retardation, malformations and to longer-term outcomes behavioral alterations and impaired cognitive functioning [6,7]. In this sense, several mechanisms have been proposed through which fever interferes with fetal development. Particularly all of them include heat shock proteins expression [8] and

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interruption of protein synthesis and enzyme production, which ultimately results in altered or dysfunctional cellular processes, such as apoptosis [6]. In this line, it has been reported that apoptosis could be determinant for normal placental development and its increase and early appearance may also be involved in the pathophysiology of pregnancy-related diseases [8,9].

Intriguingly, it has been reported that leptin, produced by placenta, as well as their receptors seem to play a possible role in the apoptotic process acting through an autocrine mechanism [10]. Physiological leptin effects in placenta include angiogenesis, growth and immunomodulation [11] as well as increase protein synthesis [12–14] and anti-apoptotic actions [12]. Furthermore placental leptin levels are increased under stressful condition, such as preeclampsia or intra-uterine growth restriction (IUGR) [15], where apoptosis is also boosted [16]. It has been suggested that this overproduction of leptin may be helpful to prevent the highly stress-mediated apoptosis of the trophoblastic cells. Although the role of leptin in preventing the apoptotic process triggered by the deprivation of serum in trophoblastic JEG-3 and Swan-71 cells is well established [12,17], the role during cellular responses to high temperature is still unknown. For this reason we aimed to investigate the effect of leptin in the apoptosis triggered by elevated temperature (40 °C and 42 °C) in human placental explants [18,19]. More specifically, we investigated the leptin effect on apoptosis by studying the protein cleavage of caspase-3 as well as the p53 expression, the master key regulator of death signaling, and several of its downstream proteins such as Mdm-2, p21, Bax and Bcl-2.

2. Materials and methods

2.1. Placental explants collection and processing

Term placentas from uncomplicated pregnancies (n = 10) were obtained after cesarean section delivery following normal term pregnancies in the Virgen Macarena University Hospital. None of the patients had previous history of diabetes mellitus or any known endocrinopathy. Subject characteristics were mean maternal age at delivery (26.0 years ± 7.0), mean infant birth weight (3095 g ± 69), mean placenta weight (525 g ± 81). Subject characteristics were similar with regards to gestational age (39.5 weeks ± 0.7).

Human placentas were obtained after cesarean section and immediately suspended in ice-cold PBS and transported to the laboratory, where they were washed two to three times in sterile PBS to remove excess blood. Villous tissue free of visible infarct, calcification, or hematoma was sampled from at least five cotyledons at a distance midway between the chorionic and basal plates. These core parts of cotyledons were cut into multiple cubic segments (10- to 15-mg wet weight) and thoroughly rinsed with cold DMEN-F12 medium pH 7.4 (137 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, and 4 mM NaHCO3). None of the donor patients suffered from anomalous pregnancy. This study was approved by the local ethical committee (Comité Local de Etica en Investigación del Hospital Universitario Virgen Macarena), and the patients' written consent was obtained. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

2.2. Treatments of placental explants

Placental explants were randomly distributed in tubes containing 1 ml of DMEM-F12 medium 0% FBS (n = 1 explant/tube, three replicates per treatment). Placental explants were maintained in a shaking water bath at 37 °C, 40 °C and 42 °C during 3 h in the presence or absence of 10 nM leptin (Sigma Chemical Co.). Explants were removed from the bath, centrifuged for 2 min at 2000 × g at 4 °C, and resuspended in 500 μl of lysis buffer [1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 50 mM NaF, 10 mM pyrophosphate and protease inhibitor cocktail) during 30 min at 4 °C on an orbital shaker and later centrifuged at 10 000 × g for 20 min. Supernatants were analyzed by Western blot analysis.

2.3. Western blot analysis

Total cell lysates were prepared in lysis buffer. The lysates were centrifuged at 10 000 × g for 10 min to remove cellular debris. Total protein levels were determined by the bicinchoninic acid method (Thermo Scientific), using bovine serum albumin as standard. 50 μg protein were loaded in each lane. Lysates were mixed with Laemmli's sample buffer containing 2% sodium dodecyl sulfate and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS-PAGE on a 12% gel, and electrophoretically transferred to a nitrocellulose membrane (Hybond; Amersham Pharmacia Biotech, Piscataway, NJ) thereafter. Membranes were equilibrated in 1 × PBS, and nonspecific binding sites were blocked by 5% nonfat milk in PBS at room temperature for 1 h. The membranes were then immunoblotted with polyclonal rabbit anti-Caspase-3 (8G10) (1:1000, Cell Signaling; #9665), monoclonal mouse anti-cytokeratin-18 neo-epitope M30 (1:1500, Peviva prod.10700), monoclonal mouse anti-p53 (1:1000, Santa Cruz; sc-126), polyclonal rabbit anti phospho Ser46 p53 (1:1000, Cell Signaling; #2521), polyclonal goat anti-p53AIP1 (1:3000, Santa Cruz; sc-14095), polyclonal rabbit anti-p21 (1:1000, Santa Cruz; sc-756), polyclonal rabbit anti-Mdm-2 (1:1000, Santa Cruz; sc-965), polyclonal rabbit anti-Bax (1:1000, Santa Cruz; sc-493), or polyclonal rabbit anti-BCL-2 (1:1000, Epitomics; #1017-1).

Membranes were stripped and loading controls were performed by immunoblotting the same membranes with monoclonal mouse anti-β tubulin (1:2500, Santa Cruz; sc-5274), except for the p53 and phospho Ser46 immunoblots, whose loading control was determined loading the same amount of samples in a different gel. The antibodies were detected using horseradish peroxidase-linked anti-rabbit/anti-mouse immunoglobulin (1:12,000, Amersham; NA934/NA931), anti-goat immunoglobulin (1:15,000, life technologies; Q[22603) and visualized using a highly sensitive chemiluminescence system (Supersignal; Pierce). Quantification of protein bands was determined by densitometry using Image Gauge version 3.12 software (ScienceLab, Fuji Photo Film Co., Ltd.).

2.4. Quantitative real-time RT-PCR assay

Abundance of p53 mRNA was determined by quantitative real time RT-PCR reaction (qRT-PCR). Total RNA was extracted from placental explants using TRISURE reagent, according to the manufacture's instructions (Bioline Co). Concentration and purity of the isolated RNA were estimated by spectrophotometry at 260 and 280 nm. For cDNA synthesis, 5 μg of total RNA was reverse-transcribed at 50 °C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche). Quantitative real time PCR reaction was performed using the following primers based on the sequences of the NCBI GenBank database: p53, forward, 5'GGAAGAGAATCTCCGCAA3'; reverse, 5'AGCTCTCGGA ACATCTGGAAG3'; cyclophilin, forward, 5'CTTCCCCGATACTTCA3'; reverse, 5'TCTTGGTGCTACCTC 3'; p53AIP1, forward 5'GGGGACTTC TCAGGTCGTGT3'; reverse, 5'TGGACTTCTTGATGCCCCGA3'; p21, forward, 5'GATGGCACCAGAGGTGGTTA3'; reverse, 5'TCCCGAAA TATTGGGGAAAG3'; Mdm-2, forward, 5'TTACCCAGGCTG

GAGTGCAG3'; reverse, 5'GAGAATGGTGCGAACCCG3'

qRT-PCR Master Mix Reagent kit was obtained from Roche (Fast Start universal SYBR Green) and PCR reactions were performed on a Chromo 4 DNA Engine (BioRad). A typical reaction contained 10 μM of forward and reverse primer, 3 μl of cDNA and the final reaction volume was 25 μl. The reaction was initiated by preheating at 50 °C for 2 min, followed by heating at 95 °C for 10 min. Subsequently, 41 amplification cycles were carried out as follows: denaturation 15 s at 95 °C and 1 min annealing and extension 1 min at 59 °C. The threshold cycle (C_T), from each well was determined by the Opticon Monitor 3 Program. Relative quantification was calculated using the $2^{-\Delta\Delta Ct}$ method [20]. For the treated samples, evaluation of $2^{-\Delta\Delta ct}$ indicates the fold change in gene expression, normalized to a housekeeping gene (cyclophilin), and relative to the untreated control. Melting curves in the qPCR experiments showed a single PCR product (p53 or p53AIP1 at a different temperature than the reference gene (supplementary data).

2.5. Data analysis

In placental explants, immunoblot are a representative experiment from the 10 placentas studied. Results are expressed as the mean ± SD. The statistical significance was assessed by ANOVA followed by Bonferroni's multiple comparison post hoc test and was calculated using the GraphPad Instat computer program (GraphPad, San Diego, CA). A P value less than 0.05 was considered statistically significant.

3. Results

3.1. Leptin diminishes apoptotic effect of high temperature in human placental explants

We have previously reported that leptin has a trophic effect (promoting cellular growth and survival) in trophoblastic JEG-3 and BeWo cells, preventing the apoptosis promoted by serum deprivation [12,17]. Now, we have further analyzed the anti-apoptotic effect of leptin on the trophoblast inducing apoptosis by high temperature. We incubated placental explants at 37 °C, 40 °C and 42 °C, in the presence or absence of leptin (10 nM) to study the possible role of leptin preventing the high-temperature induced apoptosis in the trophoblast. 10 nM leptin is the leptin concentration with maximal effects in trophoblast as previously reported [13]. Time-response experiments (30 min, 1 h, 2 h, 2.5 h, 3 h, 4 h, 5 h and 6 h incubation) at high temperature yielded 3 h incubation as optimal to study the effect on apoptosis (data not shown). Apoptosis was investigated by determination of Caspase-3 activated form by Western blot. As shown in Fig. 1A, placental explants incubated at 40 °C and 42 °C increased Caspase-3 activated form compared with placental explants incubated at 37 °C. Moreover, treatment with leptin 10 nM significantly reduced Caspase-3 activation in placental explants similarly to previously reported leptin effects preventing the apoptosis promoted by serum deprivation [12].

In order to investigate whether the increased apoptosis and leptin effects are, indeed, occurring in trophoblasts cells, we also tested one of neoepitopes of Cytokeratin-18 (CK-18) (the cleavage site on CK-18 of caspases), identified by a monoclonal antibody (M30), which has been proposed as an specific apoptosis marker of trophoblast cells in human placenta [21]. As shown in Fig. 1B, placental explants incubated at 40 °C and 42 °C increased the 21 KDa fragment of CK-18 activated form compared with placental explants incubated at 37 °C. Moreover, treatment with leptin 10 nM significantly reduced CK-18 activation in placental explants similarly to the results obtained from caspase 3 experiments.

3.2. Leptin impairs the increase in p53 expression and downstream effectors produced by high temperature in explants of placental villi

To further explore the effect of temperature-induced apoptosis, we next focused on the expression of p53, a pivotal regulatory protein in the apoptotic pathway, which accumulates in cells in response to DNA damage, oncogene activation and other stressful stimuli [22]. Moreover, we also analyzed the phosphorylation of p53 at Ser-46, which has been shown to be involved in the regulation of apoptosis by transactivating apoptosis genes such as p53AIP1 [23], and the expression of Mdm-2 (a negative feedback regulator of p53) and p21 (a marker of p53 activity) [17,24].

As it is shown in Fig. 2A, the high temperature (40 °C and 42 °C) did not significantly increased the p53 protein amount. However p53 mRNA levels were increased by high temperature incubation (Fig. 2B). Moreover an increased p53 phosphorylation at Ser-46 (Fig. 2A), as well as, P53AIP1 (Fig. 3), Mdm-2 (Fig. 4) and p21 (Fig. 5) expression were observed. Treatment with leptin 10 nM significantly prevented the increase in p53 expression at mRNA levels, as well as the p53 phosphorylation at Ser-46 (Fig. 2A). Moreover, leptin significantly prevented the increase of P53AIP1 (Fig. 3), Mdm-2 (Fig. 4) and p21 (Fig. 5) expression promoted by both 40 °C and 42 °C incubation. These results suggest a strong anti-apoptotic response of leptin to high temperature in human placental explants.

3.3. Leptin enhances Bcl-2/Bax relationship in placental explants incubated at 40 °C and 42 °C

p53 also promotes Bax, a pro-apoptotic mitochondrial pore protein [25]. That is why, we next investigated the effect of high temperature, 40 °C and 42 °C in the Bax expression as well as Bcl-2 expression, an anti-apoptotic member of the Bcl-2 family which function as major regulator of the intrinsic apoptotic pathway [26]. Similarly, placental explants were incubated with or without leptin 10 nM to further characterize anti-apoptotic effect of leptin in placental explants.

As showed in Fig. 6B, high temperature decreased the Bcl-2/Bax ratio, both at 40 °C and 42 °C. Leptin treatment at 10 nM significantly increased Bcl-2/Bax ratio, consistent with the antiapoptotic effect of leptin.

4. Discussion

Normal placentation and placental development are critical for a successful pregnancy and mediate important steps necessary for fetal development [27]. However, foetus may be especially vulnerable to chemical and physical insults during defined stages of development. In particular, it has been reported that exposure to maternal fever during pregnancy can cause abortion, growth retardation and developmental defects [28]. It is known that placenta locally releases a broad spectrum of hormones and growth factors that play key roles in compensatory changes to its pathophysiological alterations. Regulators of apoptosis are now considered to have a major role in maintaining the integrity of villous trophoblast, so the study of the molecular mechanisms that regulate placental cell death is important for understanding normal development and a variety of diseases of the placenta. In this regard, leptin has been described as an important cytokine regulating trophoblast survival, promoting growth and preventing the apoptotic process in serum deprived trophoblastic cells [29]. This leptin anti-apoptotic effect seems to be mediated by p53 pathway [17]. In this work, we aimed to study the leptin effect in preventing the apoptotic process triggered by high temperature (40 °C-42 °C), as a marker of maternal fever, in human placental explants. They

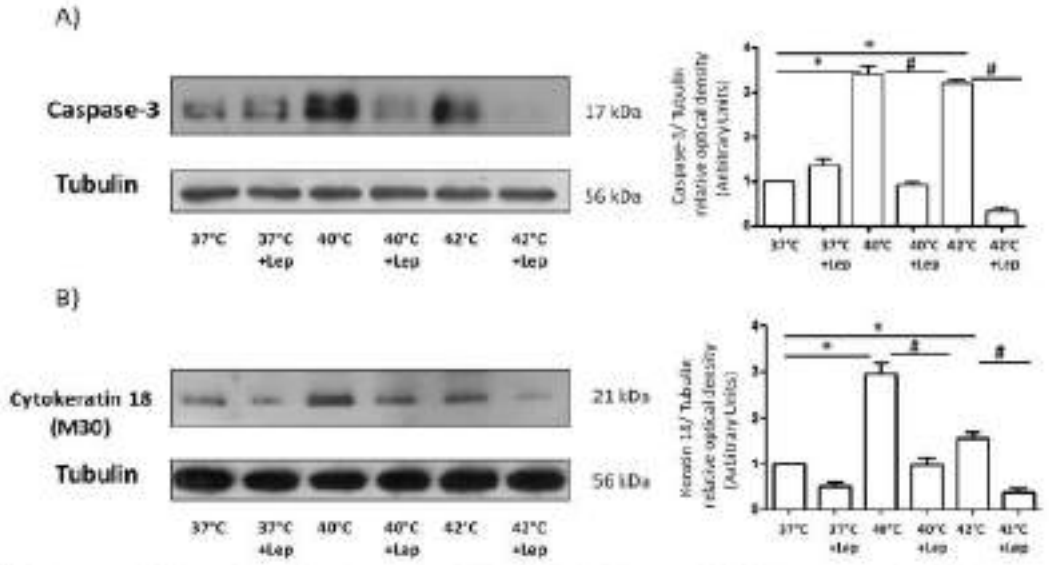

Fig. 1. Leptin diminishes apoptosis in human placental explants incubated at 40 °C and 42 °C. Caspase and CK18 destruction were determined in placental explants. Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 3 h in the presence or absence of 10 nM leptin. Caspase-3 cleaved fragment (p17 and p12) and the 21 kDa fragment of Cytokeratin-18 (CK18Asp396) was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membranes with anti-tubulin. Results are expressed as mean ± SD for three independent experiments. Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison post hoc test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.001, *p < 0.001.

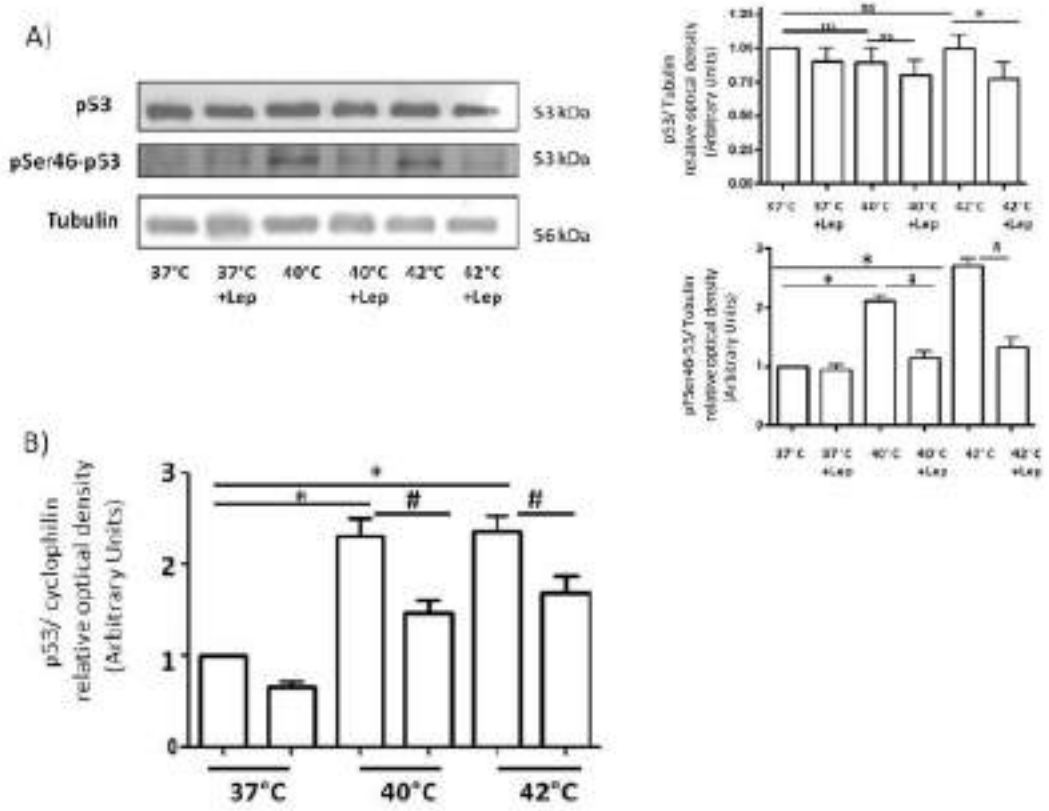

Fig. 2. Leptin diminishes p53 expression as well as Ser-46 p53 phosphorylation in human placental explants incubated at 40 °C and 42 °C. A) Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 3 h in the presence or absence of 10 nM leptin. P53 as well as phospho Ser-46 p53 were determined by Western blot analysis. Placental explants to 37 °C were used as a control. Loading controls were performed by immunoblotting with anti-tubulin antibodies. Results are expressed as mean ± SD for three independent experiments. B) p53 expression in human placental explants was determined by qRT-PCR. RNA was extracted as described in Materials and Methods. Statistical analyzes were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison post hoc test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.001, *p < 0.01.

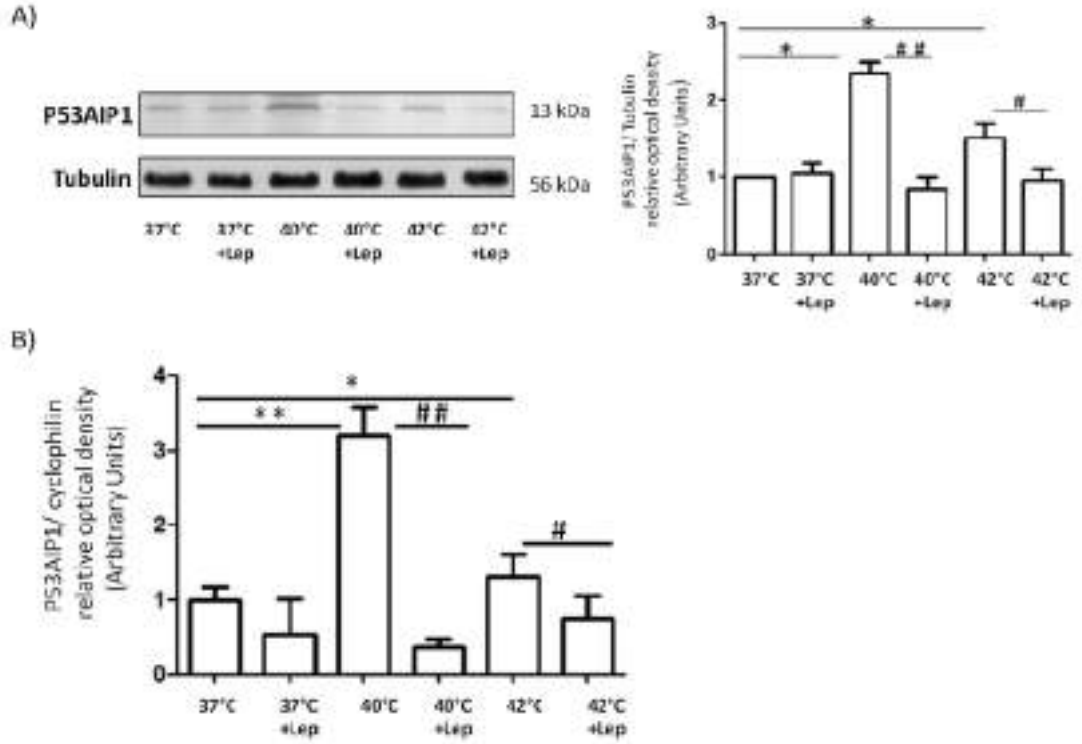

Fig 3. Leptin reduces P53AIP1 expression in placental explants incubated at 40 °C and 42 °C. P53AIP1 expression was determined in placental explants. Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 30 min in DMEM-F12 0% FBS media. After placental explants were stimulated with leptin 10 nM during 2 h. A) P53AIP1 expression was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membranes with anti-tubulin. Results are expressed as mean ± SD for three independent experiments. Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison post hoc test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.001, *p < 0.001. B) p53AIP1 expression in human placental explants was determined by qRT-PCR. RNA was extracted as described in Materials and Methods. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison post hoc test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.05, ##p < 0.01, (*) p < 0.05.

that represent a valuable model to confirm the physiological relevance of leptin in trophoblastic survival. We showed that high temperature induced apoptosis by activating caspase-3, as previously reported in the apoptotic process triggered by serum deprivation [12]. We also confirmed that result studying the appearance M30 cytokeratin 18 fragment that is only revealed after caspase cleavage of the protein in human placental trophoblast cells [21]. These findings suggest that leptin may be protective to the deleterious effects of maternal fever during pregnancy preventing the high temperature-mediated apoptosis of the trophoblastic cells. This effect may be of physiological relevance since trophoblastic cells are an important source of leptin production during pregnancy [30,31] and even more leptin is produced under stressful condition [30,32].

Trophoblastic apoptosis, as other types of cell apoptosis, includes the extrinsic and intrinsic pathways culminating in the activation of caspases. It has been reported that p53, a key component of cellular mechanisms that are activated by cellular stress, is involved in spontaneous abortion [33]. However, it remains incompletely understood how p53 is stabilized in placenta and in response to different stress signals. For that reason in this work we investigated whether this key cell cycle-signaling protein was involved in the apoptosis mediated by high temperature in human placental explants. We found a significant increase in p53 expression and phosphorylation in placental explants under high temperature conditions, but p53 protein level did not change accordingly. This may be due to increased degradation of p53 that may counterbalance the increased expression. Moreover, a decrease in p53 mRNA level and p53 phosphorylation was observed in response to 10 nM leptin, demonstrating that leptin regulates p53 pathway under high temperature conditions in human placental explants.

Under normal conditions, p53 is a short-lived protein that is highly regulated and maintained at low or undetectable levels [34,35]. After stress, such as serum deprivation [17], p53 is activated mostly at the post-translational level by a complex series of modifications that include the phosphorylation of specific residues. It was reported that p53 is serine-phosphorylated after DNA damage and other types of stress [36–38]. Therefore, it might be speculated that activation (phosphorylation) of p53 by high temperature promotes apoptosis and placental leptin may play an important role controlling this process. In this sense, we also demonstrated an increased serine-phosphorylation of p53 triggered by high temperature, as well as a significant reduced serine-phosphorylation of p53 in response to 10 nM leptin. In addition, our results suggest that phosphorylation of Ser-46 regulates the transcriptional activation of the p53AIP1 (apoptosis-inducing gene) as previously was demonstrated by Oda et al [39].

It is known that Mdm-2 is induced by activated p53, which is negatively regulated, at least in part by mediating its ubiquitination and subsequent degradation in the proteasome [34,40]. This mechanism may explain the lack of differences in p53 protein level in spite of the changes in p53 gene expression. Although little is known about leptin effect on Mdm2 levels in placenta, we demonstrated an increased Mdm-2 expression in human placental explants incubated at high temperature. In addition, leptin 10 nM

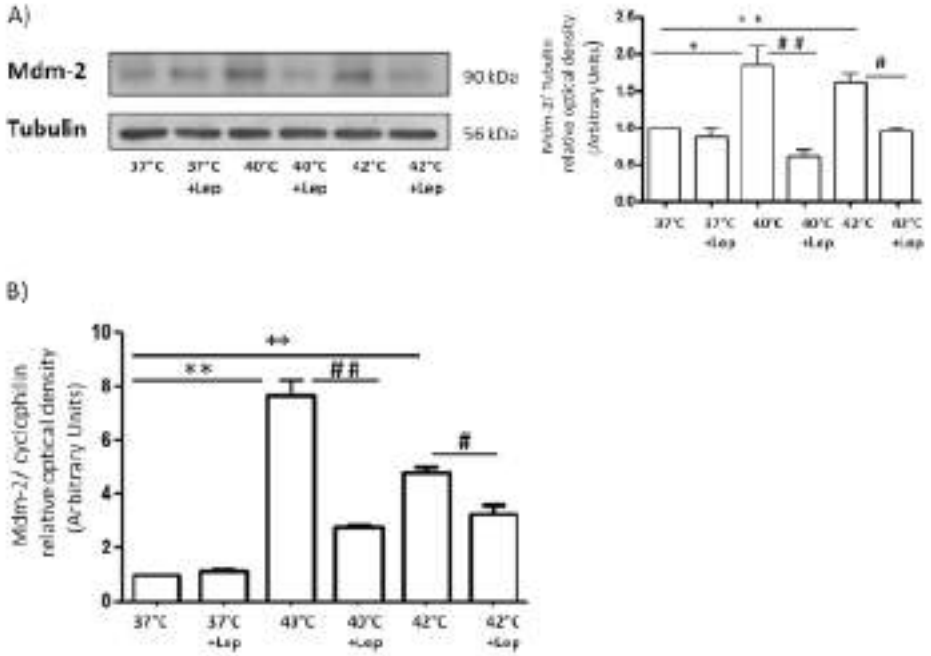

Fig. 4. Leptin diminishes Mdm-2 expression in human placental explants incubated at 40 °C and 42 °C. Mdm-2 expression was determined in placental explants. Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 30 min in DMEM-F12 0% FBS media. After placental explants were stimulated with leptin 10 nM during 5 h. A) Mdm-2 expression was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membranes with anti-tubulin. Results are expressed as mean ± SD for three independent experiments. Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison *post hoc* test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.05, ##p < 0.001, *p < 0.01, **p < 0.001. B) Mdm-2 expression in human placental explants was determined by qRT-PCR. RNA was extracted as described in Materials and Methods. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.05, ##p < 0.01, (*) p < 0.05.

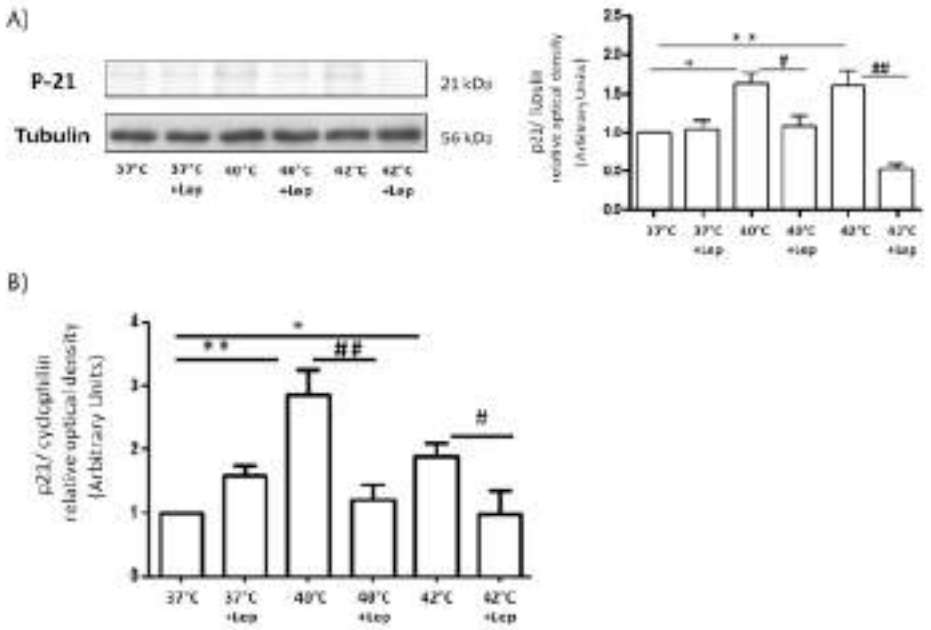

Fig. 5. Leptin diminishes p21 expression in human placental explants incubated at 40 °C and 42 °C. P21 expression was determined in placental explants. Placental explants were processed as described in Material and Methods and incubated to 37 °C, 40 °C and 42 °C during 30 min in DMEM-F12 0% FBS media. After placental explants were stimulated with leptin 10 nM during 5 h. A) p21 expression was determined by Western blot analysis. Loading controls were performed by immunoblotting the same membranes with anti-tubulin. Results are expressed as mean ± SD for three independent experiments. Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison *post hoc* test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.05, ##p < 0.001, *p < 0.01, **p < 0.001. B) p21 expression in human placental explants was determined by qRT-PCR. RNA was extracted as described in Materials and Methods. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison *post hoc* test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.05, ##p < 0.01, (*) p < 0.05.

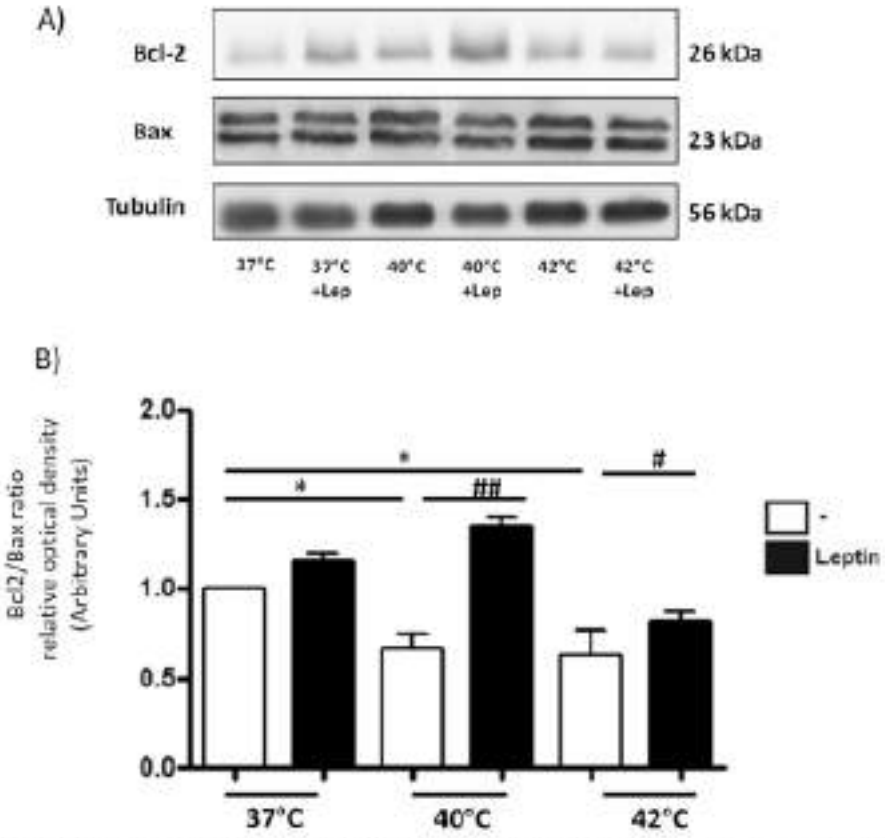

Fig 6. Leptin enhances BCl-2/Bax ratio in human placental explants incubated at 40 °C and 42 °C. Placental explants were processed as described in Materials and Methods and incubated to 37 °C, 40 °C and 42 °C during 30 min in DMEM-F12 0% FBS media. After placental explants were stimulated with leptin 10 nM during 3 h (black box). Placental extracts were prepared and proteins were separated on SDS-PAGE gels. A) BCL-2 and BAX expression were determined by Western blot analysis as indicated in the figure. Molecular weights were estimated using standard protein markers. Molecular mass (KDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting the same membranes with anti-tubulin. B) Bcl-2/Bax Ratio. Leptin increased BCL-2/BAX ratio. Results are expressed as mean ± SD for three independent experiments. Statistical analysis were performed by ANOVA and Bonferroni's multiple comparison post hoc test, relative to 40 °C and 42 °C without leptin (#) or 37 °C (*). #p < 0.05, ##p < 0.05, *p < 0.01.

not only decreases p53 expression but also Mdm-2 expression, suggesting that the leptin effect on Mdm-2 may be mediated by p53 phosphorylation level.

The p21 gene is induced by p53 in almost all cell types by a variety of stress agents, suggesting that multiple pathways can promote its activation [36,45]. Here we have found an increase in p21 expression in human placental explants incubated at high temperature as well as a reduced p21 expression in response to leptin 10 nM. Thus, our data demonstrate that maternal fever could be an important cause for p53-mediated activation of p21 and subsequent placental growth arrest, which may be counterbalanced by leptin.

Finally, it is well known that BCL-2-family proteins are central regulators of cell life and death. The first pro-apoptotic member of the family, BAX (BCL-2 Antagonist X) was identified as a BCL-2-interacting protein that opposed BCL-2 (anti apoptotic protein) and promoted apoptotic cell death [41]. Moreover, p53 also interacts with BCL-2 at the outer mitochondrial membrane to promote the oligomerization of Bax. This in turn drive the formation of pores in the mitochondrial membrane, resulting in the release of cytochrome c and other apoptotic activators from the mitochondria [42,43]. Moreover, mitochondria function is the main operations center in which the intrinsic apoptotic pathway [44]. We observed a reduced BCL-2/BAX ratio in human placental explants incubated at high temperature, which was increased in response to 10 nM leptin. The modulation of this ratio by leptin was due principally to an increase in BCL-2 protein. Therefore, in this study we have determined an activated intrinsic apoptotic pathway when placental explants were incubated at high temperature, which may be prevented by leptin incubation. Whether extrinsic pathway is severely impaired in placental explants incubated at high temperature would require further investigation.

Taken together, our findings provide evidence for an inhibitory leptin effect on the cell apoptosis program triggered by high temperature, suggesting a trophic role of leptin in the physiology of placenta. In addition, we have provided some evidence for the possible anti-apoptotic mechanisms exerted by leptin in placenta. However, further additional studies are needed to fully explain the effect of leptin on the regulation of p53 expression and BCL-2-family proteins in response to hyperthermia. This temperature-induced apoptosis may provide a model to further understand certain placental pathologies associated with fever, and may unravel new therapeutic targets. Finally, leptin seems to reduce the high temperature promoted apoptosis in trophoblast cells from human placental villi, by preventing p53 signaling.

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Conflict of interest

Authors have no conflict of interest to declare.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2016.03.009.

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REGULAR ARTICLE

Leptin protects placental cells from apoptosis induced by acidic stress

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Abstract

Development of the human placenta is critical for a successful pregnancy. The placenta allows the exchange of oxygen and carbon dioxide and is crucial to manage acid-base balance within a narrow pH. It is known that low pH levels are a risk of apoptosis in several tissues. However, there has been little discussion about the effect of acidic stress in the placenta. Leptin is produced by the placenta with a trophic autocrine effect. Previous results of our group have demonstrated that leptin prevents apoptosis of trophoblast cells under different stress conditions such as serum deprivation and hyperthermia. The purpose of the present work is to evaluate acidic stress consequences in trophoblast explant survival and to determine leptin action in these conditions. For this objective, term human trophoblast explants were cultured at physiological pH (pH 7.4) and at acidic pH (pH 6.8) in the presence or absence of leptin. Western blot assays were performed to study the abundance of active caspase-3 and the p89 fragment of PARP-1. Pro-apoptotic and pro-survival members of Bcl-2 family, as Bax, t-Bid, and Bcl-2, were studied. Moreover, p53 pathway was also evaluated including Mdm-2, the main p53 regulator. Active caspase-3 and cleaved PARP-1 abundances were increased at low extracellular pH. Moreover, t-Bid levels were also augmented as well as p53 expression and phosphorylation on S46. Leptin treatment prevents the consequences of acidosis, decreasing p53 expression and increasing Mdm-2 expression. In summary, this work demonstrated for first time that low pH induces apoptosis of human trophoblast explants involving apoptotic intrinsic pathway, and leptin impairs this effect.

Keywords Placenta · Acidic stress · Apoptosis · Leptin

Introduction

Development of the human placenta is critical for embryonic progress and successful pregnancy outcome, since it allows metabolic exchange across this interface and it works as an endocrine tissue (Knöfler and Pollheimer 2013). During pregnancy, the fetus depends on the mother for placental exchange of oxygen and carbon dioxide. For the placenta, it is crucial to manage acid-base balance within a narrow pH range in order to reduce adverse effects on fetal growth and development (Bobrow and Soothill 1999; Omo-Aghoja 2014). The normal blood pH is strongly regulated between 7.35 and 7.45. Acidocis means a high hydrogen ion concentration in the tissues and the cutoff level to define acidosis in adults is a pH of less than 7.35, but after labor and normal delivery, much lower values occur in the fetus, reaching pH 7.00 with no subsequent ill effects (Bobrow and Soothill 1999). Acidosis is a measurable outcome in the newborn that reflects the fetal environment before delivery and can be perceived as low umbilical pH (Allanson et al. 2016).

In normal pregnancy, a low oxygen (normal, 18 mmHg, (2.5%) at 8 weeks) environment in the placenta is physiological and necessary during early placentation, but when it occurs later in gestation (normal, ~ 60 mmHg (8.5%) at 12 weeks), it is pathological and associated with common complications of pregnancy (Pringle et al. 2009; Rodesch

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et al. 1992). An essential and often neglected aspect of hypoxia is the accumulation of lactic acid as the end product of glycolysis. Excess H+ ions resulting from an increased glycolytic rate are pumped outside the cell, inevitably causing acidification of the extracellular milieu. The role of hypoxia-inducing apoptosis via the production of acidosis has been previously demonstrated (Dong et al. 2014). That is why that hypoxia and acidosis are risky and could trigger intra-uterine death of the fetus (Yang and Wang 1995). The fetus exposed antenatally to chronic hypoxia and acidosis is much more at risk of associated long-term morbidity. As a consequence, antenatal events are much more important than intra- or postpartum events. For example, it was demonstrated that chronic fetal acidosis reduces neurodevelopment (Bobrow and Soothill 1999). At the same time, several maternal and fetal diseases have been related to cord blood acidosis at birth, and maternal-fetal illness could also affect placental anatomy and function (Avagliano et al. 2015). Moreover, it was suggested that during preeclampsia (PE), there could be maternal abnormalities of acid-base status (Ortner et al. 2015).

Leptin, a peptide of 16 kDa, is secreted by adipose tissue and modulates satiety and energy homeostasis (Zhang et al. 1994; Houseknecht et al. 1998). Leptin is also produced by the placenta, and it was demonstrated that leptin has several reproductive functions (Masuzaki et al. 1997). In the placenta, leptin promotes trophoblast invasion, immunomodulation, angiogenesis, protein synthesis, and growth (Frühbeck et al. 1998; Barrientos et al. 2015; Perez-Perez et al. 2015; Schanton et al. 2017). Additionally, previous works of our group have demonstrated that leptin exerts an anti-apoptotic effect on trophoblast cells increasing its importance during the first steps of pregnancy (Perez-Perez et al. 2008; Toro et al. 2014; Pérez-Pérez et al. 2016). In this line, apoptosis is determinant for correct placentation; however; its enhancement or early appearance may produce pregnancy-related diseases (Huppertz and Herrler 2005; Huppertz et al. 2006; Sharp et al. 2010). Moreover, leptin expression is increased under stressful condition as well as PE and intra-uterine growth restriction (IUGR), where apoptosis levels are altered (Heazell et al. 2011; Tzschoppe et al. 2011; Pérez-Pérez et al. 2017b).

The p53 tumor suppressor protein has well-stablished roles in monitoring various types of stress signals, as heat shock, hypoxia, and DNA damage, by activating specific transcriptional targets that control cell cycle arrest and apoptosis (Sohr and Engeland 2011). The p53 protein is modified by several posttranslational modifications including phosphorylation, acetylation, methylation, and ubiquitination (Meek and Anderson 2009). In this sense, phosphorylation of S46 is critical for p53-mediated induction of pro-apoptotic genes (Dai and Gu 2010). Otherwise, Mdm-2 is an E3 ubiquitin ligase implicated in p53 degradation. Thus, the increase in Mdm-2 expression is in accordance with its negative regulatory effect on p53 levels (Eischen and Lozano 2014).

Mitochondria play a central role in the integration and circulation of death signals initiating inside the cells. Apoptotic stimuli result in the formation of pores at mitochondrial membranes, resulting in the decrease in the mitochondrial transmembrane potential and release of pro-apoptotic proteins (Sinha et al. 2013). Low oxygen levels cause major changes in mitochondrial structure and dynamics, ultimately leading to defective mitochondrial function, reduced ATP supply, and activation of cell death pathways (Khacho et al. 2014). Apoptotic mitochondrial events are controlled by the Bcl-2 family proteins which can be of two types: pro- and anti-apoptotic (Sinha et al. 2013). The pro-apoptotic subfamily is classified into the multidomain group and the BH3-only group. Bax is one of the crucial pro-apoptotic members that modulates mitochondrial outer membrane permeability and the release of cytochrome c leading to activation of downstream apoptotic pathway (Brenner and Mak 2009). Bid, a BH3-only member, is essential for initiation of apoptotic signaling (Elmore 2007; Kaufmann et al. 2012). Truncated Bid (t-Bid) is generated through the cleavage of Bid by caspase-8 and triggers the oligomerization of Bax (Sinha et al. 2013). Bcl-2 is a pro-survival member which can attenuate Bax effects (Brenner and Mak 2009; Basanez et al. 2012). On the other hand, caspases are crucial mediators of apoptosis and caspase-mediated apoptotic cell death is accomplished through the cleavage of several key proteins required for cellular functioning and survival. PARP-1 is one of several known cellular substrates of caspases and cleavage of PARP-1 by caspases is considered to be a hallmark of apoptosis. Particularly, the cleavage of PARP-1 by caspase-3 results in the formation of two specific fragments: a 89 kDa catalytic fragment (cPARP-1) and a 24 kDa DNA-binding domain (Chaitanya et al. 2010).

Recently, it was reported that low pH induce apoptosis in tumor cells, indicating that acidic stress could activate distinct apoptotic events (Sharma et al. 2015). To our knowledge, the effect of acidic stress on human placenta was not studied yet. In this line, we hypothesized that low pH produces placental cells apoptosis and leptin might regulate this effect. The purpose of this paper is to evaluate acidic stress consequences in trophoblast cell survival and to determine leptin action in these conditions. For this aim, human trophoblast explants were cultured at pH 7.4 and 6.8, and hallmarks of apoptosis, such as caspase-3 and the p89 fragment of PARP-1, were studied in the presence or absence of leptin. Also, p53 pathway and mitochondrial intermediaries, as Bax, Bcl-2, and t-Bid, were evaluated.

Materials and methods

Trophoblast explants collection and processing

Placentas from uncomplicated pregnancies ($n = 6$) were obtained after cesarean section delivery following normal

term pregnancies in the Virgen Macarena University Hospital. Subject characteristics were mean maternal age at delivery (28.0 years ± 9.0), mean body mass index (25.8 kg/cm^2 ± 2.1), mean gestational age (39.1 weeks ± 1.1), mean infant birth weight (3250.2 g ± 98.6), and mean placenta weight (501 g ± 58). Human placentas were immediately suspended in ice-cold PBS and transported to the laboratory, where they were washed 2–3 times in sterile PBS to remove excess blood. Villous tissue free of visible infarct, calcification, or hematoma was sampled from at least five cotyledons at a distance midway between the chorionic and basal plates. These core parts of cotyledons were cut into multiple cubic segments (10–15 mg wet weight) and thoroughly rinsed with cold DMEM-F12 medium pH 7.4 (137 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgSO4, 0.3 mM Na2HPO4, 0.4 mM KH2PO4, and 4 mM NaHCO3). This study was approved by the local ethical committee (Comité Local de Ética en Investigación del Hospital Universitario Virgen de Macarena), and the patients written consent was obtained.

Treatments of trophoblast explants

Trophoblast explants were randomly distributed in tubes containing 1 ml of DMEM-F12 medium 0% FBS (equal amount of trophoblast explant per tube, three replicates per treatment) and maintained in a normoxia chamber at 37 °C under a 5% CO_2 environment. Trophoblast explants were incubated in DMEM-F12 0% FBS pH 7.4 and pH 6.8 in the absence or presence of leptin (10 nM, based on previous studies 10 nM (Perez-Perez et al. 2008, 2009, 2010)) during 5 h. The acidity of solutions was measured with an electronic "pHmeter." Trophoblast explants were removed from the bath, centrifuged for 2 min at 2000*g* at 4 °C, and resuspended in 500 μl of lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and 10 mg/ml PMSF) during 30 min at 4 °C on an orbital shaker and later centrifuged at 10000*g* for 20 min. Supernatants were analyzed by Western blot.

Western blot analysis

Total cell lysates were prepared in lysis buffer. The lysates were centrifuged at 10000*g* for 10 min to remove cellular debris. The protein concentration of the supernatant was determined by the Bradford colorimetric assay, with bovine serum albumin (BSA) as standard. Lysates were mixed with Laemmli's sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS-PAGE on a 12% gel, and electrophoretically transferred to a nitrocellulose membrane (Hybond, Amersham Pharmacia). Membranes were equilibrated in 1× PBS and non-specific binding sites were blocked by 5% non-fat milk in PBS at room temperature for 1 h. The membranes were then immunoblotted with monoclonal rabbit anti-caspase-3 (200 μg/ml, 1:1000, Santa Cruz), monoclonal rabbit anti-PARP (200 μg/ml, 1:1000, Santa Cruz), monoclonal mouse anti-p53 (200 μg/ml, 1:5000, Santa Cruz), polyclonal rabbit anti-phospho Ser-46 p53 (1:1000, Cell Signaling), monoclonal mouse anti-Mdm-2 (1:1000, Oncogene), polyclonal rabbit t-Bid (polyclonal rabbit anti-Bax (200 μg/ml, 1:1000, Santa Cruz), and polyclonal rabbit anti-Bcl-2 (1:1000, Epitomics). Loading controls were performed by immunoblotting the same membranes with polyclonal rabbit anti-GAPDH (6.6 μg/ml, 1:2500, Calbiochem) or monoclonal rabbit anti-α Tubulin (200 μg/ml, 1:2500, Santa Cruz). The antibodies were detected using horseradish peroxidase–linked goat anti-rabbit/anti-mouse IgG (1:12000, Amersham) and visualized using a highly sensitive chemiluminescence system (Supersignal, Pierce). Quantification of protein bands was determined by densitometry using Image Gauge version 3.12 software (ScienceLab, Fuji Photo Film Co., Ltd.).

Data analysis

Experiments were repeated separately at least three times to assure reproducible results. Results are expressed as the mean ± standard deviation (S.D.). The statistical significance was assessed by Student's test or, when multiple comparisons were necessary, by ANOVA followed by Bonferroni's post hoc test. Differences between groups were considered significant at P value < 0.05 using the GraphPad Instat computer program (San Diego, CA, USA).

Results

Low pH induces apoptosis in human trophoblast explants and leptin prevents this effect

As a first step, we investigated the apoptotic effect of acidic pH on human trophoblast explants by determination of caspase-3 activated form and cleaved PARP-1. For this aim, trophoblast explants were cultured in DMEM-F12 media at pH 7.4 (control pH) and pH 6.8 during 5 h. Western blot assays showed that incubation of trophoblast explants at pH 6.8 increased caspase-3 activation (5.75-fold increment), and leptin reversed this effect reaching similar values to control at pH 7.4 (Fig. 1a). Moreover, leptin regulation on PARP-1 cleavage was studied. As shown in Fig. 1b, in explants cultured at pH 6.8, the p89 fragment of PARP-1 abundance is significantly higher in comparison to control, indicating exacerbated apoptosis in this condition. When leptin was added, a

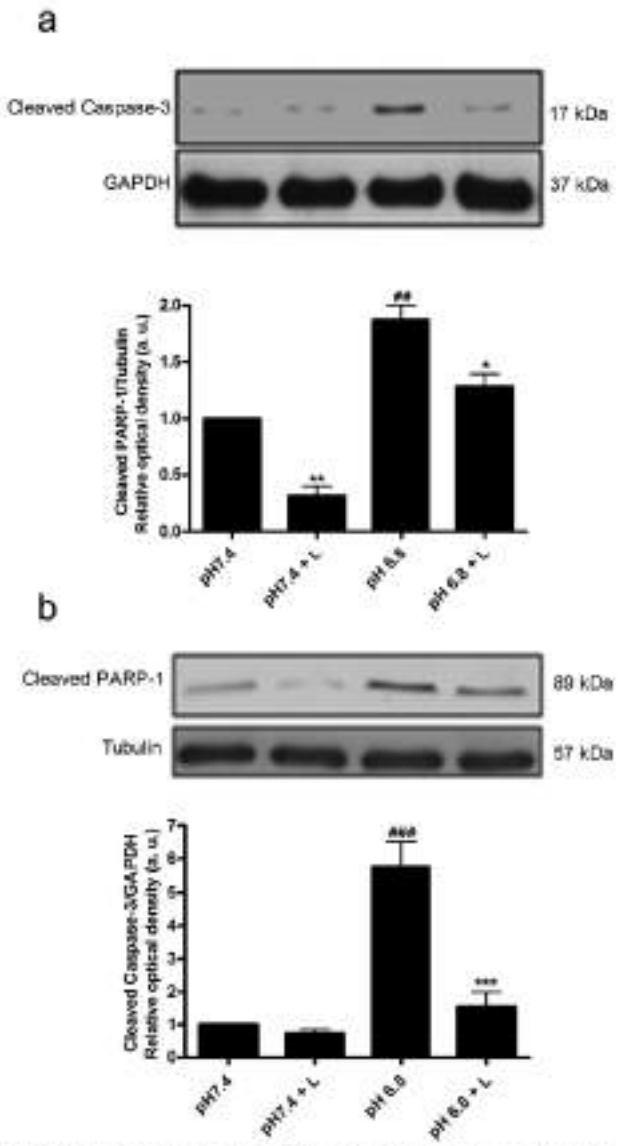


Fig. 1 Leptin reduces apoptosis in human trophoblast explants cultured at low pH. Human trophoblast explants were processed as described in "Materials and methods" and cultured in DMEM-F12 media at pH 7.4 or 6.8 during 5 h in the presence or absence of 10 nM leptin (L). Caspase-3 cleaved fragment (a) and the p89 fragment of PARP-1 (b) were determined by Western blot analysis. Cell extracts were prepared as indicated in "Materials and methods." Molecular weights were estimated using standard protein markers, and molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting the same membranes with anti-GAPDH or anti-Tubulin. Band densitometry is shown in the lower panels. Results are expressed as mean ± SD for the three independent experiments. Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison post hoc test, relative to pH 7.4 without leptin (*) or pH 6.8 without leptin (#). $^{**}p < 0.001$; $^{***}p < 0.001$

significant decrease of cPARP-1 expression is observed at pH 6.8 as well as pH 7.4. These results suggest that an acidic environment promotes apoptosis of human trophoblast explants, and leptin attenuates this effect.

Leptin regulates the balance of apoptotic mitochondrial intermediaries in acidic conditions

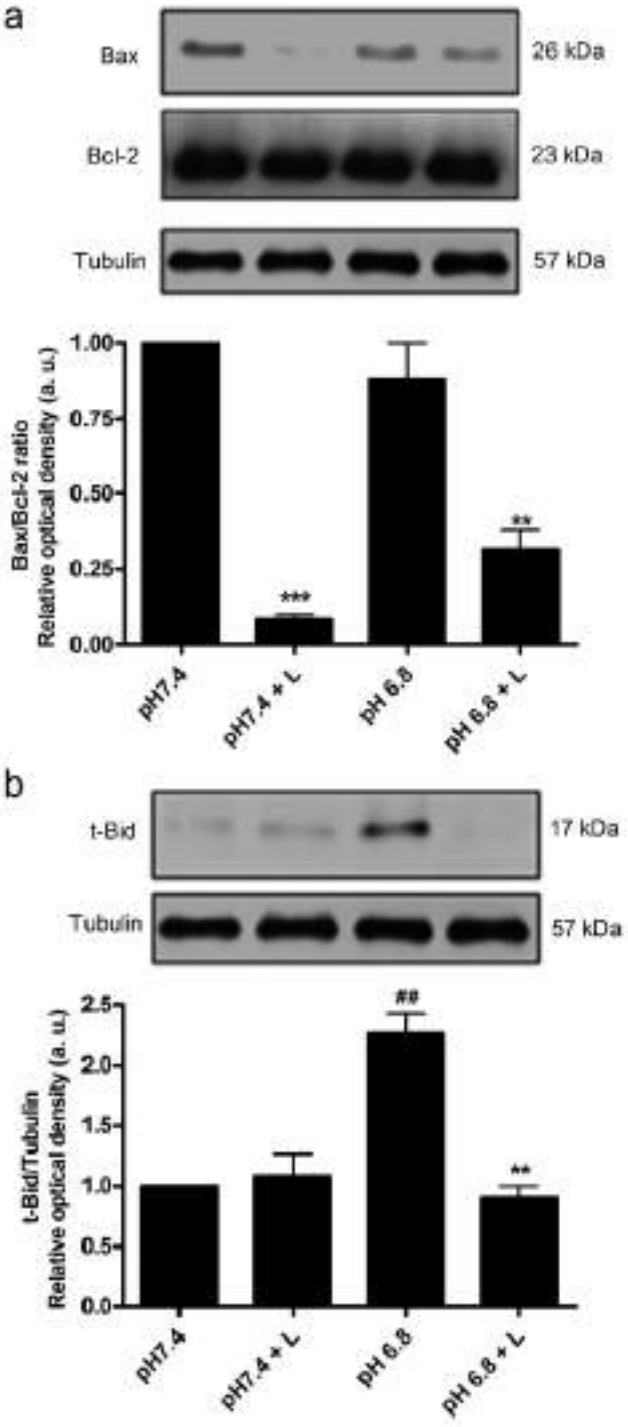
Next, we decided to evaluate whether the expression of these apoptotic intermediaries changes when trophoblast explants are cultured at pH 6.8. We found that low pH did not significantly alter Bcl-2 and Bax expressions. However, leptin treatment considerably reduced Bax expression at both pH, observing a greater effect at pH 7.4. Bax:Bcl-2 ratio is analyzed in Fig. 2a. On the other hand, we evaluated Bid activation by studying t-Bid expression. As shown in Fig. 2b, low pH produced an increase in t-Bid abundance (2.25-fold increment) and leptin reduced its expression to values comparable to the control at pH 7.4. These findings suggest that the intrinsic apoptotic pathway is involved in apoptosis triggered by acidosis, and leptin exerts an anti-apoptotic effect.

Acidic stress increases p53 expression and phosphorylation, and leptin inhibits this pro-apoptotic action

We continued the study of pH-induced apoptosis by analyzing p53 expression and its phosphorylation in S46. Western blot assays were performed, and we found that media acidification generated a 2.1-fold upregulation of p53 expression (Fig. 3a), accordingly with the increment in apoptosis described above. It could be observed that leptin treatment diminished p53 expression levels to 50% at control pH and to 66% at low pH. Then, we analyzed the levels of S46 p53 (Fig. 3a') and found that low pH incremented p53 phosphorylation (1.45-fold increment), and independently of the pH evaluated, leptin markedly decreased p53 phosphorylation. Moreover, at pH 6.8, a greater effect on pS46 p53 was observed, reaching values lower than at pH 7.4 (Fig. 3a'). These results reinforce that apoptosis is triggered by acidic pH in trophoblast explants and demonstrate that p53 pathway is activated in acidic stress conditions. Also, these findings reinforced the notion of leptin as a cytokine capable to regulate p53 pathway under acidosis.

Leptin boosts Mdm-2 in an acidic state

We decided to evaluate if low pH regulates Mdm-2 expression. As shown in Fig. 4, acidic pH generated a 2.85-fold increment in Mdm-2 levels, and in these conditions, leptin further increased in approximately two times Mdm-2 expression. However, at control pH, leptin did not significantly modify Mdm-2 levels. These results suggest that Mdm-2 expression could be upregulated by acidic pH. These findings may suggest that in acidic conditions, leptin is downregulating p53 levels through increasing Mdm-2 levels (Fig. 5).



◀ Fig. 2 Bax:Bcl-2 ratio and t-Bid expression are decreased by leptin acidic conditions. Human trophoblast explants were processed as described in "Materials and methods" and cultured in DMEM-F12 media at pH 7.4 or 6.8 during 5 h in the presence or absence of 10 nM leptin (L). Bax and Bcl-2 expressions were determined by Western blot, and Bax:Bcl-2 ratio was estimated (a). The truncated form of Bid (t-Bid) was determined by Western blot analysis (b). Cell extracts were prepared as indicated in "Materials and methods." Molecular weights were estimated using standard protein markers, and molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting the same membranes with anti-Tubulin. Band densitometry is shown in the lower panels. Results are expressed as mean ± SD for three the independent experiments. Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison post hoc test, relative to pH 7.4 without leptin (*) or pH 6.8 without leptin (#). $^{*,\#}p < 0.01$; $^{***}p < 0.001$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$

Discussion

The placenta is a complex fetal organ that performs pleiotropic functions during fetal growth (Desoye and Hauguel-de Mouzon 2007). Normal fetal development is dependent upon a sufficient oxygen, nutrient, and waste exchange through the placenta (Wulff et al. 2003). As the demand of the developing fetus for oxygen increase, the capacity of the maternal blood vessels to supply this must be altered radically and deficiencies in this process are associated with several dangerous pregnancy complications (Cartwright et al. 2010). In early pregnancy, a moderately hypoxic environment is crucial for appropriate embryonic development since normal proliferation and differentiation of trophoblastic cells may be driven by low-oxygen concentration in the decidua (James et al. 2006; Castro-Parodi et al. 2013). Oxygen enrichment of fetal blood is promoted by partial pressure differences in the feto-maternal circulation. When the placenta is exposed to lower pressure of O_2 (PO_2) during invasion, physiologic placental remodeling is impaired (Cartwright et al. 2007). Thus, several conditions in the mother could be linked to sub-optimal oxygen supply to the feto-placental unit, and the biological mechanisms may vary. A mother with anemia and with heart or with lung diseases or diabetes has reduced the ability to provide her fetus with sufficient oxygen. Older mothers also may not be the best oxygen providers to their offspring (Eskild et al. 2016). Hemoglobin affinity for oxygen is determined by the pH and partial pressure of carbon dioxide (PCO_2); for example, when PCO_2 is incremented or the pH is reduced, hemoglobin affinity for oxygen decreases producing acidosis. Thereby, acidic environment could impair the correct development of the fetus as well as the anatomy and function of the placenta (Bobrow and Soothill 1999; Avagliano et al. 2015).

In recent years, the importance of apoptotic cascade for the correct function of the trophoblast has become evident. It is well-stablished that apoptotic process is a naturally occurring event in the placenta and has a major role in maintaining the integrity of villous trophoblasts (Levy and Nelson 2000; Huppertz et al. 2006; Heazell et al. 2011). The cells of vital organs have been demonstrated to be at risk of apoptosis at low pH levels (Lin et al. 2016). There are various reports about low extracellular pH effect in tumor cells since these cells retain lower pH than that of normal tissues (Vaupel et al. 1989). To date, there has been very few studies about the consequences of acidosis during pregnancy, and to our knowledge, the consequences of acidic stress in apoptosis of

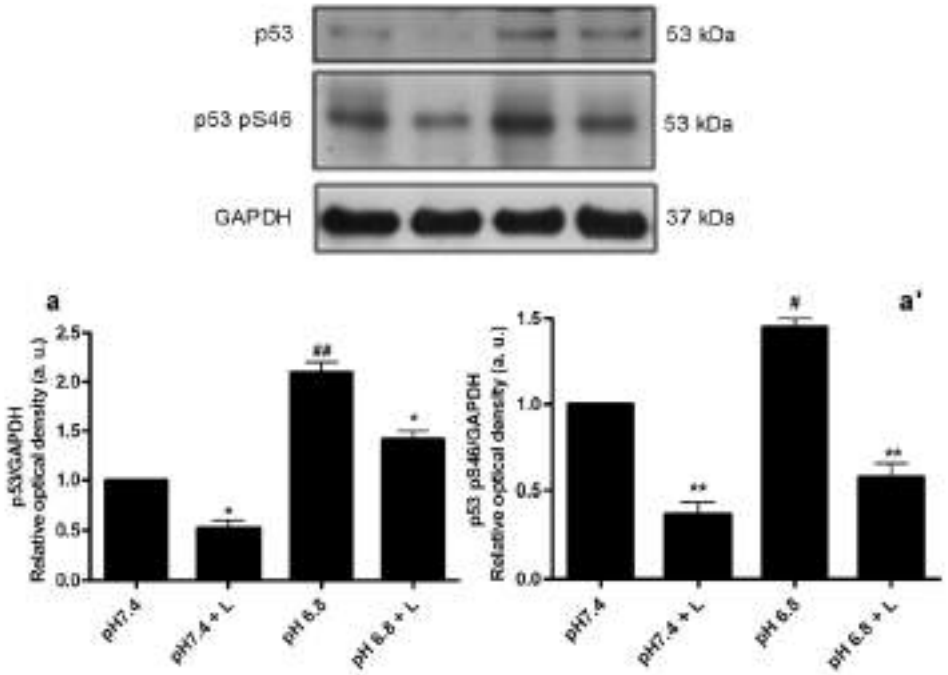


Fig. 3 Acidic stress increases p53 and pS46 p53 expressions, and leptin impairs this effect. Human trophoblast explants were processed as described in "Materials and methods" and cultured in DMEM-F12 media at pH 7.4 or 6.8 during 5 h in the presence or absence of 10 nM leptin (L). p53 (a) and pS46 p53 (a') expressions were determined by Western blot analysis. Cell extracts were prepared as indicated in "Materials and methods." Molecular weights were estimated using standard protein markers, and molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting the same membranes with anti-GAPDH. Band densitometry is shown in the lower panels. Results are expressed as mean ± SD for three the independent experiments. Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison post hoc test, relative to pH 7.4 without leptin (*) or pH 6.8 without leptin (#), $^{*}p < 0.05$; $^{**}p < 0.01$; $^{\#}p < 0.05$; $^{\#\#}p < 0.01$

placental cells were not researched. Based on the importance of the acid-base balance for the success of the gestation and the little discussion available about low pH effect on placental cells, this research attempted to evaluate a possible effect of acidosis on trophoblast survival and the participation of leptin as a placental cytokine. For this aim, human trophoblast explants were cultured at physiological pH (pH 7.4) and at low pH (pH 6.8) in the presence or absence of leptin.

In the present work, we first decided to study the effect of low extracellular pH in placental cells by evaluating caspase-3 and cPARP-1, two apoptosis hallmarks. PARP-1 is a nuclear DNA-binding protein involved in DNA repair and apoptosis which is cleavage by caspase-3 during early apoptosis (Chaitanya et al. 2010). We found that acidic stress increased the expression of caspase-3 active form, as well as the abundance of the p89 fragment of PARP-1, suggesting that low pH induces apoptosis on placental cells. These results are in agreement with Aoyama et al. (2005) who found that acidosis increased caspase-3 activation as well as caspase-12 mRNA and protein expressions in astrocytes. Moreover, it was recently reported that acidic extracellular pH induces cleavage of caspase-9 and PARP in Jurkat T lymphocytes, results that support our findings (Kim et al. 2017). On the other hand, when leptin was added to culture media, apoptosis was strongly reduced suggesting a pro-survival effect of leptin. This evidence reinforces the anti-apoptotic leptin effect in the placenta described by several previous works of our group (Magarinos et al. 2007; Perez-Perez et al. 2008; Toro et al. 2014; Pérez-Pérez et al. 2016).

Mitochondria are crucial, multifunctional organelles which actively regulate cellular homeostasis and are directly involved in triggering different and complexly interconnected programs promoting cell survival or death (Apostolova et al. 2011). Mitochondrial remodeling by acidosis, through the activation of a dual program that modulates mitochondrial dynamics and architecture, represents a novel and physiological pathway that sustains mitochondrial integrity and ATP production despite oxygen limitations (Khacho et al. 2014). Since the balance between the pro-apoptotic and anti-apoptotic members of the Bcl-2 family proteins and their up- and downregulations usually determine the fate of the cells by either undergoing apoptosis or surviving in an organ pathophysiology (Sinha et al. 2013), we decided to analyze the intrinsic apoptotic pathway in acidic conditions. We found that pro- and anti-apoptotic intermediaries like Bax and Bcl-2 are not influenced by low pH, but t-Bid abundance is augmented in acidic stress conditions. These results suggest that intrinsic apoptotic pathway might be involved in low pH-

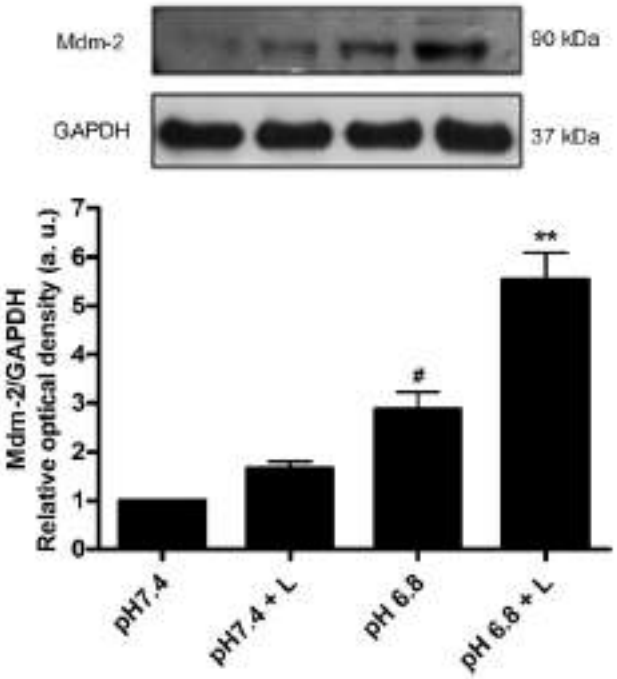


Fig. 4 Leptin increases Mdm-2 at low pH. Human trophoblast explants were processed as describe in "Materials and methods" and cultured in DMEM-F12 media at pH 7.4 or 6.8 during 5 h in the presence or absence of 10 nM leptin (L). Mdm-2 expression was determined by Western blot analysis. Cell extracts were prepared as indicated in "Materials and methods." Molecular weights were estimated using standard protein markers, and molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting the same membranes with anti-GAPDH. Band densitometry is shown in the lower panels. Results are expressed as mean ± SD for three the independent experiments. Statistical analyses were performed by ANOVA and Bonferroni's multiple comparison post hoc test, relative to pH 7.4 without leptin (*) or pH 6.8 without leptin (#),**$p < 0.01$; *$p < 0.05$

induced placental cell apoptosis. In this regard, Sharma et al. (2015) have described that acidosis-triggered apoptosis of Raji cells and suggested that apoptosis induction is associated with Bax. Otherwise, Kim et al. (2017) have investigated Bid levels in low pH conditions but they found that t-Bid bands remained intact. We consider that the observed differences could be due to the varied pH analyzed. So, it could be thought that at different acidic conditions, distinct actors of intrinsic pathway are regulated. In this line, we propose that pH 6.8 is sufficient to the cell to regulate t-Bid levels, which are related with Bax localization and oligomerization (Eskes et al. 2000), while at more extreme conditions, Bax expression regulation could be necessary. Independently of the pH assayed, we found that leptin reduce Bax/Bcl-2 ratio. We also detected a diminution of t-Bid abundance when leptin was added at pH 6.8. These results reinforce the leptin anti-apoptotic effect described previously. Additionally, results of our group demonstrated that in serum deprivation and hyperthermia conditions, leptin decreases the expression of pro-apoptotic proteins while increases the levels of anti-apoptotic intermediaries involved in apoptotic intrinsic pathway (Toro et al. 2014; Pérez-Pérez et al. 2016).

p53 is a transcription factor which is activated in response to different stress stimuli to allow growth arrest and apoptosis (Brady and Attardi 2010). It was reported that p53 could play an important role in cellular differentiation and in the control of the invasion of trophoblastic cells, highlighting the importance of this protein for placental development (Cohen et al. 2007). To further examine acidosis effect on placental cell apoptosis, we decided to evaluate p53 pathway. There was an increase of p53 expression associated with acidic pH, indicating that low pH affects p53 levels. Very little was found in the literature on the question of p53 signaling at acidic conditions. In this line, Lin et al. (2016) described that low pH exposure of cardiomyocytes increments p53 and caspase-3 mRNA levels, and Sharma et al. (2015) reported that NF- B translocation to nucleus in response to acidic environment could indicate that p53 is regulated by NF- B in Raji cells. At the same time, when leptin was added, p53 expression was decreased. Similar effects were perceived when phosphorylation on S46 of p53 was analyzed, since we found that low pH increases their levels while leptin treatment diminishes them. At this point, the present work produced results which corroborate the findings of previous works of our group where we described a p53 downregulation by leptin (Toro et al. 2014, 2015). Another important finding was that acidic stress also affects Mdm-2 expression. Mdm-2 constitutes a feedback loop in which p53 activates Mdm-2 gene expression, increasing Mdm-2 protein levels which then bind p53 and inhibits its activity (Inoue et al. 2005). Our results demonstrated that low pH increments Mdm-2 expression, and leptin increases even more such levels. These results are consistent with the increment of p53 expression and phosphorylation described above as Mdm-2 expression is regulated by p53. On the other hand, leptin-induced Mdm-2 expression does not seem to be mediated by p53, which is downregulated. In fact, the upregulation of Mdm-2 may contribute to the leptin effect downregulating p53 levels. Mdm-2 expression is known to be upregulated by signaling, such as PI3K pathway (Gottlieb et al. 2002), which is activated by leptin in human trophoblast (Perez-Perez et al. 2010). Besides, it is known that stress conditions could induce the inhibition of p53 nuclear export, promoting p53 accumulation. Moreover, other mechanisms enhancing the nuclear import rate or the disrupting the interaction between p53 and its cytoplasmic-binding partners could be involved (Inoue et al. 2005). Further research with more focus in Mdm-2 regulation and p53 localization should be done to clearly understand leptin regulation of p53 pathway.

During hypoxia, restricted gas exchange increments carbon dioxide concentration leading acidosis. PE is a disorder associated with maternal hypertension, reduction in placental

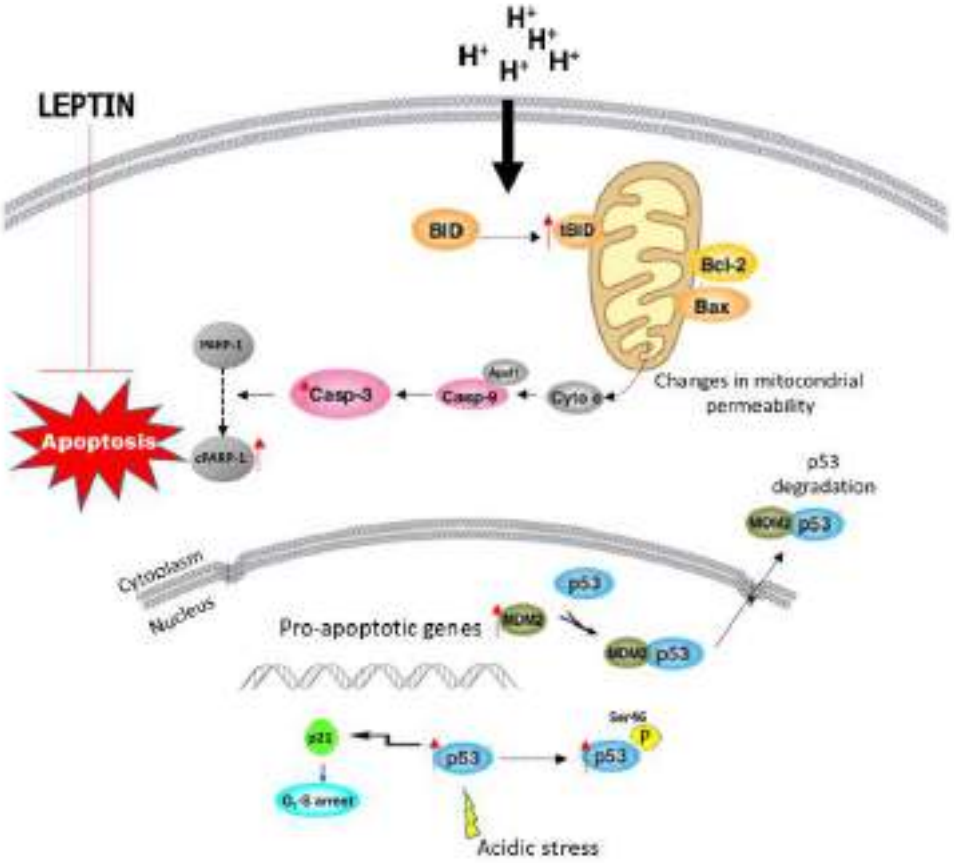


Fig. 5 Proposed model. Apoptotic intrinsic pathway and p53 signaling pathway are induced by acidic stress in human trophoblast cells (red arrows indicate the increased expression of pro-apoptotic intermediaries during acidosis). Leptin treatment reduces low extracellular pH effects and, thereby, prevents apoptosis

blood flow, and placental hypoxia. Placentas from preeclamptic women show vascular abnormalities and inflammation compared to placentas from healthy pregnancies, suggesting a role for inflammation in the disease (Harmon et al. 2016). Besides, chronic inflammation in obese women produces mitochondrial dysfunction in the trophoblast. Expression of leptin is strongly associated with various inflammatory responses and the immune system (Pérez-Pérez et al. 2017a, b) and plays crucial role in the pathophysiology of obesity and development of diabetes mellitus and insulin resistance (Rehman et al. 2018). In this sense, PE is characterized by increased levels of leptin (Mise et al. 1998; Grosfeld et al. 2001; Pérez-Pérez et al. 2017a, b). Grosfeld et al. reported that leptin gene expression is upregulated under hypoxic conditions in BeWo cells. In addition, Meißner et al. (2005) measured leptin levels during hypoxia in JAr cells and found that leptin production is increased at an oxygen tension of 1%. They also investigated leptin role on apoptosis and their results suggested that leptin does not influence apoptotic pathways in JAr cell line under hypoxic and non-hypoxic conditions. That evidence is not in agreement with our previous results that position leptin as an anti-apoptotic hormone in the placenta; however, the difference could be due both to the experimental model and to the higher leptin doses used. In this line, most of the studies that investigated the role of leptin in reproduction have used supra-physiological leptin concentrations, and this may have resulted in conflicting results to determine the role of leptin (Herrid et al. 2014). Nevertheless, the mechanism involved in trophoblast response to hypoxia and leptin role in this context has not been sufficiently explored. Nevertheless, we thought that acidosis could promote inflammation during PE, and leptin might have a protective role that could explain the observed leptin upregulation in this pathology. In this line, we propose that inducing acidic stress could be a good approach to advance in the study of leptin association with PE.

In this work, we have evaluated how alteration of acid-base balance affects placental cell survival. Our findings, for the first time, provide evidence for induction of apoptosis by acidic stress in the placenta. In this context, we have demonstrated that leptin exerts a pro-survival action, in agreement with our previous results. However, these results not only reflect that leptin protects from apoptosis but also highlight its pleiotropic effects on different pregnancy aspects. Particularly, we demonstrated that leptin could regulate the intrinsic apoptotic pathway and p53 signaling in an acidic environment. This is a very important observation, because in reviewing literature, no data was found on the association between low pH and leptin expression or effects.

It is widely accepted that leptin plays an integral role in the normal physiology of the reproductive system and has a wide

range of biological functions on trophoblast cells involved in successful establishment of pregnancy. Moreover, the present work also brings knowledge about leptin effects in a possible pathophysiologic context, which could be useful for designing novel therapeutic strategies using leptin. To address this issue is very important since a number of evidence suggested that leptin might have potential as a treatment for diverse pathologies including the malfunctioning of the reproductive system (Pérez-Pérez et al. 2017a, b).

Finally, it should be noticed that in this study, only trophoblast explants which are not exclusively comprised by trophoblast cells were employed. However, we consider that the study of human placenta tissue is really important because trophoblast explants represent an interesting physiological model. In the next stage of our research, we plan to complement this work by using a trophoblast cell line. In addition, future studies with more focus on p53 post-translational modifications and localization will be interesting to fully explain p53 signaling regulation by leptin. Moreover, we considered that the study of signaling pathways involved in leptin anti-apoptotic effect will be very interesting to better understanding the mechanisms involved on leptin action.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Increased Expression of Aquaporin 9 in Trophoblast From Gestational Diabetic Patients

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Key words
- gestational diabetes mellitus
- aquaporins
- AQP9
- placenta

Abstract

Gestational diabetes mellitus is the most frequent pathophysiological alteration in pregnancy, increasing the incidence of complications in both mother and fetus. The macrosomia that occurs in these fetuses may be related with some changes in nutrient transport mechanism in placenta. The presence of aquaporin 9, an aquaglyceroporin, has previously been demonstrated in placenta. We raised the question whether aquaporin 9 expression may be upregulated in placenta from gestational diabetes, thus providing a faster transport of glycerol and water through placenta. We studied 21 placentas (13 controls and 8 gestational diabetes) from cesarean delivery at term. The expression of aquaporin 9 was analyzed by quantitative PCR, immunoblot, and immunohistochemistry. The median values from quantitative PCR were compared by nonparametric tests for independent samples (Mann-Whitney U-test). We have found that trophoblast from gestational diabetes express higher amount of aquaporin 9, which was found statistically significant ($p < 0.05$). The increase in aquaporin 9 expression was confirmed by immunoblot, and localization in the syncytiotrophoblast was checked by immunohistochemistry. The increase in aquaporin 9 expression in placenta from gestational diabetes may contribute to the higher transport rate in this pathology of pregnancy.

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Introduction

Aquaporins (AQPs) are a family of integral membrane proteins. There are 13 known AQPs in mammals. Their structure is tetrameric with monomers (<30kDa) containing 6 membrane-spanning helical domains surrounding an aqueous pore [1, 2]. Three subgroups of AQPs have been described according to their structure and functional properties: The "classical aquaporins", which only permeate water; the "aquaglyceroporins" that comprise AQP3, 7, 9, and 10, which are also permeable to urea and glycerol, and the "super-aquaporins", AQP11 and 12, which are localized in the cytoplasm and whose permeability has not yet been fully determined [3]. In the particular case of AQP9, it can also facilitate the flux of neutral solutes such as monocarboxylates, purines, and pyrimidines.

AQPs are also expressed in placenta and fetal membranes where they seem to play an important role in amniotic fluid volume regulation. Previously, Damiano and colleagues demonstrated that the localization of AQP9 was not only in apical and basal membranes but also in the cytoplasm of human preeclamptic placenta, which was found increased 2.5-fold compared with normal term placentas. Moreover, they found that there was a lack of functionality of AQP9 for water and mannitol transport, but there is evidence that this aquaporin is involved in the excretion of urea across syncytiotrophoblast of human placenta from mother to fetus [4, 5]. It is known that a proper development of pregnancy depends on high-quality ovulation, successful fertilization, normal embryonic and fetal development, and homeostasis of amniotic fluid. Throughout pregnancy, AQPs are expressed in placenta, uterus, adnexa, brain, urinary system, and the lacrimal gland. The regional and temporal regulation of AQPs plays important roles in normal pregnancy, fetal growth, and homeostasis of amniotic fluid volume. The pregnant phenotypes of aquaporin-knockout mice provide direct evidence that AQPs deficiency results in adverse outcome of pregnancy. Changes in the expression regulation of aquaporins are seen in preeclampsia, abnormal amniotic fluid volume,

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chorioamnionitis, and maternal undernourished pregnancy [6]. Gestational diabetes mellitus (GDM) is the most frequent pregnancy alteration, affecting 4–8% of all pregnancies [7,8], and increasing the risk of both the mother and the fetus for adverse events. In vitro studies showed an upregulation in transport system for some amino acids in placenta. The alteration of placenta function may be the reason for abnormal fetal growth [9] observed in this pathology of pregnancy [10]. Women with GDM have increased plasma leptin levels [11]. Besides, insulin levels are also increased in GDM, and hyperinsulinemia may mediate increase of leptin synthesis in placenta [12]. In this sense, our group has recently described increased leptin and leptin receptor expression in placentas obtained from GDM [13], and insulin induces leptin expression in trophoblastic cells, enhancing the activity of leptin promoter region [14]. Increased insulin and leptin levels may modulate the expression of AQP9, as it was previously described in placenta [15], as well as adipocytes and hepatocytes [16]. Even though the functional role of AQPs in placenta remains to be elucidated, its expression and regulation in abnormal pregnancy may point to new potential therapeutic targets for the pathology of pregnancy. In this context, increased AQP9 expression has been found in preeclamptic placenta [5]. We raised the hypothesis that AQP9 expression may also be increased in GDM. In the present study, we have examined the expression of AQP9 in human syncytiotrophoblast of placentas from normal and GDM pregnancies.

Subjects and Methods

Subjects

This study was approved by the local ethical committee and informed written consent was obtained from all subjects before the collection of samples. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

Term placentas (n = 21, 13 from healthy pregnancies and 8 from GDM) were obtained after programed cesarean section by the Obstetric-Gynecology service of the Hospital Virgen Macarena. The patients were diagnosed with GDM based on ADA criteria: Glycemia fasting >92 mg/dl (5.1 mmol/l); glycemia 1-h post glucose intake >180 mg/dl (10 mmol/l); glycemia 2-h post glucose intake >153 mg/dl (8.5 mmol/l). Clinical data are shown in ◉ **Table 1**.

Human placentas were immediately suspended in ice-cold phosphate-buffered saline (PBS) and transported to the laboratory (10–20 min) for being processed within 1–2 h after delivery as previously described [17]. Briefly, placentas were washed 2–3 times in sterile PBS to remove excess blood. Villous tissues, free of visible infarct, calcification, or hematoma were sampled from at least 5 cotyledons at a distance midway between the chorionic and basal plates. These core parts of cotyledons were cut into multiple cubic segments (10–15 mg wet weight) and thoroughly rinsed with cold DMEN-F12 medium pH 7.4 (137 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, and 4 mM $NaHCO_3$).

Table 1 Subject characteristics of healthier cases and GDM.

Characteristics	Healthy (n = 13)	GDM (n = 8)	p-Value
Maternal age at delivery (years)	28.1 ± 6.0	34.8 ± 5.0	< 0.05
Gestational age at delivery (weeks)	39.5 ± 0.7	37.6 ± 1.4	ns
Infant weight (g)	3 120 ± 67.2	3545 ± 285	< 0.05
Placenta weight (g)	430 ± 57	575 ± 88	< 0.05
IMC (kg/cm²)	26	28.4	ns

Values are mean ± SD. ns: Not significant

Quantitative real time PCR assay

Total RNA was extracted from placental samples using Trisure reagent, according to the manufacturer's instructions (Bioline Co). Concentration and purity of the isolated RNA were estimated by spectrophotometry at 260 and 280 nm with purity in A_{260}/A_{280} ratio around 2.0.

For cDNA synthesis, 5 µg of total RNA was reverse-transcribed at 55 °C during 1 h using the Transcriptor first Strand cDNA synthesis Kit (Roche). qPCR reaction was performed using the following primers sequences: AQP-9 sense: 5'GAAAAGACTGAGCCAGAG GAA3', AQP-9 Antisense: 5'AGACCCTCATTGTCTGGGTCTA3' and cyclophylin, forward primer: 5'CTTCCCCGATACTTCA3' and reverse primer: 5'TCTTGGTGCTACCTC3'.

RT-qPCR Master Mix Reagent kit was "SensiMixTM Plus SYBR Kit" (Quantace), and PCR reactions were performed on MJ Mini BioRAD (Bio Rad).

The reaction was initiated by preheating at 95 °C for 10 min. Subsequently, 45 amplification cycles were carried out as follows: denaturation 15 s at 95 °C, 30 s annealing at 58 °C, and 30 s extension at 72 °C. The threshold cycle (CT) from each well was determined by the Bio RAD CFX Manager Program. Relative quantification was calculated using the $2^{-\Delta CT}$ method, using cyclophylin as control expression. For qPCR, samples were run in triplicates with 5% intra-assay variability, and 11% interassay variability.

Western blot analysis

Total protein level was determined with "BCA Protein Assay Kit" (Pierce, Rockford, IL, USA) by the bicinchoninic acid method (Thermo Scientific), using bovine serum albumin as standard (BSA, Sigma Chemical Co, St. Louis, USA).

Supernatants were mixed with Laemmli's sample buffer containing 2% SDS and 30 mM β-mercaptoethanol, boiled for 5 min, resolved by SDS-PAGE on a 10% gel, using Prestained Molecular Markers Broad Range (Bio Rad) as PM marker. After that, the samples were electrophoretically transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences).

Membranes were equilibrated in 1× PBS and nonspecific binding sites were blocked by albumin solution (5%) in PBS at room temperature for 30 min. Membranes were then immunoblotted with polyclonal antibodies that detect human AQP9 (1:1 000, AlphaDiagnostic). Loading controls were performed by immunoblotting the same membranes with monoclonal anti-Tubulin (1:1000, Santa Cruz Biotechnology Inc.). Membranes were then incubated with secondary antibodies using horseradish peroxidase-linked anti-mouse/anti-rabbit immunoglobulins. Bound horseradish peroxidase was visualized by a highly sensitive chemiluminescence system (Super Signal from Pierce). The bands obtained in the blots were scanned and analyzed by the PCBAS 2.0 program (Raytest, Straubenhardt, Germany). The relative optical density of the different bands was normalized by the corresponding intensity of the GAPDH immunoblot in each individual experiment.

Immunohistochemistry

Human villous tissues were cut into small pieces, fixed overnight in 10 per cent formaldehyde in 0.1 M sodium phosphate

buffer, pH 7.4, dehydrated, and embedded in paraffin. Paraffin sections (4µM) were cut and mounted on 2% silanized slides, dried, dewaxed and rehydrated. Tissue slices were incubated 30min in 3% H_2O_2/methanol to block endogenous peroxidase, and washed with PBS. All subsequent steps were carried out in a humidified chamber. Non-specific binding sites were blocked by incubation at room temperature in serum solution 1:20 for 20min. Before removal of the serum solution, sections were incubated 18–24h at 2–8°C with anti-AQP9 antibodies at a dilution of 1:500. The sections were then washed in PBS (3×, 5min each) and incubated with their respective secondary antibodies. The manufacturer's instructions were followed for the sequential incubation and durations for the exposure to the secondary antibodies. After washing with PBS, the sections were incubated with diaminobenzidine substrate kit (Dako, Carpinteria, CA, USA) for 5min that resulted in a brown-colored precipitate at the antigen-antibody binding sites and the reaction was stopped in distilled water. After removing the slides from water, all the sections were dehydrated and one drop of aqueous mounting medium (Dako Paramount) was applied, and the sections were cover-slipped.

Finally, the immunohistochemical specimens were examined using a Leica Laborlux S Microscope (Leica Microsystem GmbH, Wetzlar, Germany) with a Nikon DS12 photo digital system (Nikon Corp, Tokyo, Japan). Each sample was analyzed with a double-blind system by 2 different operators under magnifications up to 100×. A cell was considered as positive when it demonstrated distinct brown surface staining. Representative sites in each sample were photographed at 100× magnification and captured with a software system (CS3, version 10.0.1; Adobe Photoshop, San Jose, CA, USA).

Statistical analysis

In placental explants, immunoblot are a representative experiment from the 21 placentas studied (13 from healthy pregnancies and 8 from GDM). Results are expressed as mean±SD. The statistical significance was assessed by ANOVA followed by Bonferroni's multiple comparison post hoc test and was calculated using the GraphPad Instat computer program (GraphPad, San Diego, CA, USA). A p-value less than 0.05 was considered statistically significant.

Results

Since we have hypothesized that AQP9 may be upregulated in placenta from gestational diabetes, we have investigated the AQP9 expression in placenta from gestational diabetic women compared to that from control women, using quantitative PCR. PCR data were analysed using the CFX Manager™ Software Version 1.5 of BIO RAD program and then were subjected to statistical analysis by GraphPad Instat computer program. As shown in ⊙ **Fig. 1**, samples of placentas of women with GDM have a significant higher expression of AQP9 gene compared with control placentas.

In order to assess whether the increase in AQP9 gene expression produced higher amount of AQP9 protein, we carried out immunoblot analysis. ⊙ **Fig. 2** shows a representative immunoblot of 2 gestational diabetic samples compared with 2 control samples. We have found that trophoblast from gestational diabetes patients express higher amount of aquaporin 9 than trophoblast

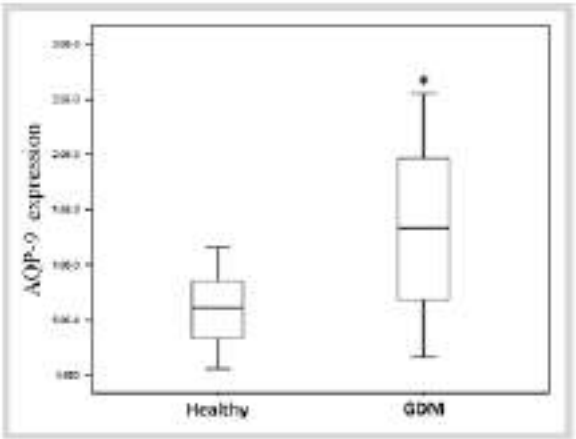

Fig. 1 Increased expression of Aquaporin 9 (AQP9) in placentas from pregnancies complicated with gestational diabetes mellitus (GDM) compared with placentas from healthy pregnancies. Placental samples were obtained from 13 control placentas and 8 placentas from GDM. AQP-9 mRNA was quantified with qRT-PCR. RNA was extracted as described in Materials and Methods. Cyclophilin was used as internal standard. Statistical analyses were performed by ANOVA. Asterisks indicate significant differences from the control according to Bonferroni's multiple comparison post hoc test. * $p<0.05$ indicates significant differences from the control.

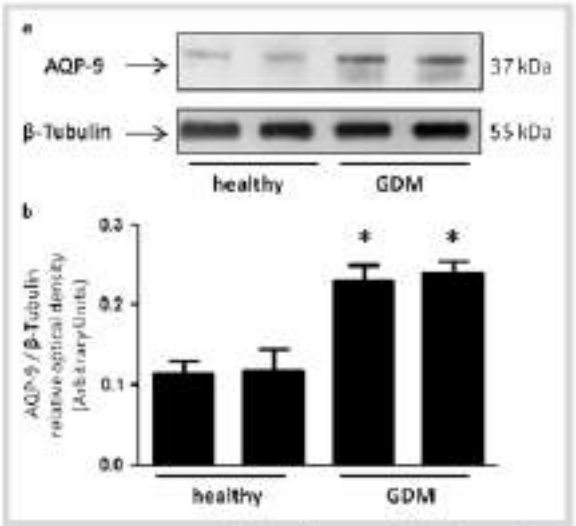

Fig. 2 AQP9 expression in healthy and gestational diabetic placental (GDM) explants. **a** Placental extracts were prepared and proteins were separated on SDS-PAGE gels. AQP-9 was determined by Western blot analysis. Molecular weights were estimated using standard protein markers. Molecular mass (kDa) is indicated at the right of the blot. Loading controls were performed by immunoblotting in the same membranes with anti-tubulin. A representative immunoblot shows a 37 kDa band corresponding to the glycosylated form of AQP9 in normal and GDM placental explants. **b** Densitometry of immunoblots containing AQP9 protein level expression was performed, and after normalization for β-tubulin, the values were plotted as AQP9/β-tubulin relative ratio. Each plotted value corresponds to the mean± SD obtained from five 5 independent experiments ($p<0.05$).

Fig. 3 Localization of AQP-9 in healthy and GDM placenta: Representative sections from Healthy and GDM placental explants evidencing the AQP-9 expression. **a** Negative controls were performed by omitting the primary antibody and replaced by a non-immune rabbit serum. Immunohistochemistry revealed specific labeling in the apical membrane of syncytiotrophoblast cells from healthy placental explants. In placentas from patients with GDM, AQP-9 labeling was stronger in the apical membrane. Magnification × 400. (Color figure available online only).

from control patients. These results are in line with those obtained by qPCR.

The localization of leptin expression in control and GDM women trophoblasts was verified by immunohistochemistry. AQP9 was expressed in the syncytiotrophoblast as previously described. ◉ **Fig. 3** shows immunohistochemistry analysis for one placenta cut from a healthy sample and one section of placenta from women with GDM. Data reveal that the expression level of AQP9 is present in the syncytiotrophoblast, and placenta samples from women with GDM express higher amount of AQP9 than those obtained from healthy placentas.

Discussion

Gestational diabetes is a health problem that affects both the mother and the child, increasing the risk of death during the perinatal period. The main pathophysiological complications of GDM are due to fetal macrosomia, which is accompanied by a larger size and weight of the placenta to support the increased needs of the macrosomic fetus. Fetal macrosomia requires a greater availability of nutrients, which are provided by various transport systems, such as amino acids, fatty acids, and glucose. Another important nutrient for energy provision is glycerol, transported by some AQPs, such as AQP3, AQP7 and AQP9.

Our research group has been working on the role of leptin in the growth and metabolism of the placenta [18] where the trophic action of leptin may mediate an increase in the size of the placenta from DMG, since both the expression of leptin and its receptor are increased [13]. These effects appear to be mediated by activation of protein synthesis by acting on the PI3K and MAPK pathways, which converge at the point of activation of protein translation signaling [18]. Moreover, leptin seems to increase AQP9 expression in trophoblast explants in vitro (data not shown).

In this context, it seemed reasonable to consider that the transport system of glycerol, namely AQP9 trophoblast was overexpressed in placentas from women with GDM, and the leptin/leptin receptor system could play an important role, since the system is activated in the placenta from GDM. We have found that, indeed, the amount of AQP9 mRNA, as well as protein amount in trophoblasts from placentas of women with GDM is higher than that observed in placental trophoblasts from control women. Besides, the AQP9 localization was confirmed in syncytiotrophoblast as previously described [5].

The increased expression of AQP9 in the syncytiotrophoblast from placentas with GDM could mediate the increased transport of glycerol to the fetus, thus contributing to attend the increased energy intake requirements in the macrosomic fetus. On the other hand, we cannot exclude the possible role of other AQPs, such as AQP3, which is known to be present in the syncytiotrophoblast. In any case, the increased expression of AQP9 may contribute to the increase in water and/or glycerol transport. Regarding the underlying mechanisms producing this increase in AQP9, we may speculate that a possible candidate may be leptin, whose plasma levels are increased in GDM. In this context, the possible effect of leptin on AQP9 expression in vitro is being currently investigated. Regardless of the mechanism, with the limitation of the number of samples, the present data strongly suggest the increased expression of AQP9 in trophoblast from GDM and thus, the increased glycerol transport to the fetus may help to cover the increase in energy needs that may occur in GDM.

Acknowledgements

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Conflict of Interest

The authors declare that they have no conflicts of interest in the authorship or publication of this contribution.

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Los resultados del **objetivo 7** están publicados en el artículo:

Leptin upregulates aquaporin 9 expression in human placenta in vitro.
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ORIGINAL ARTICLE

Leptin upregulates aquaporin 9 expression in human placenta *in vitro*

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ABSTRACT

Aquaporins are integral membrane proteins that have permeability functions in many tissues. Aquaporin 9 may transport not only water but also small molecules, such as glycerol, monocarboxylates, purines and pyrimidines. Aquaporin 9 is expressed in syncytiotrophoblast of human term placenta, and it may contribute to the embryonic/fetal growth and survival. We have previously found that Aquaporin 9 expression levels seem to be increased in placenta from gestational diabetes. Since leptin plasma levels and leptin expression are increased in placenta from gestational diabetes, we aimed to study the possible role of leptin on Aquaporin 9 expression in human placenta *in vitro*. The present work shows that leptin produces a dose-dependent increase of Aquaporin 9 expression, resulting in an increase in Aquaporin-9 protein in human trophoblast explants.

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Trophoblastic cells; pregnancy; transporter; GDM; AQP9; Leptin

Introduction

Placenta is a critical organ responsible for nutrient uptake, waste elimination, and gas exchange between mother and fetus [1]. Consequently, placental dysfunction can lead to a number of adverse fetal outcomes, including abnormal fetal growth [2,3]. Gestational diabetes mellitus (GDM) is the most frequent pregnancy alteration, affecting 4–8% of all the pregnancies [4,5]. Leptin is a peptide hormone that centrally regulates energy metabolism [6], but also regulates other systems including reproduction [7]. Leptin is produced by adipocytes, but also by trophoblast cells, acting as a trophic factor [8,9]. That is why plasma leptin levels are increased during pregnancy [10]. Besides, plasma leptin levels have been found increased in women with GDM [1,11]. Hyperinsulinemia observed in GDM may contribute to the increase of leptin synthesis in placenta [12,13], since insulin increases the expression of leptin in trophoblastic cells. We have previously studied the role of leptin in the growth and metabolism of the placenta [8], and both leptin and its receptor are overexpressed in placenta from GDM [14]. Previously, our group had found that the AQP9 expression is also increased in syncytiotrophoblast from placentas of GDM patients [15]. Aquaporins (AQPs) are a family of integral membrane proteins with water permeability functions. AQP9 can also facilitate the flux of neutral solutes such as glycerol, monocarboxylates, purines and pyrimidines [16,17]. AQPs are expressed in placenta and fetal membranes, where they seem to play an important role in amniotic fluid regulation. There is evidence that AQP9 is also involved in the excretion of urea across syncytiotrophoblast of human placenta from mother to fetus [18–20]. AQP9 expression has been found to be positively regulated by insulin [21]. Changes in the expression regulation of AQPs are seen in preeclampsia, abnormal amniotic fluid volume, chorioamnionitis and maternal undernourished pregnancy [22], thus pointing to a new therapeutic target in pathological pregnancies. In this context, our group has found that the amount of AQP9 mRNA as well as AQP9 protein level in trophoblast from placentas of women with GDM is higher than that observed in control placenta trophoblast [15]. Since leptin plasma levels and leptin expression are increased in placenta from GDM [8,14], now we aimed to study the possible role of leptin on AQP9 expression in human placenta *in vitro*.

Material and methods

The present research has been performed in accordance with the Declaration of Helsinki and has been approved by the local Institutional Review Board (Virgen Macarena University Hospital IRB) and samples were obtained with prior informed written consent from all subjects. All mandatory laboratory health and safety procedures have been complied with in the course of conducting the experimental work reported in this paper.

Placental explants collection and processing

Term placentas from non GDM patients and non hypertense patients, were obtained after programed cesarean section. Placentas were immediately suspended in ice cold phosphate buffered saline (PBS) and transported to the laboratory for being processed within 1–2 h after delivery as previously described [23].

In vitro studies with leptin stimulation

The experiments were performed in duplicate from five independent placentas. The explants were incubated during 6 h at

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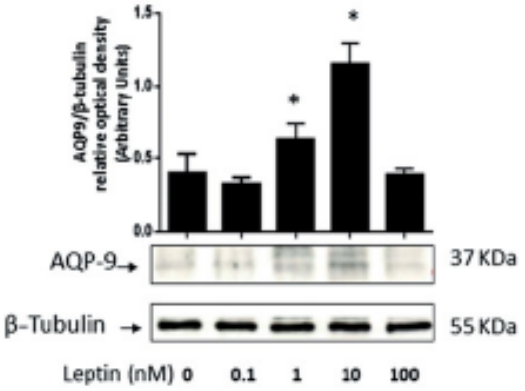

Figure 1. Leptin treatment increases the amount of AQP9 in human placental trophoblast explants. Placental explants were incubated with increasing concentrations of leptin (0, 0.1, 1, 10, 100nM) for 6h. Placental extracts were prepared and proteins were separated on SDS PAGE gels. AQP9 was determined by Western blot analysis. Loading controls were performed by immunoblotting in the same membranes with anti β tubulin. A representative immunoblot shows a 37 kDa band corresponding to the glycosylated form of AQP9 placental explants. Densitometry of immunoblots containing AQP9 protein level expression was performed, and after normalization for β tubulin, the values were plotted as AQP9/β tubulin relative ratio. Results shown are from a representative experiment and are expressed as means+SD for three independent experiments *$p < .05$ vs. control.

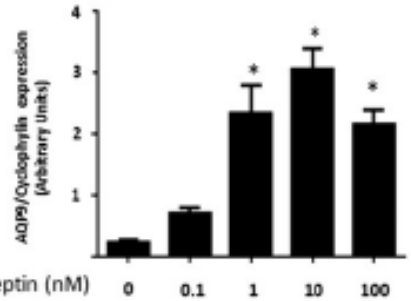

Figure 2. Leptin treatment increases the AQP9 expression in human placental trophoblast explants. Placental explants were incubated with increasing concentrations of leptin (0, 0.1, 1, 10, 100nM) for 6h. Total RNA was extracted and AQP9 mRNA was quantified with qRT PCR in independent experiments. Cyclophilin was used as internal standard. Results shown are from a representative experiment and are expressed as means+SD for three independent experiments *$p < .05$ vs. control.

37°C in isotonic medium DMEN F12 (0% SBF) with increasing leptin concentrations (0, 0.1, 1, 10 y 100 nM). For the immunoblotting, samples were lysed, denatured and resolved by SDS PAGE, electrophoretically transferred to a nitrocellulose membrane (Hybond ECL, AmershamBiosciences), and then incubated with polyclonal antibodies that detect human AQP9 (AlphaDiagnostic). Loading controls were performed by immunoblotting the same membranes with monoclonal anti β Tubulin (Santa Cruz Biotechnology Inc, Dallas, TX). The antibodies were detected using ECL, SuperSignal® (Thermo Scientific, Waltham, MA).

For the expression analysis by RT PCR, total RNA was extracted using Trisure® (Bioline Co, London, UK). Five micrograms of total RNA was reverse transcribed at 55°C during 1h using the Transcriptor first Strand cDNA synthesis Kit (Roche, Basel, Switzerland). qPCR reaction was performed using the primers sequences and protocol described in previous work [15]. Data were analyzed using CFX Manager™ Software version 1.5 program BIO RAD and subsequently underwent statistical analysis using the GraphPad Prism software.

Statistical analysis

In placental explants, immunoblot are a representative experiment from the 5 placentas studied. Results are expressed as mean + SD. For the expression analysis data by RT PCR, the statistical significance was assessed by ANOVA followed by Bonferroni's multiple comparisons *post hoc* test and was calculated using the GraphPad Instat computer program (GraphPad, San Diego, CA). It was considered statistically significant a p values less than .05.

Results

To study the effect of leptin on AQP9 expression in human trophoblasts, explants were incubated with increasing concentrations of leptin (from 0 to 100 nM) for 6 h. The analysis of protein expression was referenced as β Tubulin control protein. As shown in Figure 1, leptin stimulated AQP9 expression in trophoblast explants, in a dose dependent manner, with maximal effect achieved with a concentration of 10 nM and observing an effect of saturation at 100 nM. Similar dose response results have previously been found in leptin and insulin effects on human trophoblast explants such as protein synthesis [11,24].

To test whether leptin induces AQP9 gene expression, trophoblast explants were incubated in the same manner as described above, but the expression level was quantified by qRT PCR. As shown in Figure 2, the expression of AQP9 is dose dependently increased by leptin, reaching a maximum effect at 10 nM, and showing a saturation effect at 100 nM. Therefore, leptin seems to transcriptionally activate AQP9 expression in placenta.

Discussion

AQP9 has previously been found in the apical membranes of the syncytiotrophoblasts of human term placenta [19]. AQP9 is likely fundamental to the regulation of fetal water and solutes flow both in intramembranous absorption and in placental water transfer from mother to fetus. Nevertheless, it should be taken into account that AQP9 is not only permeable to water, but also to neutral solutes [25], suggesting that this channel may also be involved in metabolite diffusion and may, therefore, have a role in placenta/fetal energy metabolism.

Our previous results about the role of leptin in the growth and metabolism of the placenta trophoblastic cells [8], and the overexpression of both leptin and its receptor in placenta from GDM [14,26], suggest that leptin may mediate the increase in size of the placenta by activating both PI3K and MAPK signaling pathways [11]. We have previously found increased expression of AQP9 in the syncytiotrophoblast from placentas with GDM, and thus, AQP9 could mediate the increased transport of glycerol to the fetus to attend the increased energy requirements in the macrosomic fetus [15]. The typical macrosomia observed in newborn from GDM mother requires a greater availability of nutrients provided by different transport systems, including an important nutrient for energy provision such as glycerol, which may be transported by AQP3, AQP7 and AQP9.

In this context, our results demonstrate that leptin produces an increase in AQP9 expression by trophoblast explants *in vitro*, providing a molecular mechanism for a better nutrient transfer

and therefore, AQP9 may be a mediator of the metabolic action of leptin in placenta, and finally, leptin could mediate the increased AQP9 expression observed in GDM.

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Disclosure statement

The authors report no conflicts of interest.

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DISCUSIÓN

La leptina es una hormona pleiotrópica con un papel central en el inmunometabolismo [106–108] y en la regulación de la reproducción en general [109] y en la función reproductora en la mujer en particular [110,111] (Fig. 7)

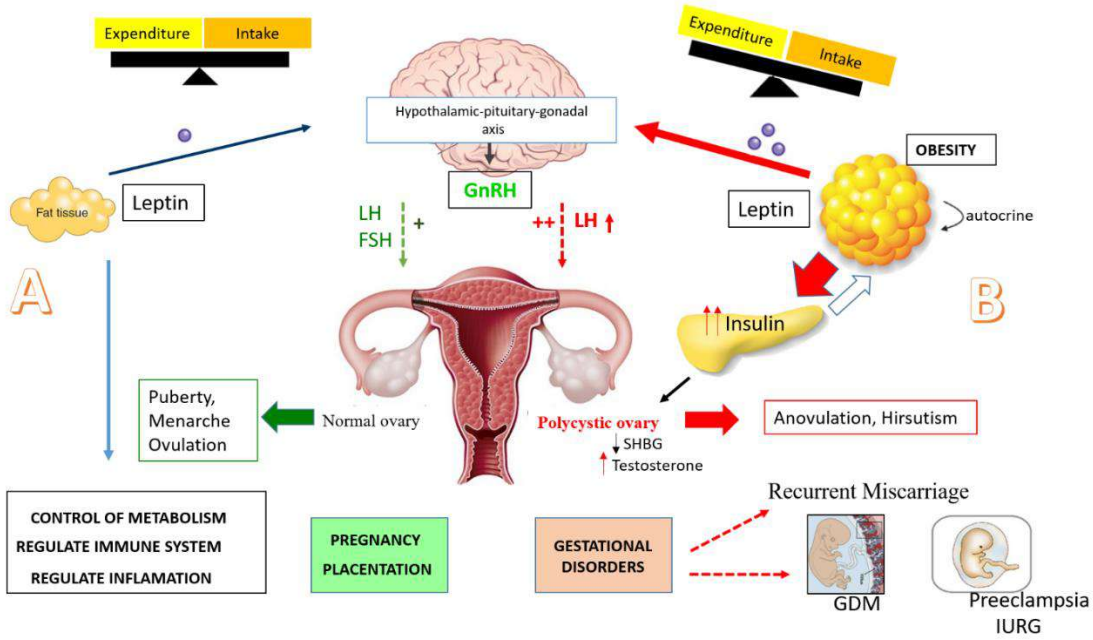

Figura 7. Papel de la leptina en la función reproductora de la mujer

El papel central de la leptina en la reproducción se ha visto ampliado a la patología de la reproducción, donde se ha destacado la participación de la leptina en la fisiopatología del PCOS y del embarazo [112,113] como hemos revisado nosotros recientemente en el tercer artículo incluido en esta Tesis Doctoral [41]. Por lo tanto, nuestro trabajo ha profundizado en el conocimiento de los mecanismos moleculares que subyacen en el PCOS y la diabetes gestacional.

Nuestro grupo viene trabajando en el campo de la leptina y su señalización desde hace más de 20 años, y una de sus aportaciones en este campo es la participación de la proteína de unión a ARN relacionada con la transducción de señales Sam68, en la señalización del receptor de leptina, lo que ha demostrado en células mononucleares sanguíneas [83,114,115], células de trofoblasto, y en cáncer de mama. Sam68 parece ser reclutado a la señalización como substrato de fosforilación en Tyr interaccionando con proteínas con dominios SH2 [82]. De hecho, la estructura de Sam68 (Fig. 7) contiene secuencias ricas en Tyr. Además, Sam68 contiene otros dominios de interacción proteína-proteína, como

secuencias ricas en prolina que pueden interaccionar con dominios SH-3 [79,116]. La participación es relevante ya que cuando disminuye su expresión la señalización se ve seriamente afectada, tal y como se ha visto en células de trofoblasto [76] o en células de cáncer de mama [78].

Por otro lado, Sam68 tiene un dominio KH de unión a ARN por lo que puede enlazar la señalización con la regulación del metabolismo del ARN incluido el splicing alternativo [75,117].

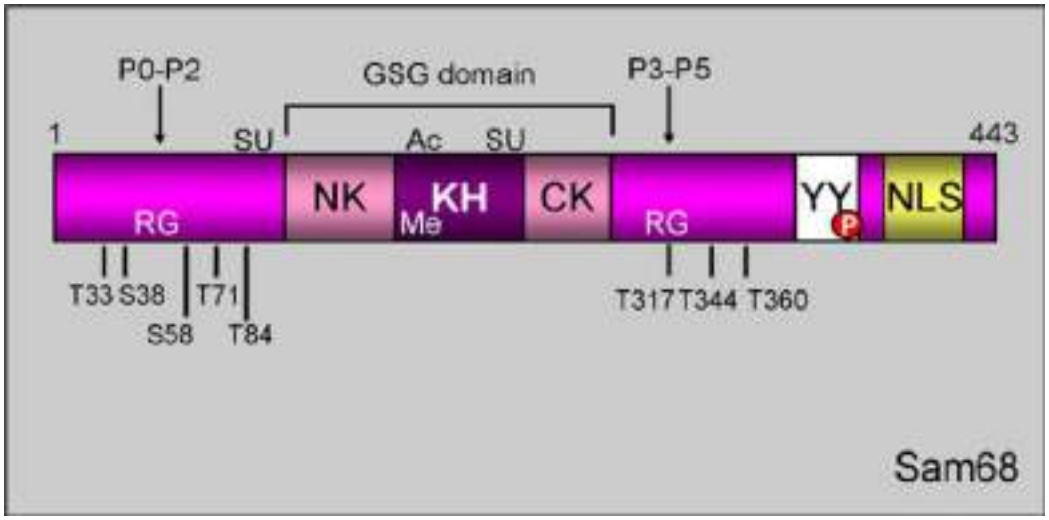

Figura 8. Estructura de la proteína Sam68 [118]

Nuestros resultados confirman el papel de Sam68 en la señalización en general [79] como se ha visto para otros receptores, como el de insulina [119], Angiotensina[120], Toll like Receptors [121], EGF[122]. BCR o TCR[123]. Nuestros resultados en células de la granulosa inciden no sólo en la participación de Sam68 en la señal del receptor de leptina, sino su necesidad para llevar a cabo su función.

Los resultados funcionales sobre la expresión de aromatasa en las células de la granulosa han contribuido a aclarar un punto importante en los mecanismos fisiopatológicos del PCOS. El defecto de expresión de aromatasa y la

consecuente disminución de la conversión de andrógenos en estrógenos está reconocida por la comunidad científica [122–124]. De hecho la inhibición de la aromatasa es un modelo experimental de PCOS en ratas [125].

Sin embargo, el papel de la leptina y la hiperleptinemia en la expresión de aromatasa ha sido controvertido. Muchos trabajos concluyen que la leptina inhibe la síntesis de estrógenos por la correlación inversa con la hiperleptinemia. Pero el efecto de la menos síntesis de estrógenos podría deberse a la resistencia a la acción de la leptina que justificaría además la hiperleptinemia. Sin embargo, hay pocos trabajos que estudien el efecto directo de la leptina en la granulosa in vitro, en especial de muestras humanas. Los trabajos que lo han hecho han combinado el efecto de la leptina sobre otras hormonas, como la insulina, el IGF-1, FSH, o los glucocorticoides [126–131], encontrando un efecto inhibidor que bien pudiera tratarse de la contraregulación de la señal de la misma manera que nuestro grupo ha demostrado el cross-talk negativo de leptina e insulina en trofoblasto de placentas humanas, en especial en DMG [98].

Nosotros hemos confirmado el efecto estimulador de la leptina sobre la expresión de aromatasa en células de la granulosa humana, como se había descrito también previamente [132]. Además, hemos demostrado que este efecto de la leptina se encuentra inhibido en la granulosa de mujeres con PCOS (Fig. 8), confirmando la resistencia a la leptina que hemos encontrado a nivel de la señalización, dependiente además de la expresión de Sam68.

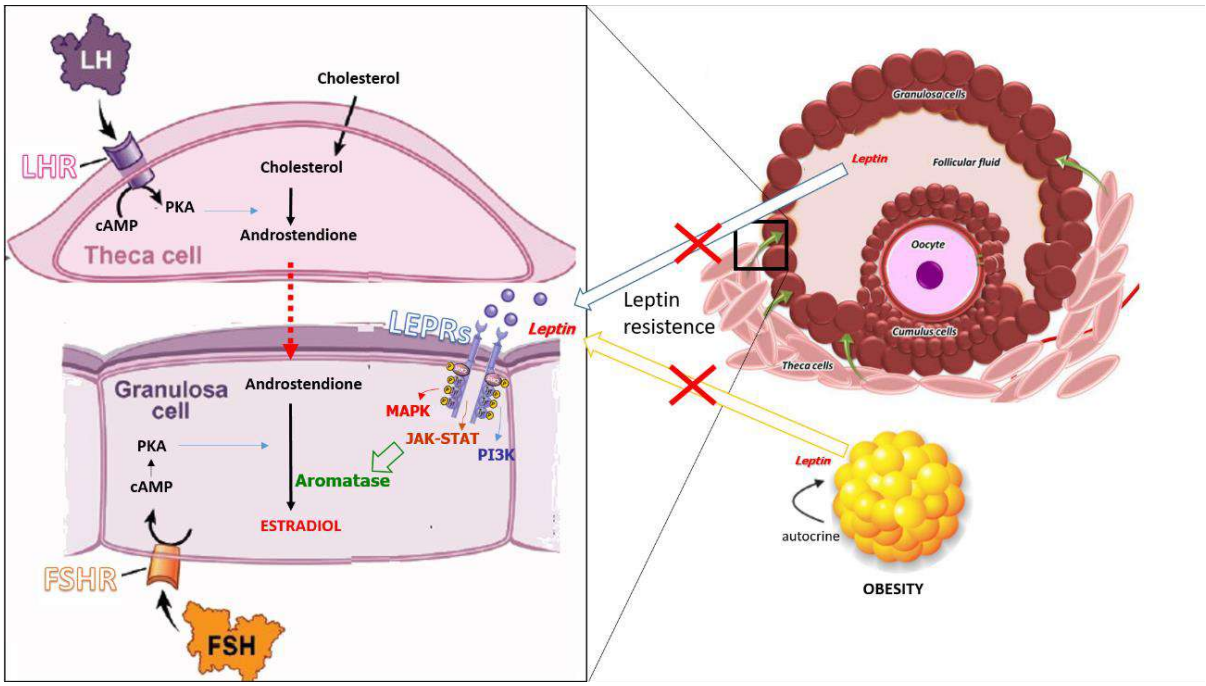

Figura 9. La leptina favorece la síntesis de estrógenos induciendo la expresión de aromatasa, que se encontraría inhibida en la resistencia a la leptina.

El papel de la leptina como una hormona trófica autocrina del trofoblasto está bien establecido, con un efecto antiapoptótico, en el que se ha utilizado fundamentalmente la retirada de suero del cultivo celular como estímulo proapoptótico. Nosotros elegimos estudiar el efecto protector de la leptina sobre la apoptosis producida por 2 efectos más fisiológicos, o más propiamente dicho, fisiopatológicos como son la hipertermia y la acidosis. Los efectos de la temperatura y el pH sobre la placenta no habían sido estudiados in vitro. En esta Tesis hemos demostrado el papel de la leptina previniendo la apoptosis producida tanto por la hipertermia como la acidosis, evitando la activación de la vía intrínseca de la apoptosis y la vía p53, que son activadas por las dos situaciones fisiopatológicas (Fig. 10).

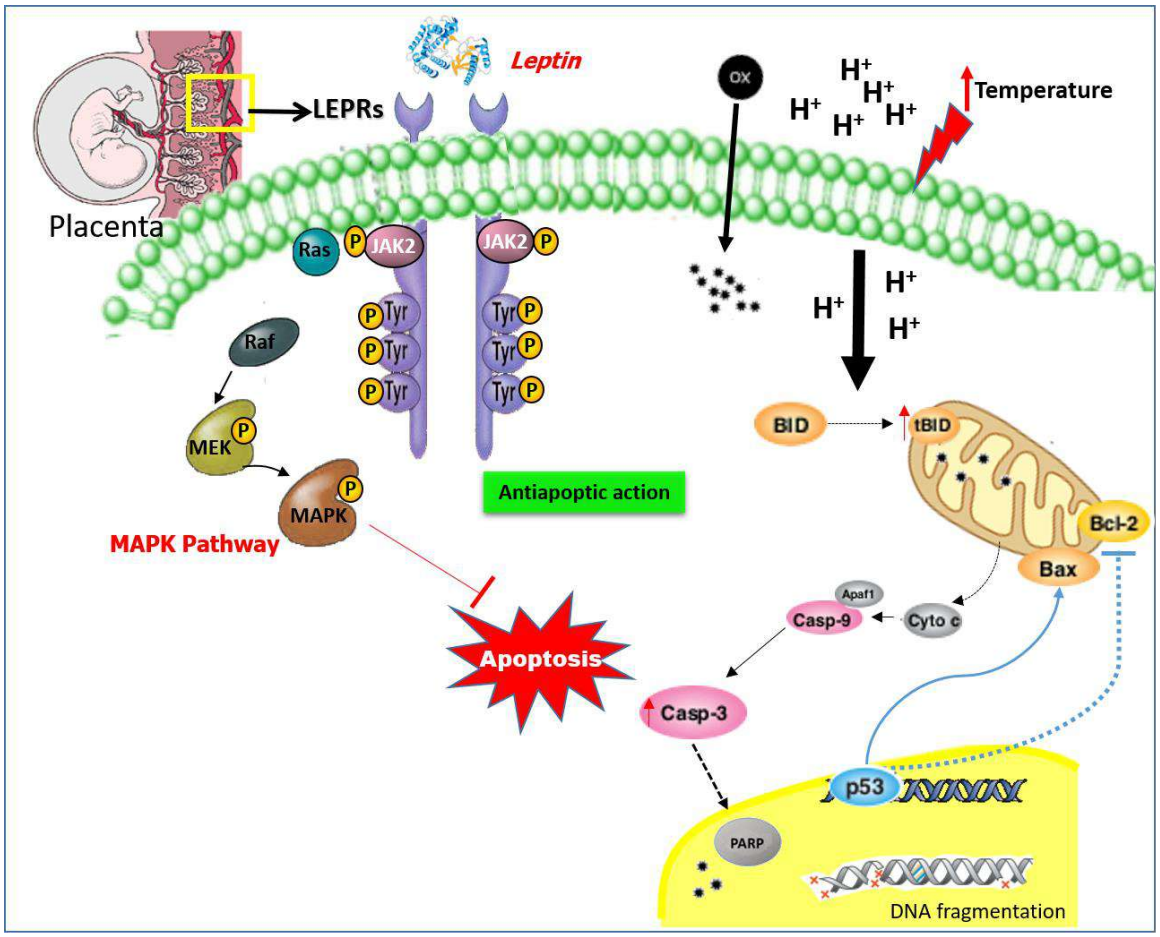

Figura 10. Papel de la leptina inhibiendo la apoptosis

Pero nuestro grupo tiene experiencia no sólo en la regulación por la leptina del crecimiento y supervivencia del trofoblasto en condiciones fisiológicas, sino también en la patología del embarazo más frecuente, la diabetes gestacional, donde los niveles de leptina están muy aumentados [133,134], debido a la expresión aumentada de leptina en el trofoblasto y de activación del receptor de leptina como se ha descrito previamente [135].

Nuestra hipótesis es que la leptina favorecería el crecimiento de la placenta y además faciitaría el crecimiento del feto aumentando el transporte de nutrientes necesarios para la macrosomía como se apuntado por otros autores [136]. En este sentido la leptina podría aumentar la expresión de transportadores de nutrientes en el trofoblasto para llevar a cabo esta función en la diabetes gestacional. Uno de esos transportadores sería la AQP-9 que permite el paso no solo de H2O sino también de glicerol, un nutriente importante para la síntesis de

triglicéridos. En esta Tesis hemos demostrado que la expresión de AQP-9 está incrementada en el trofoblasto de la placenta de mujeres con diabetes gestacional, y que la leptina podría participar, aumentando lpor la hiperleptinemia la expresión de AQP-9, como hemos demostrado in vitro con trofoblasto de placentas control (Fig. 11).

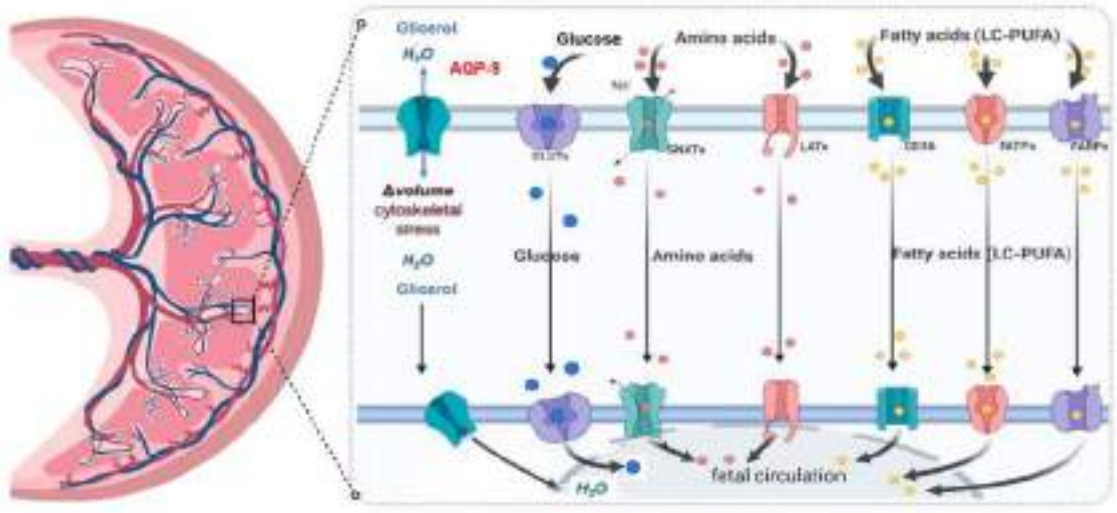

Figura 11. Los transportadores de nutrientes incluido AQP-9 contribuyen al aporte necesario de nutrientes al feto, que están aumentados en la diabetes gestacional.

Por tanto, en resumen, la leptina jugaría un importante papel regulador en la función tanto del folículo ovárico como del trofoblasto placentario, pero la resistencia a la acción de la leptina en la granulosa de la mujer con PCOS, como el exceso de acción de la leptina en la placenta de la mujer con diabetes gestacional podrían ser importantes mediadores fisiopatológicos y posibles dianas terapéuticas en estas alteraciones de la reproducción femenina [111].

CONCLUSIONES

1. **Sam68 participa de forma necesaria en la señal de la leptina en las células de la granulosa, siendo substrato de fosforilación, y regulándose positivamente su expresión por la misma señalización de la leptina**

2. **La leptina estimula in vitro la expresión de aromatasa en células de la granulosa, y ese efecto se pierde en la granulosa de mujeres con PCOS, reflejando la resistencia a la acción de la leptina**

3. **La expresión de Sam68 en la granulosa de mujeres con PCOS está descendida en comparación con la de la granulosa de mujeres control, contribuyendo por tanto a la resistencia a la acción de leptina en la granulosa de mujeres con PCOS.**

4. **La leptina previene la apoptosis de células trofoblásticas producida por la hipertermina, inhibiendo la vía de p53**

5. **La leptina previene la apoptosis de células trofoblásticas producida por la acidosis, inhibiendo la vía de p53**

6. **La expresión de AQP-9 en trofoblasto de mujeres con diabetes gestacional está aumentada en comparación con la del trofoblasto de mujeres control.**

7. **La leptina estimula in vitro la expresión de AQP-9 en las células del trofoblasto**

Por tanto, la resistencia a la leptina en la granulosa es uno de los mecanismos de mala función ovárica y la proteína Sam68 podría jugar un papel relevante.

La leptina tiene un papel protector de la placenta ante el estrés térmico y acidótico, pero el exceso de producción de leptina en la placenta de la diabetes gestacional puede contribuir no sólo al aumento del tamaño sino también de la expresión de transportadores de nutrientes como la AQP-9.

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ANEXO. Publicaciones relacionadas con la Tesis

Hemos publicado otros artículos relacionados con esta Tesis Doctoral, aunque no los hemos incluido:

Leptin and Nutrition in Gestational Diabetes.
Pérez-Pérez A, **Vilariño-García T**, Guadix P, Dueñas JL, Sánchez-Margalet V. Nutrients. 2020 Jul 2;12(7):1970. doi: 10.3390/nu12071970.

Nutritional modulation of leptin expression and leptin action in obesity and obesity-associated complications.
Montserrat-de la Paz S, Pérez-Pérez A, **Vilariño-García T**, Jiménez-Cortegana C, Muriana FJG, Millán-Linares MC, Sánchez-Margalet V. J Nutr Biochem. 2021 Mar;89:108561. doi: 10.1016/j.jnutbio.2020.108561.

Leptin stimulates DMP-1 and DSPP expression in human dental pulp via MAPK 1/3 and PI3K signaling pathways.
Martín-González J, Pérez-Pérez A, Cabanillas-Balsera D, **Vilariño-García T**, Sánchez-Margalet V, Segura-Egea JJ.Arch Oral Biol. 2019 Feb;98:126-131. doi: 10.1016/j.archoralbio.2018.11.019. Epub 2018 Nov 20.

Sam68 Mediates the Activation of Insulin and Leptin Signalling in Breast Cancer Cells.
Pérez-Pérez A, Sánchez-Jiménez F, **Vilariño-García T**, de la Cruz L, Virizuela JA, Sánchez-Margalet V.PLoS One. 2016 Jul 14;11(7):e0158218. doi: 10.1371/journal.pone.0158218. eCollection 2016.

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