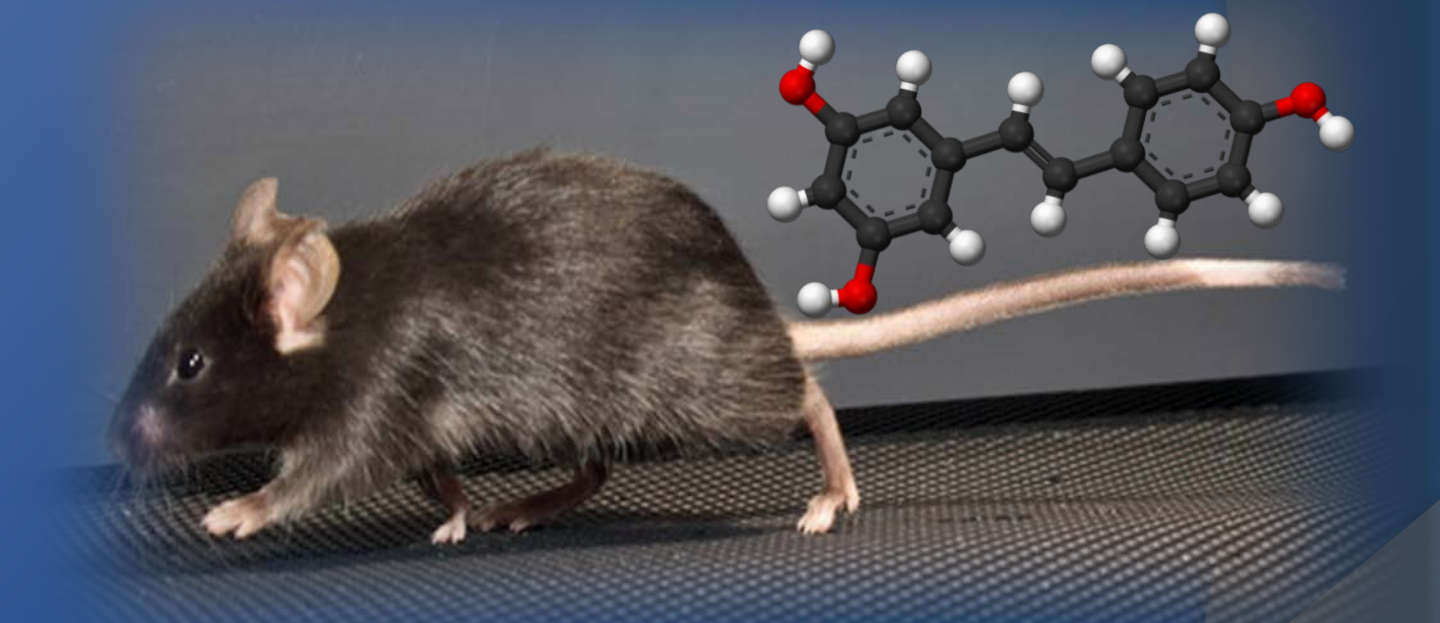
TESIS DOCTORAL

RESVERATROL AND EXERCISE REGULATE HEALTHY AGING MARKERS IN MICE



BUI THANH TUNG

DEPARTAMENTO DE FARMACOLOGÍA
FACULTAD DE FARMACIA
UNIVERSIDAD DE SEVILLA

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RESVERATROL Y EJERCICIO REGULAN MARCADORES DE ENVEJECIMIENTO SALUDABLE EN RATONES

BUI THANH TUNG

2014

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INFORMA

Que Don Bui Thanh Tung, Licenciado en Farmacia, ha realizado bajo su dirección en coordinación con el Profesor Guillermo López Lluch de la Universidad Pablo de Olavide, la tesis doctoral titulada "**Resveratrol and exercise regulate healthy aging markers in mice**", y que a su juicio reúne los méritos suficientes para optar al grado de Doctor.

Y para que conste, firmo el presente en Sevilla, a treinta de enero de dos mil catorce.

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INFORMA

Que Don Bui Thanh Tung, Licenciado en Farmacia, ha realizado bajo su dirección en coordinación con la Profesora Virginia Motilva Sánchez de la Universidad de Sevilla, la tesis doctoral titulada **"Resveratrol and exercise regulate healthy aging markers in mice",** y que a su juicio reúne los méritos suficientes para optar al grado de Doctor.

Y para que conste, firmo el presente en Sevilla a treinta de enero de dos mil catorce.

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INFORMA

Que la presente Tesis Doctoral titulada "**Resveratrol and exercise regulate healthy aging markers in mice**" realizada por Bui Thanh Tung, ha sido dirigida por la **Dra. Virginia Motilva Sánchez** y el **Dr. Guillermo López Lluch**, para aspirar al grado de Doctor en Farmacia con Mención Internacional, cumpliendo los requisitos para este tipo de trabajo.

Y para que conste, firmo el presente en Sevilla, a treinta de enero de dos mil catorce.

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TABLE

ABBREVIATIONS

4-HNE	4-Hydroxyl-2-nonenal
8-OHdG	8-Hydroxyl-2'-deoxyguanosine
ABTS	2,2'-Azino-bis(3-ethylbenzothiozoline-6-sulphonic acid)
AMs	Adhesion molecules
ASC	Apoptosis-associated speck like CARD domain containing protein
CoQ	Coenzyme Q
CASP-1	Caspase-1
CAT	Catalase
COX-2	Cyclo-oxygenase-2
CR	Calorie restriction
CRP	C-reactive protein
$CytB_5Rase$	Cytochrome b_5 reductase
DAMP	Damage associated molecular pattern
DNA	Deoxyribonucleic acid
DNPH	2,4-Dinitrophenylhydrazine
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-S-transferase
H_2O_2	Hydrogen peroxide
HPLC	High performance liquid chromatography
ICAM-1	Inter-cell adhesion molecule 1
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-1R	IL-1 receptor
iNOS	Inducible nitric oxide synthase

ABBREVIATIONS

LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRR	Leucin-rich-repeat
MAPK	Mitogen activated protein kinase
MDA	Malondialdehyde
NADPH	β-Nicotinamide adenine dinucleotide phosphate reduced
NLR	NOD-like receptors
NALP-3	NACHT, LRR, and PYD domains-containing protein 3,
NQO1	NAD(P)H:quinone oxidoreductase 1
PBS	Phosphate buffered saline
PM	Plasma membrane
PMRS	Plasma membrane redox system
PPARs	Peroxisome proliferators-activated receptors
PVDF	Polyvinylidene difluoride
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSV	Resveratrol
SOD	Superoxide dismutase
TBA	2-Thiobarbituric acid
TBARS	Thiobarbituric reacting substances
TLRs	Toll-like receptors
TNF-α	Tumour necrosis factor-α
TrxR	Thioredoxin reductase
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule 1

SUMMARY

Aging is a process very complex and multifactorial that continues to the gradual deterioration in functional capacities. It normally manifests after maturity and causes disability and death. Some common and universally accepted characteristics that manifest during the process of aging, such as, increased mortality after maturation, changes in the biochemical composition of tissues, decrease of physiological capacity, reduction of the ability to respond adaptively to environmental stimuli and increased susceptibility and higher vulnerability to diseases. The defects in the human body because aging process start to arise very early in life, even before birth.

Biogerontology, the study of the biological basis of aging, has unveiled mysteries of aging by describing age-related changes in organisms, organs, tissues, cells, and macromolecules. In this study, many theories about the causes of aging have been proposed. For example, the aging process has been attributed to molecular cross-linking, changes in immunological function, damage by free radical reactions, and activation of senescence genes. Among all the theories, the free radical theory of aging is the most widely accepted. It was developed by Harman in 1956 based on the chemical nature and ubiquitous presence of free radicals. This theory states that free radicals are the major determinants of molecular damage that causes aging. Twenty years later, Harman realized that mitochondria are a major producer of reactive oxygen species (ROS) that contribute to oxidative damage. On the contrary, antioxidant defense systems that limit the free radical have evolved. The influence of aging on antioxidant defense system has been studied extensively. A considerable number of studies indicate that one or more of the antioxidant enzymes decrease as consequence of aging. Moreover, a considerable amount of research studies support that the molecular inflammatory process plays a central role in the aging process and age-related diseases. Accumulated data strongly suggest that up-regulation of pro-inflammatory mediators (TNF-α, IL-1β, IL-6, COX-2, iNOS) are induced during the aging process.

The polyphenolic compound resveratrol is a molecule synthesized in many plants, such as peanuts, blueberries, pine nuts, and grapes. Resveratrol (RSV) has become a candidate for drug development in aging studies due its antioxidant, anticarcinogenic, and anti-inflammatory properties. Scientific evidence has highlighted its potential as an anti-aging molecule as well as a therapeutic agent for cardiovascular diseases and some types of cancers. RSV could be the most promising candidate to mimic the beneficial effects induced by a chronic and moderate calorie restriction on aging. In fact,

SUMMARY

RSV has been showed to produce many of the effects found in animals fed under calorie restriction. These effects have point to RSV as an antiaging compound.

Exercise is often said to increase the generation of ROS that are potentially harmful and, therefore, can enhance oxidative damage to nucleic acids, proteins and lipids in cells. On the other hand, it has been well recognized that regular physical activity has health benefits such as reducing risk and progression of cardiovascular diseases, type 2 diabetes mellitus, cancer and neurodegenerative diseases. Exercise contributes to health with many benefits including, increased insulin sensitivity, improved oxidative capacity, decreased risk of myocardial infarction and cardiovascular disease, lower blood pressure, improved blood lipid profile and improved endothelial function. It has been shown that regular exercise significantly prevents the loss of the mass and functions age-associated of skeletal muscle during aging. Furthermore, exercise upregulates antioxidant defense mechanisms in several tissues, presumably due to increased levels of oxidative stress that occurs during exercise. Moreover, exercise is well-known as an anti-aging intervention, may also prevent or attenuate inflammation.

This thesis focuses on the beneficial effects of RSV treatment either alone or in combination with exercise during the aging process in mouse models. We allocated male C57BL/6J mice (n=48) at 3 age groups (young, mature and old) to investigate the effects of RSV and physical activity during aging. Each age group was further divided into 4 subgroups (Control no-trained, Control trained, RSV no-trained and RSV trained). The RSV dose was estimated 16.5 mg/kg animal/day and training was performed during 6 weeks before sacrifice.

In the first study, we analyzed the effects of RSV and exercise on body weight and capacity of physical activity. Our results indicate that RSV and exercise training are able to increase muscle resistance but do not have effect on body weight of mice.

In the second study, we analyzed if RSV and exercise induce changes in endogenous antioxidant activities in the liver and if the effects of RSV and exercise depended on the age of the animal at the beginning of the intervention. Aging was accompanied by the increase in oxidative damage in the liver affecting the glutathione-dependent system. Both, RSV and exercise reversed the effect of aging and maintained the high levels of activity of glutathione, glutathione peroxidase and glutathione-S-transferase in old animals. NQO1 activity was also increased. Modulation of antioxidant activities was not completely accompanied by changes at the protein level. Whereas

GPx1 protein increased in parallel with the higher activity in old animals, NQO1 protein decreased during use of RSV although the activity was enhanced.

In the third study, we aimed to investigate the effects of RSV and exercise on inflammation in the liver during the aging process. Analysis of the liver homogenates revealed that old mice had significantly higher levels than young and mature animals of the pro-inflammatory cytokines TNF-α,IL-1β, IL-6 and IL-17, the inflammasome-related transcripts for TNF-α, IL-1β, IL-6, ASC, CASP-1, NALP-1 and NALP-3, and the COX-2 protein. Our results also indicated that exposure of old mice to RSV treatment and/or physical activity decreased these inflammatory parameters. Interestingly, we found that levels of the anti-inflammatory cytokine IL-10 also increased during aging. It could be the mechanism protective by organism itself in process of aging.

In the fourth study, we studied the antioxidant system in different tissues, including those of the brain, kidney, muscle, heart and liver. The primary focus of this study was investigating the effect of RSV and/or exercise training on these tissues in old mice. The regimen of RSV and exercise improved antioxidant system defense in the major organs of the mice, as increased activities and proteins expression of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, thioredoxin reductase, NADH-cytochrome b_5 reductase, and NAD(P)H-quinone acceptor oxidoreductase. In addition, a decreased amount of malondialdehyde was observed in the liver, hearts, and muscle tissues of mice exposed to the treatment regimen of RSV and exercise.

In the fifth study, we examined whether RSV therapy and/or exercise training can increase tissue-specific levels of CoQ, a redox molecule in mice. Our results demonstrated that, in response to aging, levels of CoQ decreased in the brain but increased in liver and muscle tissues. Furthermore, expression of CoQ in brain, liver tissue in mice can be increased by adhering to a regimen of RSV treatment and exercise.

Finally, in sixth study, proteomic 2D-DIGE analysis was used to take a global look at the effects of RSV and exercise training on protein expression in the liver during aging. The liver was investigated as a principal target due to its role in the energetic and xenobiotic metabolism of drugs. We detected intensity changes in several hundreds of spots and identified several spots from the gels. Our results showed that most of the differentially expressed proteins are associated with the electron transport chain, urea cycle, oxidoreductases enzymes, regulatory factors in mitochondrial protein synthesis, the aldehyde dehydrogenase family and antioxidant capacity. These data support

the use of RSV and exercise can be of great importance for the prevention of metabolic diseases and related to aging.

In conclusion, excessive production of ROS, reduced antioxidant defense and increased inflammation significantly contribute to aging in liver. Oxidative damage and higher inflammation are the major cause and the most important contributor to aging. RSV and exercise are capable to increase antioxidative protection, modulate the endogenous defense, regulate the inflammation. Then, they can modulate mechanisms that may potentially improve health, increase longevity and contribute to treatment of degenerative age-related diseases. Lifestyle changes including regular physical activity and with supplement of RSV may improve health and increase cellular resistance to stress, and contribute to health aging.

RESUMEN

El envejecimiento es un proceso muy complejo y multifactorial que conlleva a un deterioro gradual y progresivo del organismo. Normalmente se manifiesta después de la madurez y causa la discapacidad y la muerte. Algunas de las características comunes y universalmente aceptadas que se manifiestan durante el proceso de envejecimiento, incluyen el aumento de la mortalidad después de la maduración, los cambios en la composición bioquímica de los tejidos, la disminución progresiva de la capacidad fisiológica, la reducción de la capacidad para responder de forma adaptativa a los estímulos ambientales y el incremento de la susceptibilidad y vulnerabilidad a las enfermedades. Los defectos debido al proceso de envejecimiento empiezan a aparecer muy temprano en la vida, incluso antes de nacer.

La biogerontología, es el estudio de las bases biológicas del envejecimiento, intenta conocer sus misterios mediante la descripción de los cambios relacionados con la edad en los organismos, órganos, tejidos, células y macromoléculas. Se han propuesto muchas teorías sobre las causas del envejecimiento. Por ejemplo, desde un visión biológica, el proceso de envejecimiento se ha atribuido al *cross-linking* molecular, cambios de la función inmunológica, daños de las reacciones de radicales libres, y senescencia de genes en el ADN. Entre todas las teorías existentes hasta la fecha, la más aceptada es la teoría del envejecimiento por acción de los radicales libres. Fue desarrollado por Harman en 1956 y está basada en la naturaleza química y en la presencia ubicua de los radicales libres. Esta teoría afirma que los radicales libres son los principales determinantes de daño molecular causando el envejecimiento. Veinte años más tarde, Harman reconoció que las mitocondrias son importantes productores de las especies reactivas del oxígeno (siglas en inglés ROS, de *reactive oxigen species*), las cuales contribuyen al daño oxidativo y a partir de este concepto desarrolló su teoría del envejecimiento mitocondrial por acción de los radicales libres.

Para limitar el daño producido por las ROS la célula cuenta con un sistema de defensa antioxidante, enzimático y no enzimático. Gran cantidad de estudios indican que una o más de las enzimas antioxidantes pueden disminuir como consecuencia del envejecimiento. Más aún, una gran cantidad de investigación y estudios apoyarla suposición que el proceso inflamatorio juega también un papel central en el proceso de envejecimiento y en las enfermedades relacionadas con la edad. Los datos acumulados hasta el momento, sugieren firmemente que la regulación de mediadores pro-inflamatorios (TNF-α, IL-1β, IL-6, COX-2, iNOS) se incrementa durante el proceso de envejecimiento.

SUMMARY

El compuesto polifenólico resveratrol (RSV) es una molécula sintetizada por muchas plantas, como las uvas, arándanos, moras, cacahuetes, nueces y piñones. El RSV se ha convertido en el candidato principal para el desarrollo de fármacos, en estudios de envejecimiento, debido su acción antioxidante, anti-cancerígena, y anti-inflamatorias. La evidencia científica ha revelado su potencial como una molécula anti-envejecimiento, así como un agente terapéutico para las enfermedades cardiovasculares y algunos tipos de cánceres. El RSV podría ser el candidato más prometedor por la capacidad de mimetizar los efectos beneficiosos inducidos por la restricción calórica, sin llegar a la malnutrición.

El ejercicio físico a intensidades altas o practicado durante un tiempo prolongado aumenta la generación de ROS, y el perjuicio derivado al aumentar el daño oxidativo a los ácidos nucleídos, a las proteínas y a los lípidos en las células. Sin embargo, se ha reconocido que la actividad física regular de intensidad moderada reduce el riesgo y la progresión de las enfermedades cardiovasculares, diabetes mellitus tipo 2, cáncer o enfermedades neurodegenerativas. La práctica del ejercicio físico induce amplios beneficios para la salud tales como, el aumento de la sensibilidad a la insulina y la capacidad oxidativa, la diminución de la presión arterial, la mejora el perfil lipídico en sangre, y de la función endotelial. También se ha demostrado que el ejercicio realizado frecuentemente previene significativamente la pérdida de la masa muscular y de sus funciones durante el envejecimiento. Más aún, el ejercicio previene o atenúa el proceso de inflamación y aumenta la expresión del sistema defensa antioxidante en diversos tejidos. En este sentido, el ejercicio físico al igual que el RSV, es considerado una intervención anti-envejecimiento.

Esta tesis se enfoca en los efectos beneficiosos del tratamiento con RSV solo, o en combinación con la práctica de ejercicio, durante el proceso de envejecimiento en un modelo de ratones. Para investigar los efectos del RSV y/o del ejercicio durante el envejecimiento, dividimos los ratones machos C57BL/6J (n = 48) en 3 grupos de edad (jóvenes, adultos y viejos). Posteriormente, cada grupo de edad fue sub-dividido en 4 subgrupos (Control-no entrenado, Control-entrenado, RSV-no entrenado y RSV-entrenado). La dosis de RSV estimada fue de 16,5 mg/kg/animal/día durante 6 meses y el entrenamiento se realizó durante 6 semanas antes del sacrificio.

En el primer estudio, analizamos los efectos del RSV y ejercicio sobre el peso corporal y la capacidad de la actividad física. Nuestros resultados indican que el RSV y el ejercicio de corto plazo son capaces de aumentar la resistencia muscular, mejora la resistencia, pero no tienen efecto sobre el peso corporal de los ratones.

En el segundo estudio, analizamos si el RSV y el ejercicio inducen cambios en las actividades antioxidantes endógenas en el hígado, y si estos cambios dependen de la edad del animal. El envejecimiento fue acompañado de un aumento del daño oxidativo en el hígado, especialmente en el que afecta al sistema glutatión-dependiente. Ambos, el RSV y el ejercicio, invierten el efecto del envejecimiento y mantienen altos los niveles de la actividad de glutatión, glutatión peroxidasa y glutatión-S-transferasa en los animales viejos; la actividad de NQO1 también fue incrementada. La modulación de la actividad antioxidante no fue completamente acompañada de cambios en el nivel de proteínas: la expresión de proteína GPx1 aumentaba en paralelo con la mayor actividad en los animales viejos; sin embargo, la proteína NQO1 disminuyó durante el uso de RSV aunque su actividad aumentó.

En el tercer estudio, el objetivo fue investigar los efectos de RSV y ejercicio en la inflamación en el hígado durante el proceso de envejecimiento. El análisis de los hígado homogenizados reveló que los ratones viejos tenían los niveles de las citoquinas pro-inflamatorias TNF-α, IL-1β, IL-6 y IL-17, las transcripciones relacionadas con la activación del inflamasoma TNF-α, IL-1β, IL-6, ASC, CASP-1, NALP-1, NALP-3 y también la expresión de enzima COX-2 fueron significativamente más altos que en los animales jóvenes y adultos. Nuestros resultados mostraron que el tratamiento de RSV y ejercicio reducen estos parámetros inflamatorios. Curiosamente, se encontró que los niveles de la citoquina antiinflamatoria IL-10 también aumentaron durante el envejecimiento. Este hallazgo sugiere que el aumento de los niveles de IL-10 es un mecanismo de respuesta y protección contra el proceso inflamatorio del envejecimiento

En el cuarto estudio, se estudió el sistema antioxidante en diferentes tejidos, incluyendo cerebro, riñón, músculo, corazón y el hígado. El objetivo principal de este estudio fue investigar efecto del RSV y ejercicio físico en estos tejidos en ratones viejos. El tratamiento conjunto de RSV más ejercicio mejoró el sistema defensa antioxidante en los órganos principales de los ratones, manifestándose como un aumento en las actividades y en la expresión de las enzimas, incluyendo superóxido dismutasa, catalasa, glutatión peroxidasa, glutatión reductasa, glutatión-S-transferasa, tiorredoxina reductasa, NADH-citocromo b_5 reductasa, y NAD(P)H-quinona oxidorreductasa. Además, se observó una disminución en la cantidad de malondialdehído en el hígado, corazón, y muscular de los ratones con el tratamiento indicando un menor daño oxidativo en los lípidos.

En el quinto estudio, examinamos si el tratamiento con RSV y ejercicio físico puede aumentar la expresión CoQ, una molécula redox localizada en todos los tejidos de los ratones. Nuestros resultados demuestran que, con el envejecimiento, los niveles de CoQ_9 y CoQ_{10} disminuyeron en el

cerebro, pero aumentaron en el hígado y el músculo. Además, la expresión de CoQ_9 y CoQ_{10} en el cerebro y hígado en ratones se puede aumentar mediante a tratamiento de RSV y la práctica de ejercicio.

Por último, en el sexto estudio, el análisis proteómico 2D-DIGE se utilizó para tener una visión global de los efectos del RSV y del ejercicio en la expresión de la proteína en el hígado durante el proceso del envejecimiento. El hígado fue investigado como principal objetivo debido a su papel fundamental en el metabolismo energético y xenobiótico de fármacos. Detectamos cambios de intensidad en cientos de proteínas y logramos identificar varias de ellas a partir de los geles 2D. Nuestros resultados mostraron que la mayoría de las proteínas expresadas diferencialmente se asocian con la cadena de transporte de electrones, ciclo de la urea, enzimas oxidorreductasas, factores de regulación de la síntesis de proteína mitocondrial, la familia aldehído deshidrogenasa y la capacidad antioxidante. Estos datos apoyan que el uso del RSV y la práctica de ejercicio físico de intensidad moderada pueden ser de gran importancia para la prevención de enfermedades metabólicas y relacionadas con el envejecimiento.

En conclusión, la producción excesiva de ROS, la reducción de sistema de defensa antioxidante y el aumento de la inflamación con la edad contribuyen significativamente al envejecimiento. El daño oxidativo y el aumento de la inflamación son la causa principal y la más importante que contribuyen al envejecimiento. La capacidad del RSV y el ejercicio físico moderado de aumentar la protección de antioxidante, modular la defensa endógena, la regulación de la inflamación y los mecanismos de reparación pueden potencialmente mejorar la salud, aumentar la longevidad y contribuir al tratamiento de las enfermedades degenerativas relacionadas con la edad. Un estilo de vida que incluya la práctica diaria de ejercicio físico moderado junto a una dieta suplementada con RSV pueden mejorar la salud, aumentar la resistencia celular al estrés, y contribuir al envejecimiento saludable.

INTRODUCTION

INTRODUCTION

Aging: theories

Human have shown the desire to exceed or avoid aging. In the 16th century, the Spanish explorer Ponce De Leon searched for the fountain of youth in Florida which he hoped would slow or stop the aging process. Nowadays, there are many anti-aging products on the market. Nonetheless, it is a clear fact, that we cannot stop aging. However, we have a very limited knowledge about aging. We can recognize the changes associated with age: memory loss, wrinkles, muscles loss, and balance trouble, etc., but we do not really understand what aging is, why it happens or how to prevent it. Aging and life span characteristics are specific to individual species and vary greatly even among similar species. What is aging? Why and how we age? We would like to answer these questions. What are the specific biological mechanisms that cause aging?

It is clear that aging is not a disease (Hayflick, 2000). ¨*It is an inevitable consequence of processes characterized by age-dependent physiological functional decline. Aging is a progressive accumulation of diverse deleterious changes throughout the cells and tissues of an individual, which increase the chance of disease and death with advancing age*¨ (Harman, 2000). Aging is a progressive decline in biological function accompanied by an increased risk of degenerative disease and death. It is a complex and multifactorial process and many theories have been proposed to explain this phenomenon at the molecular, cellular, systemic and evolutionary levels.

Aging theories attempt to explain the causes and effects of the aging process. Because of the enormous complexity of the changes associated with aging, it is not surprising that many theories have been proposed to explain the where, how and why of these changes. Then, a number of theories have been developed that attempt to define and explain aging. However, none of them explains all aspects of aging.

The **evolutionary theory** argues that aging results from a decline in the force of natural selection. Because evolution acts primarily to maximize reproductive fitness in an individual, longevity is a trait to be selected only if it is beneficial for fitness (Haldane, 1941). Therefore, life span is the result of selective pressures, and may have a large degree of plasticity within an individual species, as well as among species.

The **wear and tear aging theory** posed by Dr. August Weismann (1982), suggested that aging is the result of accumulating random changes that negatively affects biological systems. Aging is a

'secondary effect' of the physiological work of cells. Aging could be the result of the accumulation of toxic byproducts; be damaged due to nuclear radiation, or other gradual deteriorative process. Many people believe that aging is simply the result of deterioration caused by wear and tear, oxidation, other molecular damage, or other unavoidable natural process that causes gradual degradation (Jin, 2010).

The **waste-product theory of aging** proposes that cellular aging is caused by the accumulation of intracellular waste which may be deleterious to the cell (Sheldrake, 1974). The theory proposes that the wastes are produced, and the cells are divided at specific rates. Once produced, the waste material cannot be destroyed and cannot be eliminated by the transport across the cell membrane. The cellular waste content increases until at the time of cell division; the accumulation is apportioned between the daughters; and each daughter receives half of the waste if the division is symmetric; thus, the waste is diluted by cell division. The waste content in the steady state is determined by the relative rates of waste production and cell division; if cell division ceases, the waste content increases without limit (Hirsch and Witten, 1991).

In the **neuroendocrine theory**, the basic hypothesis advocates the central role of hypothalamus in aging while reducing sensitivity to hormones and other signaling peptides that has been demonstrated at this level. This implies that aging could be regulated by hypothalamus and pituitary which represent the body's internal 'pacemaker' regulating physiological changes over time (Meites et al., 1987).

In the case of the **immunological theory of aging**, the ability of the immune system to the quantitatively and qualitatively produce antibodies diminishes with age. As normal immune response declines, autoimmune manifestations increase, and the immune system becomes less able to discriminate between self and non-self, resulting in an increase in autoimmune diseases. The immune system represents the most powerful mechanism to face stressors. Macrophage activation due to chronic stress may explain the age-related subclinical chronic inflammatory status (Franceschi et al., 2000).

The **catastrophic error in protein synthesis theory** is based on that the spontaneous error frequency in protein translation is generally several orders of magnitude higher than that in DNA replication and RNA transcription. Then, the role of protein errors and their feedback in biochemical pathways has been considered to be crucial with respect to aging (Holliday, 1996).

In the **somatic mutation theory**, the central concept is that the accumulation of a sufficient level of mutations in somatic cells will produce physiological decrements characteristic of aging. If mutations are the fundamental cause of age changes, they must occur randomly in time and location (Kirkwood and Proctor, 2003).

The cross-linkage theory of aging holds that age changes result when two or more macromolecules become linked covalently or by a hydrogen bond. Such linkages, said to be reversible, accumulate over time. Molecular aggregation and immobilization increases, and the resulting inert or malfunctioning molecules accumulate and become increasingly resistant to catabolic processes. DNA may thus become damaged, leading to mutation or cell death. The cross-linking hypothesis is based on the observation that with age, our proteins, DNA, and other structural molecules that develop inappropriate attachments or cross-links to one another (Hayflick, 1985).

The **free radical theory of aging** is based on the production of radical by normal biochemical activities in cell. Radicals are characterized to have one or more unpaired electrons in their outer orbital, which might make them chemically highly reactive and accordingly unstable. Some of them disintegrate in less than microseconds and can react with other molecules. They include various reactive oxygen species (ROS), such as, superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH•), and of various reactive nitrogen species (RNS), such as, nitric oxide (NO) and peroxynitrite (ONOO-). At low concentrations, they are important regulators of a variety of cellular activities such as immune function and vasodilation (Sohal and Weindruch, 1996). However, at high concentrations, they damage proteins, lipids and DNA.

Free radical theory of aging (FRTA) postulated by Harman in 1956 arose from a consideration of the aging phenomenon from the premise that a single common biochemical process may be responsible for the aging and death of all living beings (Harman, 2006). This theory proposes that the accumulation of cellular damage caused by reactive oxygen species (ROS) plays a key role in the aging process, as well as in determining organism longevity (Beckman and Ames, 1998). There is abundant evidence showing that a variety of ROS and other free radical are indeed involved in the occurrence of molecular damage which can lead to structural and functional disorders, diseases and death. This theory is based on the chemical nature of free radical reactions and their ubiquitous and prominent presence in living beings (Holmes et al., 1992). In vivo, free-radicals are highly reactive with polyunsaturated fatty acids in membranes and with nucleic acids, proteins, and polysaccharides (Dröge, 2002). When radicals react with these components, the result is an extensive damage of chemical bonds and membranes leading to polymerization, accumulation of

debris, loss of membrane and enzymatic integrity, and cell death. For most biological structures, free radical damage is closely associated with oxidative damage (Circu and Aw, 2010).

Nowadays, the most widely accepted theory is **the oxidative stress hypothesis of aging.** It currently offers the best mechanistic description of the aging process and the age-related chronic disease development (Yu, 1996). The theory holds that an increase in reactive oxygen species (ROS) during aging causes functional alterations, pathological conditions, and even death (Kregel and Zhang, 2007). The aging process is characterized by the accumulation of damaged macromolecules, considered responsible for the sequential alterations that accompany advancing age and the associated progressive increase in the chance of disease and death. Under the oxidative stress hypothesis of aging, the characteristic changes of the aging process are the net effect of redox imbalances between oxidative stress and anti-oxidative mechanisms, which include oxidative alterations in DNA, protein, and other cellular components, such as antioxidant defense systems, which cause functional deficits and many age-related degenerative diseases (Yu, 1996). Furthermore, decrease in the capacity of mechanisms that repair or eliminate damaged molecules must be added to this imbalance (Martin and Grotewiel, 2006). Furthermore, the disruption of the redox balance regulate by oxidative stress. Aging influences age-related alterations in signaling transduction and gene regulation. Thus, various cellular processes related to signaling and gene expression can be inhibited or activated in response to oxidative changes in the intracellular environment (Finkel and Holbrook, 2000; Yu, 1996)

The mitochondrial free radical theory of aging emerged in 1972 when Harman suggested that mitochondria had the right characteristics to be both the sources and the direct victims of toxic free radicals (Harman, 1972). In the subsequent years, the mitochondrial theory of aging was further refined and developed by Miquel and colleagues, who suggested that the accumulation of somatic mutations in the mtDNA induced by oxidative stress is the major contributor of aging and age-related degenerative diseases (Miquel et al., 1980). The mitochondrial theory of aging summarized that reactive oxygen species (ROS) emanating from the mitochondrial respiratory chain damages macromolecules, especially mtDNA. Consequently, the accumulation of mtDNA mutations leads to production of defective mitochondrial respiration, further increasing ROS generation and oxidative damage (Jang and Remmen, 2009). Although mitochondria contain an intricate antioxidant defense system that rapidly scavenges ROS to a non-toxic form, the balance between ROS generation and antioxidant defence is believed to be disrupted during the aging process and in age-related diseases, resulting in cumulative oxidative damage of biological macromolecules. In fact, an age-related increase in oxidative damage to lipids, proteins, and DNA has been demonstrated by several

investigators in a variety of tissues in humans and several experimental animal models (Muller et al., 2007). Mitochondrial DNA mutations progressively accumulate during life and are responsible for reduced cellular oxidative phosphorylation activity, leading to increased ROS production. The mitochondrial theory of aging can be practically demonstrated by manipulating the amount of oxidative damage to the mitochondria by increasing the expression of key antioxidant enzyme in order to reduce the oxidative insults to mitochondria augmented lifespan.

In resume, a common denominator of all these theories is the accumulation of damaged molecules that cannot be processed by the mechanism that remove cell debris. Probably, the main cause of this accumulation is an imbalance in the production of damaging compounds such as ROS and protective mechanism such as antioxidants and reparative process. The main source of ROS can be damaged mitochondria but also proinflammatory immunes process.

Aging Therapeutics

Calorie Restriction, Nutrition

During the history, humans have sought a means to longevity but few have been successful in their pursuit of an anti-aging intervention (Yu, 1996). Diet plays an important role especially when different dietary habits are considered. It has been reported that people consuming “Mediterranean diet” display a better health status with higher probability to escape some age-related diseases and to reach healthy longevity (Gibney and Gibney, 2004).

A non-genetic intervention that might be a modulating factor of the aging process is caloric restriction (CR). Mammalian life span could be significantly extended by CR was demonstrated in a rodent study published by McCay in 1935 (McCay et al., 1935). CR refers to a state in which energy intake is minimized to low-normal levels while adequate intakes of protein and micronutrients are maintained at sufficient levels to avoid malnutrition. CR typically consists of an energy intake that is 30–50% below that which is required to maintain normal body weight and adiposity and has been shown to improve health at all ages and also to slow the aging process in many eukaryotes (Colman et al., 2009). CR provides a powerful and widely applicable intervention for attenuating many age-related diseases and extending maximal life span (Masoro, 1990). CR can extend the average and maximum life span and delay the onset of age-associated changes, and has been proven in many organisms from yeast, worms, and flies to mammals (Piper and Bartke, 2008). This intervention also retards the development and severity of age-related diseases and attenuates the physiological decline associated with aging. In higher mammals, CR delays many diseases

associated with aging including cancer, diabetes, atherosclerosis, cardiovascular disease, and neurodegenerative diseases (Colman et al., 2009; Roth et al., 2001). The molecular mechanisms by which CR confers metabolic benefits are not entirely clear but have been primarily attributed to a reduction of oxidative damage to vital tissues. CR also activates SIRT1, a NAD^+-dependent deacetylase that directly regulates glucose and/or fat utilization in metabolically active tissues (Nogueiras et al., 2012). CR reduces oxidative stress in the liver (Hagopian et al., 2011), skeletal muscle (Usuki et al., 2004) and white adipose tissue (Valle et al., 2010) in rodents. Furthermore, CR is also suggested to counteract the age-associated changes by modulating the mTOR signaling pathway, IGF1/insulin signaling, adiponectin expression, DNA methylation, and histone acetylation and deacetylation (Ribarič, 2012). mTOR is a serine/threonine protein kinase of the phosphatidylinositol-3-OH kinase (PI(3)K)-related family that functions as a master regulator of cellular growth and metabolism in response to nutrient and hormonal cues (Stanfel et al., 2009). mTOR functions in two distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is thought to play a part in mediating longevity and health benefits as a result of dietary restriction. It is also logical to hypothesize that mTOR signaling, a major nutrient-sensing pathway which positively regulates anabolic processes, may at least partially be responsible for the effect of CR. Indeed, inhibition of mTOR activity, by either genetic disruption or a specific inhibitor rapamycin, has consistently been shown to extend lifespan in yeast, nematodes, fruit flies and mice (Simon et al., 2013). Furthermore, CR reduces mTORC1 activity in invertebrate organisms and in some mammalian tissues, and pharmacological or genetic disruption of mTORC1 is sufficient to extend lifespan in both invertebrates and mice under non-dietary restriction conditions (Stanfel et al., 2009). Numerous studies have linked mTORC1-regulated processes, including both reduced mRNA translation and induction of autophagy, to lifespan extension from dietary restriction in different organisms (Simon et al., 2013).

CR regulates also the peroxisome proliferator-activated receptors (PPARs) that has been is closely implicated in its anti-aging activity (Chung et al., 2011). PPARs are a family of transcription factors, PPARα, PPARβ/δ, and PPARγ; that regulate cell proliferation, differentiation, insulin sensitivity, and glucose and lipid metabolism. PPAR agonists suppress age-related oxidative stress and NF-κB activity (Sung et al., 2006). PPARs could interact with NF-κB in three modes: (1) a direct interaction between two transcription factors, (2) competition for limited co-activators, and (3) an effect on posttranslational modification.

Resveratrol

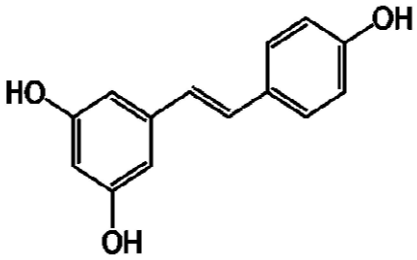


Figure 0.1. *Structure of RSV*

Resveratrol (RSV) (cis or trans-3,5,4-trihydroxystilbene) (Figure 0.1) is composed of two aromatic rings connected by a styrene double bond. In terms of its chemical and physical characteristics, it is a white solid powder with a molecular weight of 228.25 g/mol, low water solubility, photo and pH-sensitivity, and the presence of trans-and cis-isomers, with the trans-isomer being the preferred steric form. The pK_a values for the trans-isomeric mono-, di-, and tri-protonated forms are 9.3, 10, and 10.6, respectively. Both trans and cis-RSV can be detected with UV-HPLC at 308 and 288 nm, respectively (Mark et al., 2005).

RSV is a natural polyphenolic compound synthesized in a large number of plant species such as mulberries, peanuts, grapes, and is present in red wine. It has been related to the "French Paradox" (Renaud and de Lorgeril, 1992). This term was used to describe the inverse relation between coronary heart disease mortality and the predominantly red wine consumption seen in France.

RSV has recently gained popularity due to its anti-inflammatory, antioxidant, oxidative metabolic enhancing properties and the ability to mimic CR. Furthermore, the number of studies is growing to show RSV's various beneficial biological effects, including its antioxidant, anti-inflammatory activities, release of neurotransmitters and neuromodulators (Dal-Pan et al., 2011; Das and Das, 2010; Sakata et al., 2010). RSV closely resembles the effect of CR on improving oxidative stress and insulin sensitivity. RSV has also consistently been shown to improve insulin sensitivity and the associated metabolic parameters in patients with impaired glycemic control (Brasnyo et al., 2011; Crandall et al., 2012).

RSV's actions are thought to be primarily mediated through the NAD+ dependent deaceytlase, Sirt1 (Lagouge et al., 2006). RSV increases the expression and activity of Sirt1, and both RSV and Sirt1 activate PGC-1α leading to increased expression of PGC-1α target genes as well as increased expression of itself (Jackson et al., 2011). Supplementation of RSV increased PGC-1α protein expression and citrate synthase activity in the skeletal muscle of obese but otherwise healthy men, suggesting an improvement in mitochondrial efficiency (Tomé-Carneiro et al., 2012). The effect of

RSV on inducing mitochondrial biogenesis and function is likely to be mediated by an increase in both the expression and activity of PGC-1α (Um et al., 2010), a master switch of energy metabolism. SIRT1 directly modulates the transcriptional activity of PGC-1α by localizing to the promoters of its target genes (Zachary et al., 2007). Taken together, RSV promotes energy utilization at least partly by activating SIRT1/PGC-1α-dependent pathways (Lam et al., 2013). RSV has also been shown to mimic a CR that extends lifespan and stress resistance by linking to the longevity gene SIRT1 in yeast, worms, and flies.

RSV enhances the oxidative capacity of rodent skeletal muscle by increasing the expression of the components of mitochondrial electron transport chain (Price et al., 2012). In addition, RSV also increases mitochondrial density and activity in mouse liver and is associated with a concomitant increase in acetyl CoA carboxylase-1 (ACC-1) phosphorylation (Shiozaki et al., 2011). It has also been proposed that some of the beneficial effects of RSV result from phosphorylation/activation of AMP-activated protein kinase (AMPK), repressing proliferator-activated receptor gamma (PPARγ) and phosphatidylinositol 3-kinases (PI3K)/Akt (Baile et al., 2011). AMPK, a fuel sensor and master switch of energy homeostasis, is another important mediator of the metabolic benefits of RSV (Lam et al., 2013). RSV increases the phosphorylation of AMPK and ACC in C2C12 myotubes in a dose-dependent manner, indicating enhanced fat oxidation (Feige et al., 2008). Similarly, RSV supplementation increases the activity of both α1 and α2 isoforms of the catalytic subunit of AMPK in the skeletal muscle of diet-induced obese mice (Um et al., 2010). However, RSV-induced AMPK activation in the mouse skeletal muscle is also independent of SIRT1 which appears to rule out AMPK as a downstream target of SIRT1 (Um et al., 2010). The health benefits of RSV can be resumed in figure 0.2

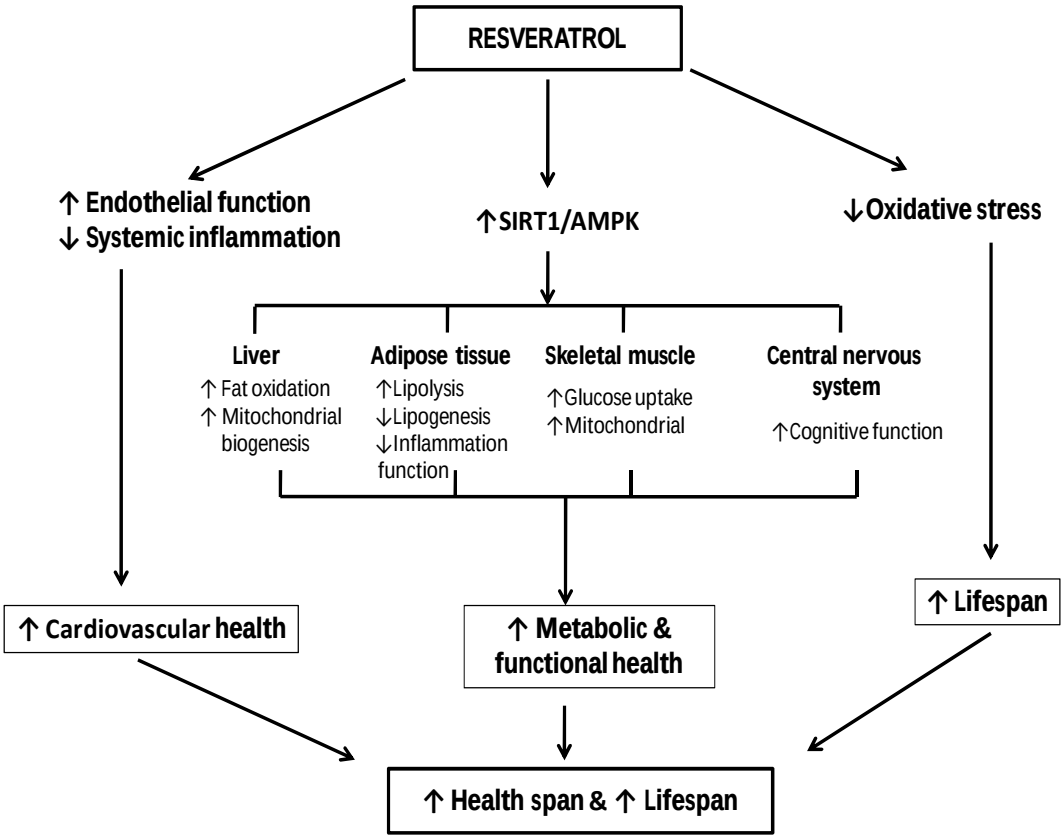


Figure 0.2. *Health benefits of RSV (Lam et al., 2013)*

Exercise

Physical exercise can improve the elderly's health. Regular physical activity reduces the deleterious effects of aging, providing an improvement in functional performance, the prevention of diseases and an increase in the longevity. Participation in regular physical activity program is an effective and secure mean to avoid and reduce the functional decline associated with aging and to improve quality of life. The recommended exercise program is multifactorial including aerobic, resistance training, exercises of balance and flexibility (Marom-Klibansky and Drory, 2002).

Physical exercise has two types: aerobic and strength exercise. Aerobic exercise is defined as a structured exercise programs involving the use of large muscle groups for extended periods of time in activities that are rhythmic in nature (Smith et al., 2010). Strength exercise involves the short periods of contractile activity (low repetition) that against a high opposing resistance. Aerobic and strength exercise collaborate with the prevention and treatment of mental diseases, which are mostly prevalent in older adults, like major depression, dementia and Parkinson's disease. Several mechanisms of neurobiological action are proposed to explain how exercise can actually reduce the

effects of aging (Deslandes, 2013). Strength exercise uses resistance that forces muscular contraction to build strength, anaerobic endurance, and skeletal muscle mass. Strength exercise often uses gravity to oppose muscle contraction.

Endurance exercise is characterized by prolonged and continuous periods of contractile activity against low resistance. Endurance exercise is the best way to improve cardiovascular function. It helps keep the heart muscle supple and the arteries flexible; lowers the resting heart rate; and boosts the heart's peak ability to deliver oxygen-rich blood to the body's tissues. Endurance exercise is best way to protect the body's metabolism from the effects of age. It reduces body fat, sensitizes the body's tissues to insulin, and lowers blood sugar levels (Harvard Men's Health Watch, 2005). And the same type of activity fights against some of the neurological and psychological changes of aging. Endurance exercise boosts mood and improves sleep, countering anxiety and depression; also improves reflex time and helps stave off age-related memory loss.

Exercise contributes towards the prevention of several cardiovascular and metabolic diseases, whilst acting as an associated treatment to medication (American College of Sports et al., 2009). Also, recent studies have showed that exercise is not only good for heart, lungs, vessels, muscles and bones, but also improves the health of brain (Deslandes et al., 2009). Exercise may contribute to the improvement of cognitive and behavioral functioning through neurobiological mechanisms necessary for neurogenesis, angiogenesis, synaptogenesis and plasticity.

Furthermore, physical activity can improve the sarcopenia in muscle, the loss of lean muscle mass occurring with advancing age. This condition often leads to a concomitant loss of strength, increases frailty and risk of falls, and an overall loss of functional independence in the elderly. Muscle protein metabolism is a dynamic process characterized by the balance between the synthesis and breakdown of muscle proteins. A disturbance of this equilibrium can lead to the loss of muscle mass and a perturbation of muscle protein turnover with aging has been proposed to play a role in the development of sarcopenia (Fry and Rasmussen, 2011).

So, as aging speeds up degenerative processes such asosteoporosis and cerebral atrophy; physical exercise increases lean body mass, bone mineral density, and the formation of new neurons and neuroplasticity (Deslandes, 2013).

Damage of Lipids, Proteins and DNA

Free radicals and other reactive species are constantly generated in vivo and cause oxidative damage to biomolecules, a process held in check only by the existence of multiple antioxidant and repair systems as well as the replacement of damaged nucleic acids, proteins and lipids.

Lipid peroxidation

Lipid peroxidation (LPO) is one of the main events induced by oxidative stress and is particularly active in biomembranes like mitochondria. Polyunsaturated fatty acids (PUFAs) are the family of the most important components of cell membranes in living systems. Free radical attack to PUFAs leads to the formation of highly reactive electrophilic aldehydes, including malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) as the most abundant products (Esterbauer et al., 1991).

The general process of lipid peroxidation consists of three stages: initiation, propagation, and termination (Figure 0.3). The initiation phase of lipid peroxidation includes hydrogen atom attraction. Several species can atract the first hydrogen atom and include the radicals: hydroxyl (OH•), alkoxyl (RO•), peroxyl (ROO•)

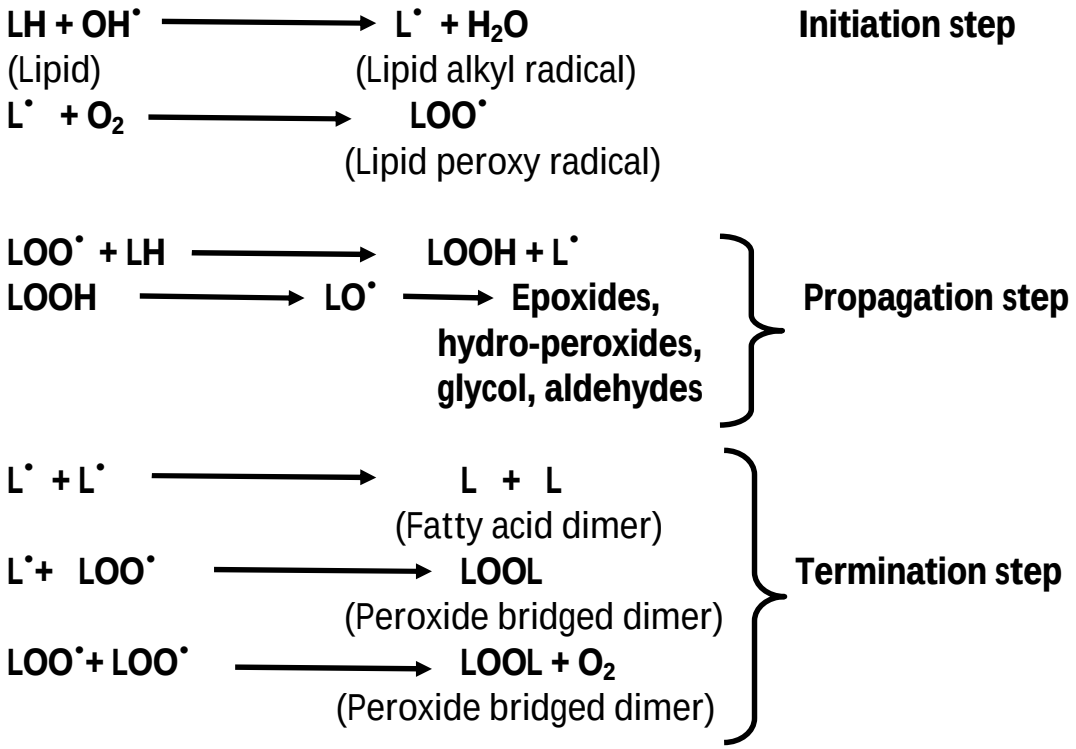


Figure 0.3. *Lipid peroxidation process. Adapted from Clark, 2008 (Clark, 2008).*

After H attraction at this position by oxidants like hydroxyl radical and activated species in oxidizing enzymes, a lipid molecule (LH) will be converted into a carbon-centered lipid radical (L•). The L• will then react with O_2 to form peroxyl radical (LOO•), which can further attack other lipid molecules during the propagation process. In addition, the LOO• can also be converted into other types of free radicals through Fenton-type reactions and β-scission (Gardner, 1989; Halliwell and Chirico, 1993).

Lipid peroxidation disrupts normal structure and function of lipid bilayers surrounding both the cell itself and membrane-bound organelles (Catalá, 2012). The LPO may alter membrane permeability, transport processes, and fluidity. LPO induces disturbance of fine structure, alteration of integrity, fluidity, and permeability, and functional loss of biomembranes, modifies low density lipoprotein (LDL) and high density lipoprotein (HDL) to pro-atherogenic and pro-inflammatory forms, and generates potentially toxic products. Further, LPO products have been shown to be mutagenic and carcinogenic (Poli et al., 2008). Lipid peroxidation is associated with the pathogenesis of a number of age-associated diseases, such as atherosclerosis, neurological diseases, cardiovascular diseases, cancer and aging.

The protective effects exerted by antioxidant toward HNE and other toxic aldehydes have been investigated by many groups to test the possibility of a therapeutic use of free radical scavengers and antioxidants against lipid peroxidation-mediated toxicity. In addition to small molecules, antioxidant enzymes such as heme oxygenase-1 (HO-1), catalase, superoxide dismutase, peroxiredoxin, and glutathione reductase have been shown to lead to a significant decrease in lipid peroxidation products (Siow et al., 2007).

Protein oxidation

Because proteins are most abundant in cells, it is not surprising that they are major targets for oxidative modifications. Exposure to ROS is known to cause a range of reversible and irreversible covalent modifications of amino acid side-chains of proteins (Ghezzi and Bonetto, 2003). ROS can attack proteins in different ways: directly at the protein backbone, amino acid residue side chains or they can lead to the formation of protein carbonyls. As a result of this damage, the affected proteins lose their biochemical functionality, protein expression is altered and finally they aggregate, resulting in different consequences for the cells (Höhn et al., 2013). Carbonyl (CO) groups (aldehydes and ketones) are produced on protein side chains (especially of Pro, Arg, Lys, and Thr) when they are oxidized. The formation of carbonyl groups occurs during normal aging and in neonates receiving oxygen ventilation (Gladstone and Levine, 1994). Protein carbonyl derivatives

can also be generated through oxidative cleavage of proteins by either the α-amidation pathway or by oxidation of glutamyl side chains, leading to formation of a peptide in which the N-terminal amino acid is blocked by ana-ketoacyl derivative (Berlett and Stadtman, 1997). Protein carbonyl content is actually the most general indicator and by far the most commonly used marker of protein oxidation.

Protein carbonylation refers to a process that forms reactive ketones or aldehydes that can be reacted by 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones. Thus, protein carbonyls in cells and tissues have been observed during oxidative stress. Increase in oxidative stress during altered homeostasis of oxidants and antioxidants leads to deleterious effect on cellular components through oxidative damages to proteins, lipids, nucleic acids. The protein carbonyl content resulted by oxidation makes the protein resistant to hydrolysis and functional inactivation of proteins in serum or plasma, cellular components, membrane proteins. Since, protein is major constituents of all forms of the biological system the exact conformation and three dimensional folding are highly connected to the protein functions, the restore of nativity of protein is crucial. Thus, critical evaluation of protein carbonyl content serves as biomarkers of protein oxidative damage in various conditions like diabetes, aging, neurodegeneration, smoking (Čolak, 2008). Accumulation of protein carbonyls has been observed in several human diseases including Alzheimer's disease, diabetes, inflammatory bowel disease, and arthritis (Beal, 2002; Chevion et al., 2000; Dalle-Donne et al., 2003).

A number of studies have shown increases in the intracellular concentrations of oxidized proteins as a function of age. Increases in protein carbonyls occur in rat hepatocytes, drosophila, brain, and kidney of mice and in brain tissue of gerbils (Sohal et al., 1993; Sohal et al., 1995; Sohal et al., 1994). In drosophila, restricting flying increases life span, and this correlates with reduced protein carbonyls (Yan and Sohal, 2000). In humans protein carbonyls increase with age in brain, muscle, and human eye lens (Mecocci et al., 1999; Pansarasa et al., 1999; Russell et al., 1987; Smith et al., 1991). The carbonyl content of human fibroblasts also increases as a function of age of the donor (Oliver et al., 1987).

DNA damage

DNA is probably the most biologically significant target of oxidative attack, and it is widely thought that continuous oxidative damage to DNA is a significant contributor to the age-related development of the major cancers, such as those of the colon, breast, rectum, and prostate. Cumulative DNA damage has been considered to be a key factor contributing both to cell aging as

well as predisposing to neoplastic transformation. During aging, mitochondrial ROS production results in DNA damage and mitochondrial dysfunction (Fraser, 2009). Oxidative stress and the resultant accumulation of ROS/RNS can lead to a number of different DNA lesions including direct modification of nucleotide bases, formation of apurinic/apyrimidinic sites, DNA single strand breaks, DNA double strand breaks and cross-linking to other molecules. Direct nucleotide modifications have been widely reported as consequences of oxidative/nitrosative damage to the cell (Bohr and Anson, 1999; Smith et al., 2013). Among numerous types of oxidative DNA damage, the formation of 8-hydroxydeoxyguanosine (8-OHdG) is a ubiquitous marker of oxidative stress. 8-OHdG, one of the oxidative DNA damage byproducts, is physiologically formed and enhanced by chemical carcinogens. During the repair of damaged DNA in vivo by exonucleases, the resulting 8-OH-dG is excreted without further metabolism into urine.

Antioxidant systems

Mammals have evolved with an elaborate defense network against oxidative stress, in which multiple antioxidant compounds and enzymes with different functions exert their respective roles. Antioxidants can be generally classified into two categories, enzymatic and non-enzymatic. The enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), supported by other auxiliary enzymes such as glutathione reductase (GR), glucose 6-phosphate dehydrogenase (G6PDH) and glutathione sulfur-transferase (GST). The non-enzymatic antioxidants include antioxidant vitamins (α-tocopherol, ascorbic acid and β-carotene), sulphydryls (mainly GSH), and a variety of low molecular weight compounds such as lipoic acid, uric acid, and ubiquinone (Halliwell, 2012; Niki, 2010; Nordberg and Arnér, 2001).

Each of the antioxidants is located in specific cellular sites and specialized in removing certain ROS, although considerable overlap and cooperation are demonstrated between them.

According to the free radical theory of aging we might expect to find a general decline of cellular antioxidant defense capacity at old age. This may be due to aging is associated with a deterioration of protein synthesis and cell differentiation capacity in most tissues.

Enzymatic Antioxidants

The primary antioxidant enzymes against superoxide radicals include SOD (EC 1.15.1.1), catalase (CAT; EC 1.11.1.6), and glutathione peroxidase (GPx; EC 1.11.1.9). These enzymes act together in the metabolic pathway of free radicals, and altered activity of one enzyme without compensatory changes in others may lead to lipid peroxidation (Figure 0.4).

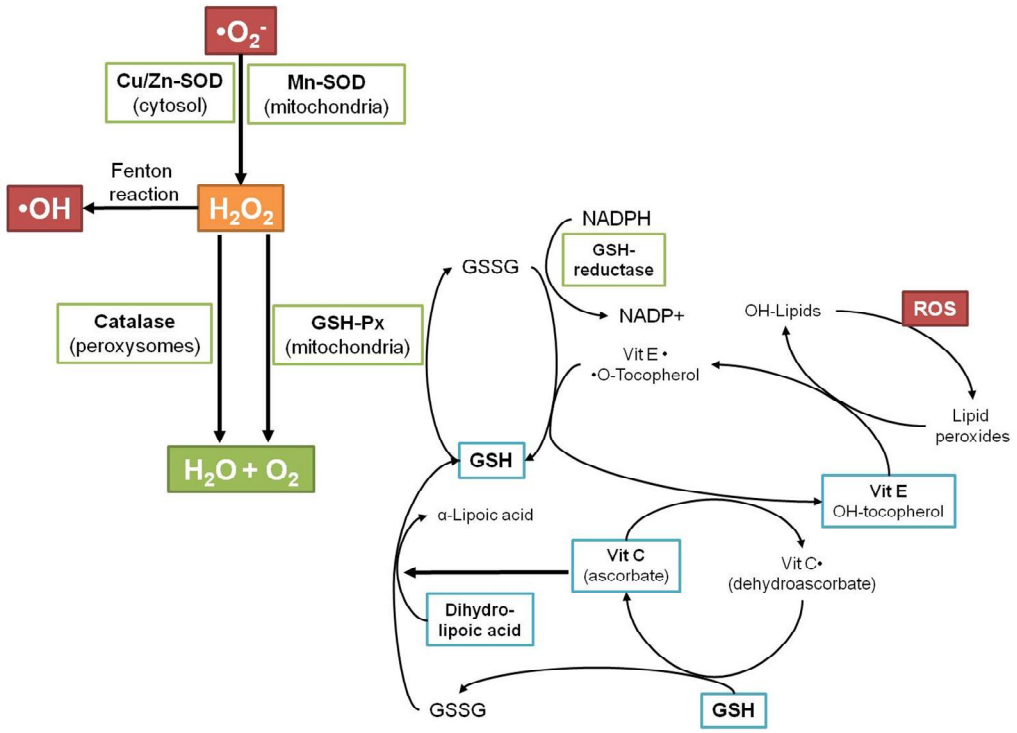


Figure 0.4. *Multiple enzymatic scavengers are utilized by the cell to limit damage from reactive oxygen species. Potentially toxic ROS are mainly removed by the enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) (Lazo-de-la-Vega-Monroy and Fernández-Mejía, 2013).*

Superoxide dismutases (SODs) are the main antioxidant enzymes that catalyze the conversion of superoxide anion (O_2•−) to hydrogen peroxide (H_2O_2) and protect cells and tissues from the reactive oxygen species (ROS) generated from endogenous and exogenous sources. Three SOD isoforms are expressed in mammalian cells: copper/zinc SOD (CuZn-SOD, SOD1), which is located in the cytoplasm (McCord and Fridovich, 1969); manganese SOD (Mn-SOD, SOD2), which is localized in the mitochondrial matrix (Weisiger and Fridovich, 1973); and extracellular SOD (EC-SOD, SOD3) (Marklund, 1982). Mitochondria are both a major source of ROS production from the respiratory chain and a major target of ROS-induced cellular injury (Daiber, 2010). Therefore, mitochondrial Mn-SOD is thought to play an important role in the cellular defense against oxidative damage by ROS.

Catalases (CAT) are heme-containing enzymes that convert hydrogen peroxide (H_2O_2) to water and O_2, and they are largely localized in subcellular organelles such as peroxisomes. Mitochondria and the endoplasmic reticulum contain little CAT. Thus, intracellular H_2O_2 cannot be eliminated unless it diffuses to the peroxisomes. Increased CAT is associated with a greater resistance to oxidant damage (Aebi, 1984; Michiels et al., 1994). Age dependent changes in catalase activity appear to

vary with tissue with reports of increased activity in muscle and decreased activity in liver (Ji et al., 1990; Terlecky et al., 2006).

Cytochrome b_5 reductase (NADH-cytochrome b_5 reductase; EC 1.6.2.2) ($CytB_5Rase$) is FAD-containing flavoprotein. The enzyme is usually bound to the endoplasmic reticulum, but has also been found bound to outer mitochondrial membranes in the liver (Sottocasa et al., 1967) and plasma membranes in erythrocytes (Choury et al., 1981). This enzyme transfers the electrons from NADH to cytochrome b_5 which plays a central role in many diverse metabolic reactions in liver such as in fatty acid desaturation, in elongation of fatty acids, in biosynthesis of cholesterol, in plasmologen synthesis, in prostaglandin synthesis and in drug metabolism involving cytochrome P450 mixed function oxidations (Arinç, 1991). A second soluble form of $CytB_5Rase$ exists in erythrocytes where it catalyzes the reduction of methemoglobin via transferring electrons to cytochrome b5 (Arinç et al., 1992). Furthermore, $CytB_5Rase$ is one of the most important components of cytochrome P450s, which play an essential role in the detoxification of xenobiotics.

NAD(P)H:quinone oxidoreductase 1 (NQO1) is an obligate two-electron reductase that is characterized by its capacity for utilizing either NADH or NADPH as a reducing cofactor and by its inhibition by dicoumarol. NQO1 reduces quinones to hydroquinones in a single two-electron step. It is primarily a cytosolic enzyme (>90%) and exists as a homodimer with one molecule of FAD per monomer. NQO1 was suggested that the enzyme may play an antioxidant role via the reduction of endogenous quinones and these compounds, when reduced, help protect cellular membranes against oxidative damage (Ross et al., 2000).

Plasma membrane redox system

It has been shown that the plasma membrane (PM) is involved in responsive of cell to oxidative stress. PM regulates cellular ion homeostasis, nutrient transport, cell adhesion, and signal transduction. It is important key for protecting cellular integrity during aging (De Cabo et al., 2004). The PM redox system (PMRS) provides electrons to recycle lipophilic antioxidants. Coenzyme Q (CoQ), in its reduced form, is a key in a PMRS. It is involved in membrane antioxidant protection, in the maintenance of intracellular redox homeostasis, regulation of cell signaling (López-Lluch et al., 2010). Furthermore, both enzymes $CytB_5Rase$ and NQO1 also contribute to the PMRS, providing the source of electrons to maintain its antioxidant properties and responsive to oxidative stress and aging (Hyun et al., 2007). The $CytB_5Rase$ transfers one electron from NADH to PM CoQ, generate CoQ•- in the PM and NQO1 reduces CoQ by a two electron mechanism using NAD(P)H in the PMRS, subsequently causing no formation of CoQ•- (López-Lluch et al., 2010) (Figure 0.5). PMRS activity regulates the NAD^+/NADH ratios of the cytosol, which is important for normal energy metabolism (Lopez-Lluch et al., 1998). The PMRS activity plays an important role

in growth and development of organisms (Crane and Navas, 1997). Moreover, the PMRS activity may maintenance the bioenergetics in cells when mitochondria activity decreases such as in aging (de Grey, 2001).

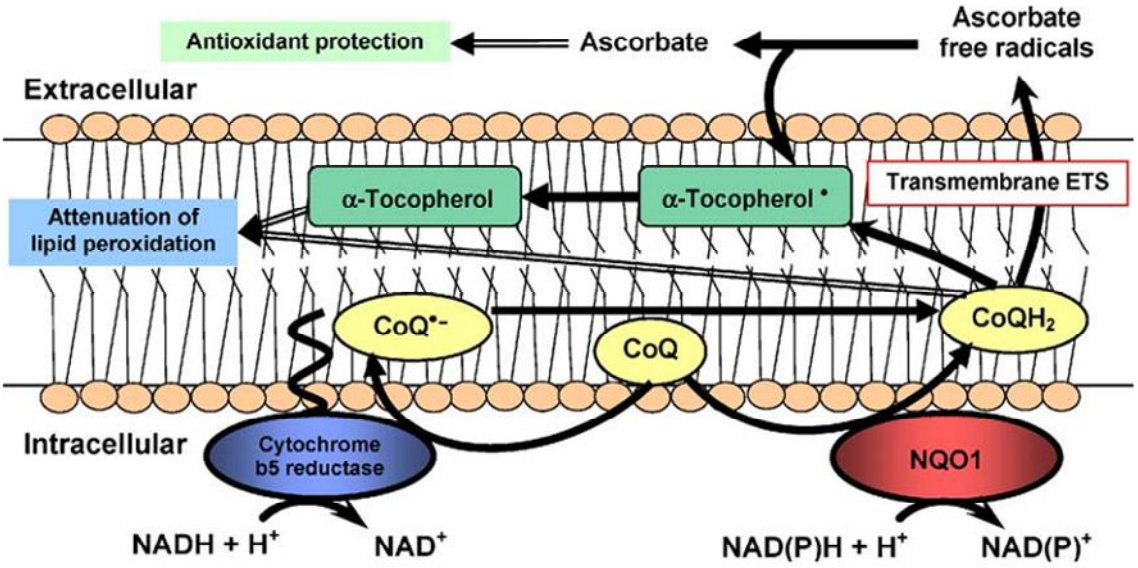


Figure 0.5. *The plasma membrane redox system (Hyun et al., 2006)*

The glutathione system, which consists of reduced glutathione (GSH) and the GSH-related enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR), is foremost among these antioxidant systems. GPx belongs to a class of enzymes that catalyze the reduction of hydrogen peroxide, phospholipid-hydroperoxide and other organic hydroxyperoxides by GSH. GSH is converted to its oxidized form (GSSG) after peroxide reduction, and GR catalyzes the NADPH-dependent conversion of GSSG to regenerate GSH, which can act as intracellular reductant (Dringen et al., 2005; Gul et al., 2000). GPx remove H_2O_2 by coupling its reduction with the oxidation of GSH. GPx can also reduce other peroxides, such as fatty acid hydroperoxides. These enzymes are present in the cytoplasm at millimolar concentrations and also present in the mitochondrial matrix. GR plays a major role in GPx and glutathione-S-transferase (GST) reactions as an adjunct in the control of peroxides and free radicals (Bompart et al., 1990). GR is a homodimeric flavoprotein and contains two FAD molecules as a prosthetic group, which is reducible by NADPH. When levels of catalase, another universally present antioxidant enzyme, are decreased, the glutathione dependant enzymes become activated (Gaetani et al., 1994).

Glutathione, which is the most important low molecular antioxidant containing group sulfhydryl, is a tripeptide antioxidant used by a variety enzyme in the detoxification of xenobiotics. It may be conjugated to toxic electrophilic substrates to render them less harmful and to facilitate their

removal from cells, or, it may be covalently added to proteins in response to stress or in the regulation of normal cellular processes (Dalle-Donne et al., 2009). GSH is synthesized in two steps. First, γ-glutamylcysteine synthetase (γ-GCS) forms a γ-peptide bond between glutamic acid and cysteine, and then GSH synthetase adds glycine. GSH prevents the oxidation of protein thiol groups, either directly by reacting with reactive species or indirectly through glutathione-S-transferases. Furthermore, glutathione-S-transferase (GST) catalyzes the conjugation of GSH to electrophilic centers on a wide variety of substrates via a sulfhydryl group, which decreases peroxide levels (Vendemiale et al., 1999). Then, GST is a group of enzymes that are important in the detoxication of many different xenobiotics in mammals. GST defends cells against the mutagenic, carcinogenic, and toxic effects of the compounds. Glucose-6-phosphate dehydrogenase helps to maintain a steady supply of metabolic intermediates such as GSH and nicotinamide adenine dinucleotide-phosphate (NADPH), which are necessary for optimum functioning of the key antioxidant enzymes.

The thioredoxin system, consisting of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH functions as an efficient general reductase for protein disulfide bonds, thereby playing an important role in a variety of cellular processes, including the formation of precursors for DNA synthesis, defenses against oxidative stress and maintenance of the generally reducing intracellular environment (Ahsan et al., 2009; Holmgren and Bjornstedt, 1995). The mammalian TrxR belong to a family of pyridine nucleotide-disulfide oxidoreductases demonstrating sequence homologies, e.g. a conserved Cys-Val-Asn-Val-Gly-Cys sequence at the catalytic center, a sequence also present in glutathione reductase (Ahsan et al., 2009). TrxR catalyzes the reduction of the active site disulfide in Trx using NADPH. There are two forms of Trx in mammalian cells: Trx1 is located primarily in the cytosol while Trx2 is the mitochondrial form of thioredoxin. Trx is a ubiquitous 12 kDa protein originally characterized as a hydrogen donor to ribonucleotide reductase but a large number of other functions for this protein have subsequently been discovered. Through the reversible formation of disulfide dithiols involving the sulfur atoms in the side chains of critical cysteine residues, Trx may regulate the activities of enzymes and transcription factors.

The nuclear E2-related factor 2

More than 20 redox-sensitive transcription factors have been found in mammalian cells, among which nuclear E2-related factor 2 (Nrf2) takes a special place. The Nrf2 plays a key role in orchestrating cellular antioxidant defenses and maintaining redox homeostasis. Accumulating evidence suggests that an age-related decline in cellular glutathione levels may be due to diminished Nrf2 activation, with several studies implicating Nrf2 as a therapeutic target in the protection against cardiovascular disease and diabetes. Nrf2 regulates transcription of genes containing

antioxidant responsive element (ARE) in their promoters. ARE is a cis-acting regulatory element or enhancer sequence, which is found in promoter regions of genes encoding phase II detoxification enzymes and antioxidant proteins. In cells, Nrf2 is constantly controlled by the repressor protein Kelch-like ECH associating protein 1 (Keap1), which is a special molecular "sensor" of changes in intracellular homeostasis (Cullinan et al., 2004). The nuclear localization of Nrf2 plays a key role for cell survival under oxidative stress condition. Upon exposure of tissues to oxidative or electrophilic stress, Nrf2 is released from Keap1. The released Nrf2 is stabilized and translocates from the cytoplasm to the nucleus, forms a heterodimer with its obligatory partner Maf, and transactivates expression of detoxification enzymes and antioxidant enzymes such as GST, NQO1, and heme oxygenase 1 (HO-1) also the coenzyme Q_{10} (Figure 0.6) (Lee and Johnson, 2004).

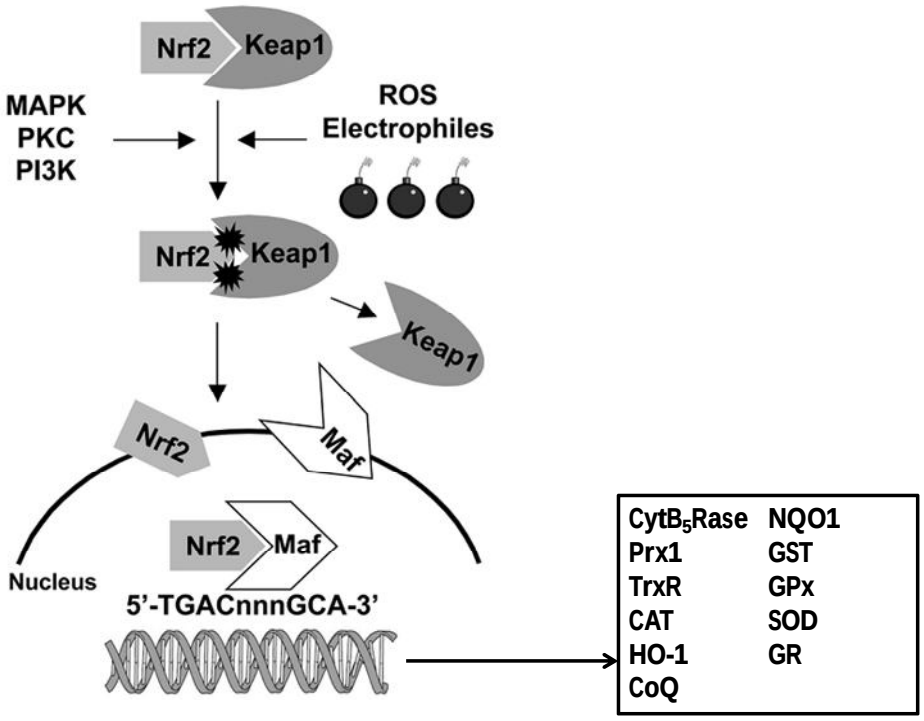


Figure 0.6. *Antioxidant responsive elemen (ARE)-driven gene expression by Nrf2. ARE activation signals dissociate the Nrf2-Keap1 complex allowing Nrf2 to translocate into the nucleus where it binds to the ARE and transcriptionally activates downstream target genes. Adapted from Lee and Johnson, 2004 (Lee and Johnson, 2004).*

Non-enzymatic Antioxidants

A major contribution to the total antioxidant capacity comes from antioxidant molecules in plasma. The non-enzymatic antioxidant components consist of molecules that interact with ROS and thereby terminate the free radical chain reactions. There are several antioxidant molecules in plasma, such

as albumin, uric acid and ascorbic acid which account for 85% of the total antioxidant capacity (Yao et al., 1998), and other antioxidants that are in blood include bilirubin, α-tocopherol and β-carotene (Yao et al., 1998; Yao et al., 2000). The most important lipid-phase antioxidant is α-tocopherol which is a powerful, lipid-soluble chain-breaking antioxidant found in lipid membranes, circulating lipoproteins and low-density lipoprotein (LDL) particles (Foy et al., 1999). Vitamin E comprises eight naturally occurring fat-soluble vitamins of which the most predominant, essential and with the highest biological activity is α-tocopherol. It is a major antioxidant in biological systems acting as a powerful chain-breaking agent through the scavenging of peroxyl radicals. Vitamin E terminates the chain reaction of lipid peroxidation in membranes and lipoproteins (Shi et al., 1999). If vitamin E is one of the major lipid soluble antioxidants, the water soluble vitamin C is one of the key aqueous phase antioxidants. Vitamin C is capable of scavenging ROS, such as superoxide, hydrogen peroxide and singlet oxygen. Vitamin C might also reduce oxidised tocopherol. Ascorbic acid itself is oxidised to dehydroascorbate and reduced back to ascorbic acid by e.g. glutathione. The concentration of ascorbic acid in plasma is clearly related to the dietary intake. Certain organs and tissues contain a higher concentration than plasma, especially the adrenal glands, brain and leukocytes (Young and Woodside, 2001).

Coenzyme-Q

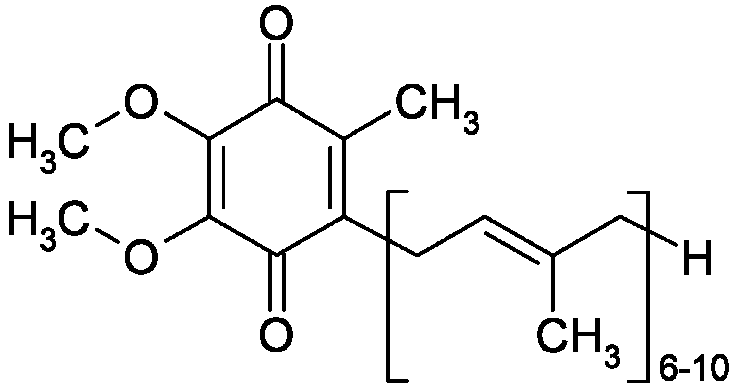


Figure 0.7. *Structure coenzyme Q*

Coenzyme Q (ubiquinone) (CoQ) (Figure 0.7) is a lipid-soluble compound composed of a redox active quinoid moiety and a hydrophobic tail. CoQ is composed of a tyrosine-derived quinone ring, linked to a polyisoprenoid side chain, consisting of 9 or 10 subunits in higher invertebrates and mammals. The predominant form of coenzyme Q in humans is CoQ_{10}, which contains 10 isoprenoid units in the tail, while the predominant form in rodents is contains 9 isoprenoid units. CoQ content in rodents as well as the ratios of CoQ_9 and CoQ_{10} vary in different organelles, tissues and species.

CoQ a vitamin-like, is an endogenous enzyme cofactor that is distributed in cellular membranes, is an essential component of the mitochondrial respiratory chain (Crane, 2001; Hargreaves, 2003). CoQ is a redox molecule (2,3-dimethoxy, 5-methyl, 6-decaprenyl benzoquinone), which exists in biochemically reduced CoQ (ubiquinol) and oxidized CoQ (ubiquinone) forms in biological tissues (Crane, 2001). CoQ is a key component for, at least, three essential systems in the cell: (1) the respiratory transport chain in mitochondria, (2) an antioxidant component in cell membranes, and (3) a key components in the maintenance of the redox homeostasis of the cell.

Due to its important role in mitochondrial and membrane functions, the redox state of CoQ (ubiquinol/ubiquinone ratio) has been suggested to be a useful biomarker of oxidative stress (Lagendijk et al., 1996; Yamamoto et al., 1998). CoQ has a functional role in many known membrane oxido-reductase systems therein; mitochondria, lysosomes, plasmalemma, Golgi apparatus.

The physiological roles of CoQ in biological systems are most well characterized in the inner mitochondrial membrane. First, CoQ is an essential cofactor in the mitochondrial electron transport chain, where it transfers electrons from complexes I and II to complex III in the mitochondrial respiratory chain and plays important roles in mitochondrial ATP production, playing a crucial role in cellular metabolism; regulating mitochondrial uncoupling proteins, the mitochondrial permeability transition pore, β-oxidation of fatty acids, nucleotide metabolism and production of reactive oxygen species (ROS) (Samorì et al., 1992; Turunen et al., 2004).

The reduced form of CoQ (ubiquinol) may act as an antioxidant by reducing reactive radicals and forms ubisemiquinone in biological systems (Forsmark-Andrée et al., 1997; Noack et al., 1994). CoQ is considered an important antioxidant because it is synthesized endogenously, is regenerated by intracellular reducing mechanisms, and is present in relatively high concentrations (Forsmark-Andrée et al., 1995; Noack et al., 1994).

Protection against oxidative damage by CoQ has been demonstrated in liposomes, low-density lipoproteins, biological membranes, proteins and DNA (Forsmark et al., 1991). CoQ is highly efficient in preventing lipid, protein and DNA oxidation and it is continuously regenerated by intracellular reduction systems. CoQ's effectiveness as lipid peroxidation inhibitor is based on its complex interaction during the process of peroxidation (Figure 0.8). The primary action is the prevention of lipid peroxyl radical ($LOO^{\bullet}$) production during the initiation process. This is the first phase of the process, where an attraction of a hydrogen atom from a methylene group of a fatty acid occurs, presupposing that it has several double bonds. $CoQH_2$ reduces the initiating perferryl radical with the formation of ubisemiquinone and H_2O_2. It is also possible that $CoQH_2$ eliminates $LOO^{\bullet}$ directly.

CoQ is also effective in preventing protein oxidation by quenching the initiating perferryl radical and functioning as a chain-breaking antioxidant, thus preventing the process of propagation. This is the second phase in lipid peroxidation, where $LOO^{\bullet}$ attracts a hydrogen atom from an additional unsaturated fatty acid, leading to formation of a carbon-centered radical ($L^{\bullet}$) and lipid hyperoxide (LOOH), which can be reoxidized to $LOO^{\bullet}$.

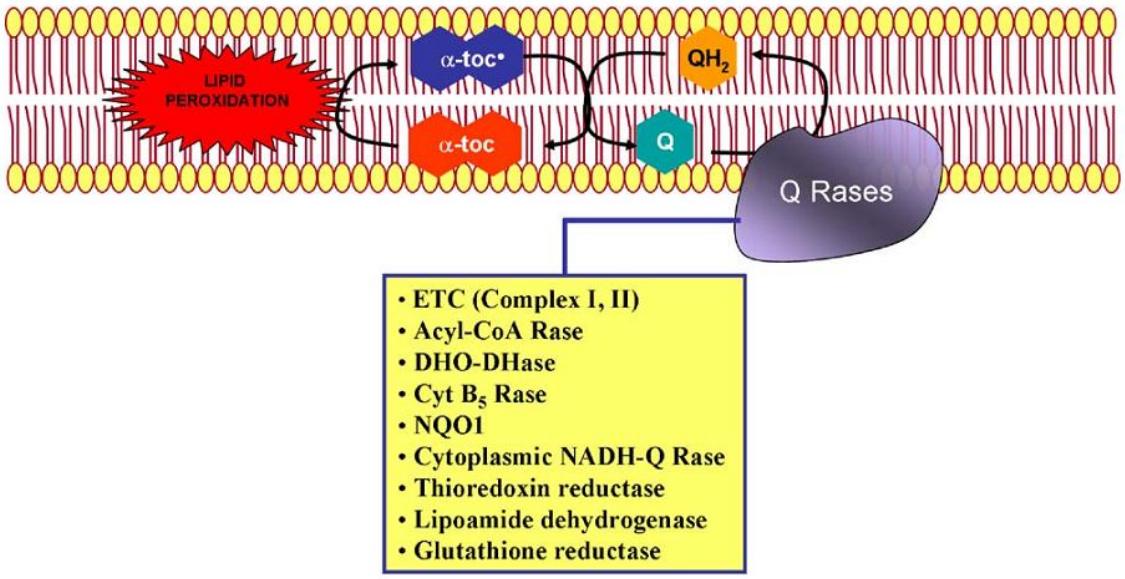


Figure 0.8. *CoQ-depending antioxidant redox cycle in cell membranes (López-Lluch et al., 2010).*

CoQ also protects DNA against oxidative damage, which is of particular interest for mitochondrial DNA. As it has been above indicated oxidative stress may damage DNA by initiating a series of metabolic reactions in the cell leading to activation of nuclease enzymes that cleave the DNA backbone (Tiano et al., 2012).

The inner mitochondrial membrane contains CoQ, which has antioxidant properties, thereby raising the issue about its respective roles in quenching the free radicals generated in inner mitochondrial membrane. The apparently paradoxical property of mitochondrial CoQ to potentially act both as a pro-oxidant and an antioxidant would seem to suggest that it may also be a modulator of the cellular redox state under physiological and/or pathological conditions, and particularly it may play a role in the aging process. CoQ is suspected to be involved in both of these age-related alterations because as an electron carrier, CoQ is a component of the oxidative phosphorylation system, and because CoQ is also an ROS generator and a quencher.

Due to these essential roles, deficiency of CoQ has been involved in several diseases most of them associated with aging process and mainly carrying neuromuscular disorders. The maintenance of

CoQ levels in these tissues is extremely important due to their high dependence to oxidative metabolism. Thus, new mechanisms to increase CoQ levels in these tissues must be developed to improve cellular activities in aging and CoQ-deficient diseases (López-Lluch et al., 2010).

Decreased levels of CoQ are found in patients with cardiomyopathies, congestive heart failure, degenerative muscle diseases. Furthermore, it has been proposed that CoQ levels decrease in all organs with aging (Kalén et al., 1989). Thus, CoQ is physiologically important as an antioxidant and has been used to treat cardiovascular disorders (Belardinelli et al., 2006; Langsjoen and Langsjoen, 1999), migraine headache (Rozen et al., 2002) and neurodegenerative disease such as Parkinsonism (Beal, 2002; Shults Clifford, 2003). In addition, it has been investigated as a treatment for cancer and the relief of some of the side effects of cancer treatment (Sakano et al., 2006).

Inflammation systems

Inflammation is a physiologic process in response to tissue damage resulting from microbial pathogen infection, chemical irritation, and/or wounding. At the very early stage of inflammation, neutrophils are the first cells to migrate to the inflammatory sites under the regulation of molecules produced by rapidly responding macrophages and mast cells prestationed in tissues. As the inflammation progresses, various types of leukocytes, lymphocytes, and other inflammatory cells are activated and attracted to the inflamed site by a signaling network involving a great number of growth factors, cytokines, and chemokines (Belardinelli et al., 2006). Inflammation may be local or systemic, and it can be classified acute or chronic. Acute inflammation is a short term non-specific response to harmful stimuli initiated by several chemical mediators that promote vascular and cellular changes characterized by vasodilatation, leakage of vasculature, and the flow of plasma and leukocytes to the site of injury, followed by rapid resolution phase and repair of the damaged tissue (Kundu and Surh, 2012). Acute inflammation is fundamentally a protective response with ultimate goal to eliminate the injury-inducing stimuli, prevent tissue damage and/or initiate repair processes. If the acute inflammatory response fails to eliminate the pathogen, the inflammatory process persists and acquires new characteristics. Chronic low-grade inflammation may result when there is inadequate resolution of acute inflammation which can prove harmful and may lead to disease (Federico et al., 2007). Many chronic inflammatory diseases that are not caused by infection or injury seem to be associated with conditions that were not present during the early evolution of humans, including the continuous availability of high-calorie nutrients, a low level of physical activity, exposure to toxic compounds, and old age. Thus, chronic inflammatory states are

associated with the homeostatic imbalance of one of several physiological systems that are not directly functionally related to host defense or tissue repair (Medzhitov, 2008).

Inflammatory processes of innate immunity are evident participants in many tissue changes of normal aging and most of the chronic degenerative diseases of aging. The aging phenomenon is associated with an increased incidence of inflammatory diseases and has some elements of the chronic inflammatory response. In addition to oxidative stress, chronic inflammatory events are known to participate in the decline in physiological functions of aging organs.

A number of signs of the chronic inflammatory response, such as a decrease in muscle protein and a rise in the plasma concentration of acute phase proteins have also been observed in senescence, accompanying a decrease in the antioxidant status (Nuttall et al., 1999) and an increased production in tumor necrosis factor-alpha (TNF-α) (Kudoh et al., 2001; Rink et al., 1998). In chronic inflammatory diseases, for example rheumatoid arthritis, an increased oxidative stress is present (Grimble, 2001). Inflammation may be considered a core process of human aging because of its involvement in baseline aging and in the major degenerative diseases of later life, atherosclerosis, Alzheimer disease, and cancer. Blood levels of C-reactive protein (CRP), interleukin (IL)-6, and other proinflammatory cytokines are risk indicators of cardiovascular events and mortality. Even in the absence of specific pathological lesions, inflammatory gene expression increases during aging in humans and animal models, in mammals and in several invertebrate models. Inflammation may prove central to therapeutic interventions for specific diseases as well as to general anti-aging strategies.

Inflammatory responses are part of the host immune defenses to pathogens and tissue responses to injury. At molecular levels, systemic infections and traumatic wounds can trigger rapid innate immune responses with hepatic secretion of IL-6 and TNF-α which mediate systemic energy metabolism and adaptive immunity. IL-1, IL-6, and TNF-α are examples of proinflammatory cytokines, while IL-10, IL-13, and the secretory leukocyte protease inhibitor fall into the category of anti-inflammatory cytokines. These pro- and anti-inflammatory mediators are synthesized and released by a wide variety of cells in response to infection, tissue damage, and other environmental and biochemical stimuli. Furthermore, TNF-α and IL-1 are not only directly cytotoxic to endothelial cells in general, but they also act as specific transcriptional activators in vascular endothelial cells.

Prostaglandins (PGs) and leukotrienes are arachidonic acid derived lipid metabolites with potent pro-inflammatory and potentially pathogenic characteristics. PG and leukotriene metabolites are important pro-inflammatory mediators and can induce ROS production, leading to tissue damage (Yu and Chung, 2001). Cyclooxygenase (COX) and lipoxygenase (LOX), key enzymes in the PG synthetic cascade, convert arachidonic acid to PGH2 and leukotrienes, and generate significant

ROS, particularly during aging, because of increased COX and LOX activities (Zou et al., 2009). Increased COX and LOX levels are an important contributor to the molecular inflammation (Chung et al., 2002; Zou et al., 2009). Furthermore, increase of pro-inflammatory PGs and decrease cytoprotective PG have been associated with aging (Choi and Yu, 1998).

TNF-α is a pleiotropic cytokine with a wide variety of functions in many cell types which is produced by a variety of cells types, including activated macrophages and lymphocytes. It stimulates proliferation of normal cells, exerts cytolytic or cytostatic activity against tumor cells, and causes inflammatory, antiviral and immunoregulatory effects. TNF-α exerts its biological activity by binding to types 1 and 2 receptors (TNFR-1 and TNFR-2) and activating several signaling pathways. Both TNF receptors have significant homology in their extracellular domains in that they contain cystein-rich domains; however, they differ structurally in their cytoplasmic domains. TNFR-1 contains a death domain (DD), whereas TNFR-2 lacks DD. TNFR-1 is more abundantly expressed, existing in most tissues and cell types and appears to be the main signaling receptor. The majority of deleterious effects produced by TNF-α seem to be mediated via this receptor. TNFR-1 mediates many actions of TNF-α, including cytokine production, activation of transcription factors like NF-κB, and apoptosis (Bhardwaj and Aggarwal, 2003). It has been described that during human aging TNF-α production and TNF-α-induced apoptosis are increased (Gupta et al., 2003). Also plasma levels of TNF-α are elevated in the elderly (Bruunsgaard et al., 2003).

IL-6 is known as “a cytokine for gerontologists” (Krabbe et al., 2004). IL-6, a pleiotropic 184 amino acid monomer, is one of the interleukins that can act both as a pro-inflammatory or anti-inflammatory cytokine and myokine (Wu and Schauss, 2012). It is usually produced by lymphocytes, macrophages, endothelial cells, fibroblasts, hepatocytes and neural tissues to stimulate immune response, especially during infection and after trauma, such as burns or other tissue damage leading to inflammation. The synthesis of IL-6 is tightly regulated and expressed at low levels in healthy individuals, however during infection, trauma or other stress, it is expressed at much higher concentrations and its level positively correlate with cause mortality, unstable angina, left ventricular dysfunction, propensity to diabetes and its complications, hypertension, obesity, renal failure and several types of cancer (Goicoechea et al., 2012). High plasma levels of IL-6 correlate with greater disability, morbidity, and mortality in the elderly (Maggio et al., 2006) and IL-6 is typically increased in serum, plasma, and spleen with aging (Ershler and Keller, 2000).

The progressive increase of oxidative stress during aging not only causes oxidative damage to cellular macromolecules, but also modulates the pattern of gene expression through functional alterations of transcription factors. Among many transcription factors, nuclear factor kappa B (NF-

κB) is known to be exquisitely sensitive to oxidants, i.e. oxidative stress. Transcriptionally active NF-κB is typically composed of a heterodimeric protein complex that contains a DNA-binding component and an acidic transactivation domain, primarily a heterodimer of p50 and RelA/p65 polypeptides. In unstimulated cells, NF-κB is bound to inhibitory proteins, the IκB family, in the cytoplasm.

NF-kB originally identified as a B cell specific transactivator of the immunoglobulin gene, is involved in key reactions of inflammatory, acute phase and immune responses. The NF-κB family is comprised of at least five well-characterized proteins referred to as p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), c-Rel and RelB that can form various homo- and heterodimeric combinations with different transcriptional activities (Baeuerle and Baltimore, 1996). NF-κB is activated by a wide variety of stimuli, including infection, inflammation, and oxidative stress such as H_2O_2, pro-inflammatory cytokines (TNF-α, IL-1, IL-6), LPS, ionizing irradiation, and viral infection (Hae Young et al., 2005). The activation of NF-κB is responsible for the transcription of many pro-inflammatory proteins like TNF-α; interleukins, like IL-1, IL-2, and IL-6; chemokines; adhesion molecular, like ICAM-1, VCAM-1, and E-selectin; and enzymes, like iNOS and COX-2 (Hae Young et al., 2005).

During aging, NF-κB modulates signaling for oxidative stress-induced inflammation. Gene expression of pro-inflammatory IL-1β, IL-6, TNF-α, COX-2, LOX and iNOS is enhanced by NF-κB, during aging (Chung et al., 2006). Other pro-inflammatory mediators, such as adhesion molecules (VCAM-1, ICAM-1, P-, E-selectin), are all up-regulated through NF-κB activation in the aorta during the aging process (Zou et al., 2003). NF-κB activation and increased ROS production during aging are suppressed by calorie restriction (Kim et al., 2002).

The inflammasome

The term ‘‘inflammasome’’, introduced by Tschopp and colleagues (Martinon et al., 2002) refers to intracellular multiprotein sensors which can recognize a large set of danger signals, induced either by pathogens or cellular stress, and once activated, they subsequently stimulate inflammatory responses, via NOD-like receptors (Martinon et al., 2009; Salminen et al., 2012). Since many inflammasome activators are common factors in the aging process itself, and even more frequent in many age-related diseases, there is an emerging field of inflammasomes in the aging process and associated diseases.

Nod like receptors (NLRs) contain a C-terminal leucin-rich-repeat (LRR) domain that plays a role in the recognition of ligands, a central NACHT domain that is responsible for the oligomerization and dNTPase activity, and an N-terminal CARD, pyrin (PYD), BIR (baculoviral inhibitory repeat),

or acidic transactivation domain. NLRs have been grouped into subfamilies by either the NACHT domain or the N-terminal domain. Several, but not all, NLRs play a role in the formation of a multiprotein complex called the inflammasome. In addition, non-NLR proteins, such as AIM2 can also form a complex with caspase-1 (Martinon et al., 2009; Szabo and Csak, 2012).

The sensor, NLR, forms a complex with the effector molecule, pro-caspase-1, with or without the contribution of an adapter molecule, such as the apoptosis-associated speck like CARD domain containing protein (ASC) (Bauernfeind et al., 2011; Ye and Ting, 2008). Inflammasome priming and activation lead to autoactivation of caspase-1 that in turn cleaves pro-IL-1β, pro-IL-18 into their mature form, inactivates IL-33 and regulates cell death and survival. Inflammasome activation leads to auto-activation of the 45 kDa inactive pro-caspase-1 precursor into p20 and p10 subunits that form the active caspase-1, resulting in the cleavage of pro-IL-1 pro-IL-18 into mature forms, and inactivation of IL-33 (Cayrol and Girard, 2009).

The four main prototypes of inflammasomes are NALP-1, NALP-3, NLRC4 and AIM2. They have different recognition sites and ligand specificity and all culminate in caspase-1 activation.

NALP-3 (NACHT, LRR, and PYD domains-containing protein 3, cryoporin) (or NLRP-3) was first described by Hoffman et al (2001) who discovered four single mutations in the NALP-3 gene, in families with familial cold autoinflammatory syndrome and Muckle–Wells syndrome, which lead to increased IL-1β production (Hoffman et al., 2001). Later, Agostini et al. reported that NALP-3 forms an IL-1β-processing inflammasome complex (Agostini et al., 2004). To date, NALP-3 is the most fully characterized member of the inflammasome family. It consists of the NOD-like receptor NALP-3, the adaptor molecule ASC, and the effector molecule pro-caspase-1. Since NALP-3 does not contain a CARD domain, the presence of the adaptor molecule is necessary for the complex formation (Schroder and Tschopp, 2010).

In the case of NALP-3, the activated receptor interacts with the adaptor protein ASC which recruits the inflammatory caspase-1 (CASP-1) to the complex which subsequently oligomerizes into penta- or heptameric inflammasomes. CASP-1 is the common effector molecule in inflammasomes which cleaves the inactive precursors of two proinflammatory cytokines, i.e. IL-1β and IL-18, into their mature forms which are then secreted from cells. In addition to CASP-1, some other inflammatory caspases, e.g. CASP-4, CASP-5 and CASP-12, can also process the pro-forms of these cytokine.IL-1β is a proinflammatory cytokine, a central regulator of inflammation that binds to the IL-1 receptor (IL-1R) to exert its broad biological effects. IL-1R also recognizes IL-1α and binds IL-1R antagonist (IL-1Rα), the latter inhibiting IL-1R activation. IL-18 activates natural killer (NK) cells to produce IFNγ while IL-33 is a chromatin-associated cytokine of the IL-1 family that drives Th2

responses (Dinarello, 2009). The full-length active IL-33 is cleaved and inactivated by caspase-1 (Cayrol and Girard, 2009).

The expression of NALP-3 is tightly regulated at the transcriptional level via NF-κB (Bauernfeind et al., 2011; Bauernfeind et al., 2009). It should be noted that different cellular stresses and the aging process can stimulate NF-κB signaling (Piva et al., 2006) and probably enhance the priming and potentiation of the inflammasome activation.

The major pathways have been implicated in NALP-3 inflammasome activation induced by a wide variety of activators (Figure 0.9). First, the increased level of ROS induced by oxidative stress was one of the first stimuli which was demonstrated to trigger NALP-3 activation and promote CASP-1 -dependent IL-1β secretion. This is based on the observation that inhibitors or scavengers that block mitochondrial ROS or NADPH oxidase suppress inflammasome activation. ROS induction may represent a common pathway from different cellular insults. For example, large particles (Fubini and Hubbard, 2003) and ATP (Cruz et al., 2007) that are known ‘‘inflammasome-activators’’, induce ROS production.

Second, NALP-3 activation is induced by crystals or large particles such as silica, asbestos, aluminium, amyloid, monosodium urate, and cholesterol. It has been shown that disruption of lysosomes by chemical damage or lysosomal damage after phagocytosis of these large particles induces NALP-3 inflammasome activation (Duewell et al., 2010). Several studies have indicated that the release of cathepsin B after lysosomal damage can activate NALP-3. Consistent with the role of lysosomal damage in inflammasome activation, the role of a lysosomal protease, cathepsin B, has been implicated in certain forms of NALP-3 activation (Tschopp and Schroder, 2010). Lysosomal destabilization is also associated with the NALP-3 activation induced by cholesterol crystals inmacrophages. During aging, lipofuscin accumulates in lysosomes of post-mitotic cells. These insoluble aggregates may physically cause lysosomal damage. Release of cathepsin B from ruptured lysosomes can trigger inflammasomal activation. Furthermore, lysosomes are iron-rich organelles and thus sensitive to ROS-mediated oxidation of proteins and lipids (Kurz et al., 2011), which can also induce lysosomal membrane rupture. Then, the membrane damage can lead to the release of cathepsin B, which is known to stimulate inflammasomes. For instance, cholesterol crystals activate NALP-3 inflammasomes via cathepsin B release in human macrophages (Rajamaki et al., 2010).

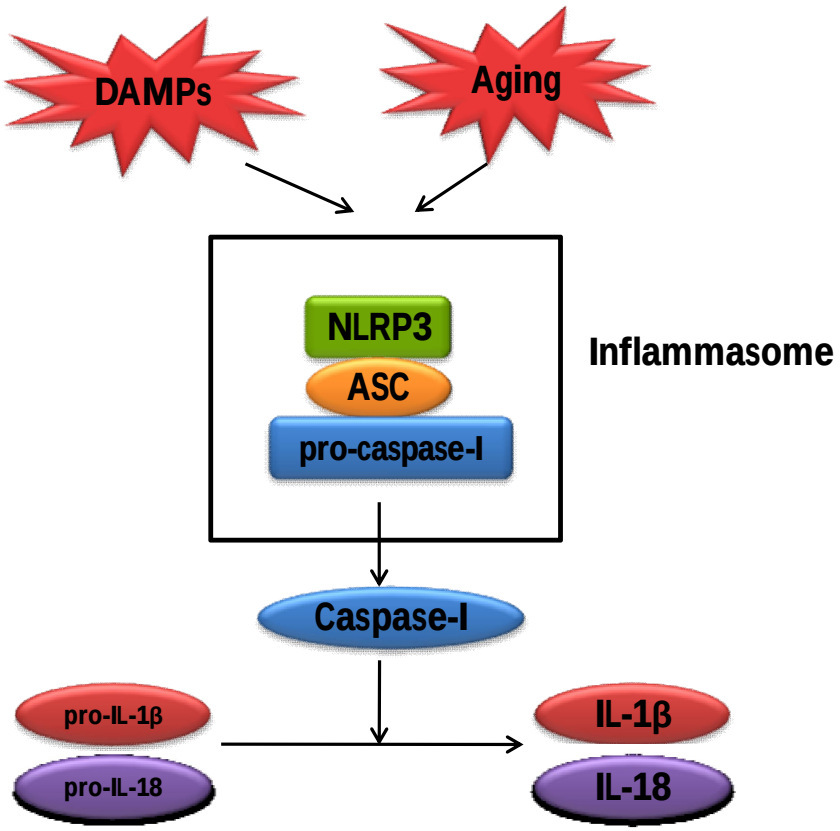


Figure 0.9. *Inflammasome activation by danger signals. Formation of NALP-3 inflammasome requires the adaptor protein ASC and results in the activation of the cystein protease caspase-1. Active caspase-1 processes inactive pro-IL-1β and pro-IL-18 into its biologically active cytokine. Mature cytokines will be subsequently released to the extracellular. (DAMPs: damage associated molecular patterns).*

Third, extracellular ATP sensed by the P2X7 purinergic receptor results in potassium efflux and recruitment of pannexin that induces NALP-3 activation. Pannexin is a membrane pore that allows the delivery of extracellular PAMPs and DAMPs into the cytosol (Kanneganti et al., 2007). NALP-3 inflammasomes could be activated by the efflux of potassium in human monocytes. For instance, extracellular ATP, released from damaged cells, can induce potassium efflux by activating the ATP-gated P2X7 receptors (Petrilli et al., 2007). The exact mechanism of inflammasome activation induced by potassium efflux is not known, but it seems that it is also associated with a ROS-dependent activation mechanism.

The ROS are important molecules causing oxidative stress. Increased oxidative stress has been associated with the aging process (Kregel and Zhang, 2007). The increased ROS production during aging enhances the accumulation of the DAMPs (Salminen et al., 2012). DAMPs activate inflammatory cascades and the inflammasome. Tschopp et al. (2010) showed that NLRP3

inflammasomes was actived by via ROS production (Tschopp and Schroder, 2010). Because mitochondrial is source of ROS, then mitochondria actively involves in the activation of NLRP3. Increased mitochondrial production of ROS stimulated NLRP3 whereas the inhibition of ROS production, significantly suppressed the stimulation of NLRP3 (Zhou et al., 2011). Thus, increases in oxidative stress with aging may also contribute to the development of chronic inflammation and disease. Furthermore, the inflammasome activated by a wide range of DAMPs generates the ROS. The ROS are produced by NALP3 activators and they are important secondary messengers signaling NALP3 inflammasome activation (Martinon, 2010). It has been showed that ROS production by NALP3 activators involves NADPH-oxidases (Bedard and Krause, 2007). Thus, increasing ROS during aging activates the inflammasomes, in particular NLRP3 whereas the chronic inflammation in aging activates the inflammasome then subsequently generates the ROS.

OBJECTIVES

OBJECTIVES

The general objective of this study is to investigate the potential health benefits of exercise and RSV treatment either alone or in combination in mouse models in aging. This main objective can be divided in five partial objectives.

Antioxidant study in liver at different ages of mice

The effects of physical activity training and RSV treatment on biomarkers of aging were investigated. Oxidative stress and antioxidant status markers including lipid peroxidation [malondialdehyde (MDA)], protein oxidation (carbonyl protein), glutathione total, sulfhydryl group and activities and level of protein expression of various antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), cytochrome b_5 reductase ($CytB_5Rase$), NAD(P)H-quinone oxidoreductase (NQO1) and thioredoxin reductase (TrxR)] were determined in liver collected of three age groups: young, mature and old of mice.

Inflammation study

The effects of exercise training and RSV treatment on biomarkers of inflammatory response were studied. The liver tissue samples from three age groups of mice were collected and analysed for TNF-α, IL-1β, IL-6 and IL-10, COX-2 and inflammasome NALP-3 as markers of the inflammatory response.

Antioxidant study in different tissues of old mice

The effects of physical activity training and RSV treatment on biomarkers of aging were investigated. Oxidative stress and antioxidant status markers including lipid peroxidation [malondialdehyde (MDA)], glutathione total, sulfhydryl group and activities and level of protein expression of various antioxidant enzymes [catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), cytochrome b_5 reductase ($CytB_5Rase$), NAD(P)H-quinone oxidoreductase (NQO1) and thioredoxin reductase (TrxR)] were determined in brains, muscles, kidneys, hearts and livers of old mice.

Coenzyme Q study

OBJECTIVES

The level of coenzyme Q was studied in brain, muscle and liver of three age groups of mice to investigate effects of RSV treatment and physical activity during the aging process.

Protein expression in liver by 2D-DIGE

This study also investigates the effects of RSV treatment and exercise training on changes in protein expression that occur in the liver during the aging process.

OBJETIVOS

El objetivo general de esta tesis es investigar los beneficios potenciales del tratamiento con resveratrol sobre la salud, solo o en combinación con el ejercicio en modelos de ratones durante el envejecimiento. El objetivo principal se puede dividir en cinco objetivos específicos:

Estudiar las enzimas antioxidantes en muestras de hígado a diferentes edades

Se investigaron los efectos del ejercicio y el tratamiento del RSV en marcadores de envejecimiento. Los marcadores del estrés oxidativo y sistema antioxidante incluyendo la peroxidación lipídica [malondialdehído (MDA)], la oxidación de proteínas (proteína carbonilo), glutatión total, grupo sulfhidrilo y sus actividades y el nivel de expresión de proteínas de varias enzimas antioxidantes [catalasa (CAT), superóxido dismutasa (SOD), glutatión peroxidasa (GPx), glutatión reductasa (GR), glutatión-S-transferasa (GST), citocromo b_5 reductasa ($CytB_5Rase$), NAD(P)H-quinona oxidorreductasa (NQO1) y la tiorredoxina reductasa (TrxR)] fueron determinados en muestras de hígado de tres grupos de edad: jóvenes, adultos y viejos.

Estudiar la respuesta del sistema inflamatorio en muestras de hígado a diferentes edades

Se estudiaron los efectos del tratamiento de RSV y/o el ejercicio en los marcadores de la respuesta inflamatoria. El hígado de tres grupos de edad de los ratones fue recogido y analizado para determinar el nivel del TNF-α, IL-1β, IL-6 e IL-10, la COX-2 y el inflamasoma NALP-3 como marcadores de la respuesta inflamatoria.

Estudiar enzimas del sistema antioxidante en diferentes tejidos de los ratones viejos

Se investigaron los efectos del tratamiento del RSV y/o el ejercicio en los marcadores del envejecimiento. Los marcadores de estrés oxidativo y el estado antioxidante incluyendo la peroxidación lipídica [malondialdehído (MDA)], el glutatión total, el grupo sulfhidrilo y las actividades y sus niveles de expresión de proteínas de varias enzimas antioxidantes [catalasa (CAT), superóxido dismutasa (SOD), glutatión peroxidasa (GPx), glutatión reductasa (GR), glutatión-S-transferasa (GST), citocromo b_5 reductasa ($CytB_5Rase$), NAD(P)H-quinona oxidorreductasa

(NQO1) y la tiorredoxina reductasa (TrxR)] fueron determinados en el cerebro, el músculo, el riñón, el corazón y el hígado de los ratones viejos.

Estudiar los niveles de la coenzima Q en diferentes tejidos de los ratones a distintas edades

Se estudiaron el nivel de la coenzima Q en el cerebro, el músculo y el hígado de los tres grupos de edad de los ratones para investigar los efectos del tratamiento de RSV y/o el ejercicio durante el proceso de envejecimiento.

Estudiar la expresión de proteínas en muestras de hígado a distintas edades mediante 2D-DIGE

Este estudio también investigó los efectos del tratamiento del RSV y/o el ejercicio sobre los cambios en la expresión de proteínas que se producen en el hígado durante el proceso de envejecimiento.

MATERIAL AND METHODS

MATERIAL AND METHODS

Animals and feeding regimens

Male C57BL/6J mice (Charles River, France) were used in our study. RSV treatment and exercise started at three different ages, 2 months (young group), 12 months (mature group) and 18 months (old group) (Figure 0.10). A total of 48 mice were used. Animals were housed into enriched environmental conditions in groups of 4 animals per polycarbonate cage in a colony room under a 12 h light/dark cycle (12:00 AM – 12:00 PM) under controlled temperature (22 ± 3ºC) and humidity. All animals were maintained accordingly to a protocol approved by the Ethical Committee of the University Pablo de Olavide and following the international rules for animal research.

Just before starting, animals were randomly divided in two sedentary groups: Control and RSV. Control group received water-containing ethanol as vehicle (180 µL ethanol/100 mL H_2O) whereas the group RSV received water with RSV (180 µL of 1 mg/10 mL trans-RSV in ethanol/100 mL H_2O) (Cayman Chemicals, USA) in opaque bottles to avoid light-dependent decomposition. RSV stability was determined and drinking water was changed twice a week for both groups. Taken into consideration an average drinking of 4-5 ml/day and the weight of the animals, the dose of RSV was around 500 µg/animal/day (16,5 mg/kg/day). In any case, the animals had free access to food (Teklad Global Diet Chow 2014S, Harlan Tekland, USA). Under these conditions, animals were maintained for 4.5 months. After this time mice of each group were randomly divided again in two groups: non-trained (NT: sedentary) and trained (T) animals (Figure 0.10). After a week of adaptation the mice began to exercise for 1.5 months. Training was performed in a rodent treadmill (Treadmill Columbus 1055M-E50, Cibertec SA) with 8% of inclination, 20 min/day, 5 day/week for 6 weeks. Training protocol consisted of a 3 min warm-up, followed by a training bout in which running speed was gradually increased to 20 m/min. Three days after from the last training procedure, animals were killed by cervical dislocation; liver, brain, kidney, muscle and heart were dissected and frozen in liquid nitrogen, and stored in -80^{o}C until analysis. At the end of the experiments the age of animals was 8 (young group), 18 (mature group) and 24 (old group) months respectively (Figure 0.10).

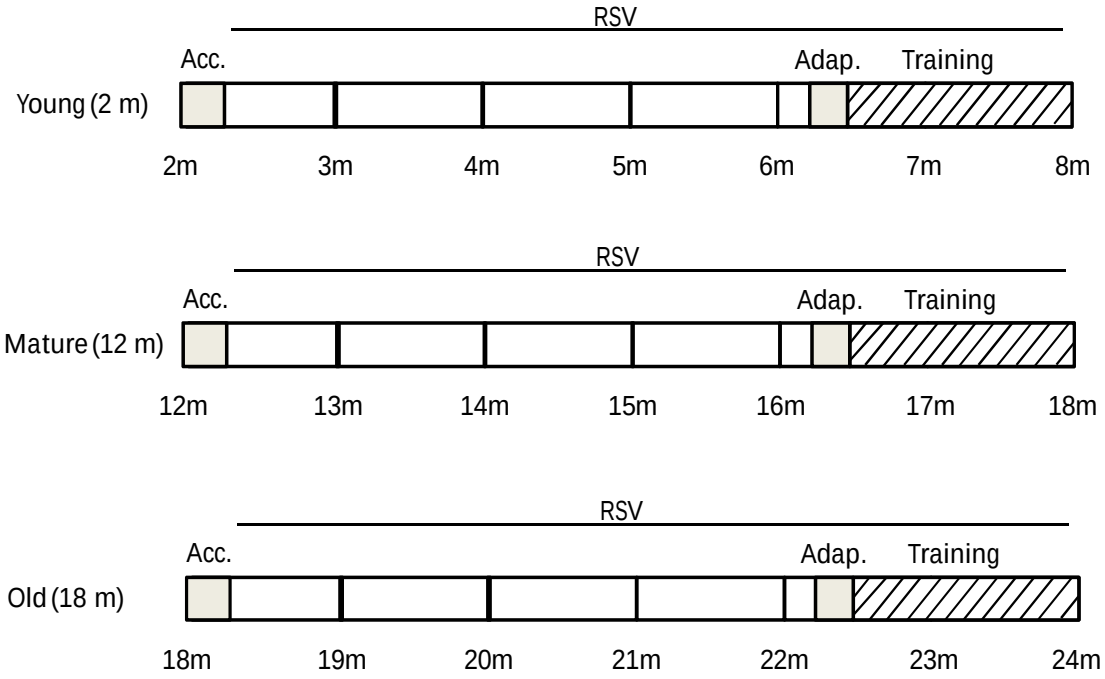


Figure 0.10. *Chronological scheme of procedures with mice groups. Animals received RSV at 2, 12 and 18 months of age and maintained during 6 months. Training was initiated just after 4 months and a week of age starting with a week of adaptation. After six months of treatment with RSV and/or 1.5 months of training animals were sacrificed and samples obtained and processed for determinations (Tung et al., 2013).*

Endurance exercise test

The animals were exposed to extenuating physical exercise on treadmill associated with electric stimuli, without inclination and began at 7 m/min, fastening speed by 5 m/min every 5 min. The final exercise intensity was estimated at the moment the animal stopped for more than 5 seconds under electric stimuli without trying to move back to the treadmill.

Tissue homogenization

For enzymatic activities and Western Blotting (WB) procedures, frozen tissue was homogenized in 9 volumes of ice-cold tissue lysis buffer containing 2 mM Tris-HCl, 20 mM Hepes, 1 mM EDTA, 70 mM Sucrose and 220 mM Manitol and 1 mM PMSF with protease inhibitors (Roche, Spain). Homogenates were centrifuged at 1,000 × g for 10 min at 4°C. For lipid peroxidation procedure, frozen tissue was homogenized in 9 volumes of ice-cold tissue lysis buffer only containing 20 mM Tris-HCl. And for detection of protein carbonyl groups we worked with the same buffer as used for activities and WB procedures but containing 1% of 2-mercaptoethanol to further prevent the protein

oxidation. Pellets containing un-lysed cells and cellular debris were discarded. Protein concentration was determined by Bradford's method (Bradford, 1976).

Lipid peroxidation assay

Lipid peroxidation assay was performed by determining the reaction of malonaldehyde with two molecules of 1-methyl-2-phenylindole at 45°C (Gérard-Monnier et al., 1998). The reaction mixture consisted of 0.64 mL of 10.3 mM 1-methyl-2-phenylindole, 0.2 mL of sample and 10 µL of 2 µg/mL butylated hydroxytoluene. After mixing by vortex, 0.15 mL of 37% v/v HCl was added. Mixture was incubated at 45°C for 45 min and centrifuged at 10,000 g for 10 min. Cleared supernatant absorbance was determined at 586 nm. A calibration curve prepared from 1,1,3,3-tetramethoxypropane (Sigma, Spain) was used for calculation. Peroxidized lipids are shown as nmol MDA equivalents/mg protein.

Slot blotting for detection of protein carbonyl groups

Protein carbonilation was performed as indicated by Robinson (Robinson et al., 1999), based on a combination of 2,4-dinitrophenylhydrazine (DNPH) derivatization and dot blotting. Blanks were prepared by treatment with 20 mM $NaBH_4$ and incubation at 37°C for 90 min. Then samples and corresponding blanks were prepared at final concentration 0.5 mg/mL by diluting in70% trifluoroacetic acid. One µL protein samples were slot-blotted onto a polyvinylidene difluoride (PVDF) membrane. PVDF membrane was incubated with 50 mL of 0.1 mg/mL DNPH in acetic acid for 15 min, then washed extensively in acetic acid (3x5 min) and immersed in a solution of 7% acetic acid, 10%methanol for 15 min at room temperature. Membrane was washed with deionized water four times for 5 min each. Then, the membrane was incubated in SYPRO Ruby blot stain reagent for 15 min. After washing with deionized water (3 x 1 min) SYPRO Ruby fluorescence was monitored for quantification of the total protein loading. After that, membrane was blocked with 5% skim milk dissolved in 0.5 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween-20 for 1 h at room temperature. Further, it was incubated with the primary antibody anti-DNP (Sigma) at a 1:5000 dilution overnight at 4^oC. After three washes with Tris-buffered saline with 0.1% Tween-20, it was incubated with secondary horseradish peroxidase conjugated goat anti-rabbit antibody (Sigma, Spain) in TBST with 5% skim milk at a 1:10000 dilution for 1 h at room temperature. Slot blot detection was developed using an enhanced chemiluminescence detection substrate Immobilon ^{TM}Western Chemiluminescent HRP Substrate (Millipore, USA). Carbonylated proteins were visualized by the ChemiDoc™ XRS+ System and compiled with Image Lab™ 4.0.1 Software (Bio-Rad Laboratories, USA) for quantification.

Sulfhydryl groups determination

Protein SH groups were estimated by Ellman's method (Ellman, 1959). The assay was performed in a plate 96 wells Sterilin (Fisher Scientific) where 10 µL of homogenate was transferred to each well containing 180 µL of 0.1 M buffer sodium phosphate pH 8.0, 1 mM EDTA; 10 µL of 10 mM 5,5-dithiobis (2-nitrobenzoic acid) (DTNB). Absorbance was measured at 412 nm in Omega Microplate Reader (BMG Labtech, Germany) after 15 min incubation at room temperature. The sulfhydryl group content was determined from a standard curve in which L-cystein (Sigma, Spain) standard equivalents present (0, 25, 50, 100 and 200 nmol) were plotted against the absorbance. The amount of sulfhydryl group was reported as nmol per mg total protein.

Antioxidant activities determination

Endogenous liver antioxidant activities were determined as indicated below.

Determination of Superoxide Dismutase (SOD) activity

Total SOD activity in tissue homogenates was determined following the procedure of Marklund and Marklund with some modifications. The method is based on the ability of SOD to inhibit the autoxidation of pyrogallol. In 970 µL of buffer (100 mM Tris-HCl, 1 mM EDTA, pH 8.2), 10 µL of homogenates and 20 µL pyrogallol 13 mM were mixed. Assay was performed in thermostated cuvettes at 25°C and changes of absorption were recorded by a spectrophotometer (EVO 210, Thermo-Fisher, USA) in triplicate at 420 nm. One unit of SOD activity was defined as the amount of enzyme can inhibit the auto-oxidation of 50% the total pyrogallol in the reaction (Marklund and Marklund, 1974).

Determination of Catalase (CAT) activity

CAT activity was measured in triplicate according to the method of Aebi (Aebi, 1984) by monitoring the disappearance of H_2O_2 at 240 nm. Thirty µL homogenate was suspended in 2.5 mL of 50 mM phosphate buffer (pH 7.0). Assay started by adding 0.5 mL of 0.1 M hydrogen peroxide solution and absorbance at 240 nm was recorded every 10 s during 2 min and used to calculate CAT activity. Hydrogen peroxide solution was substituted by phosphate buffer in the negative control. CAT activity was determined by using the mmolar extinction coefficient 39.4 mM^{-1} for H_2O_2 and was expressed nmol of hydrogen peroxide converted per min and per mg total protein where 1 U activity is equal to 1 µmol H_2O_2 converted to H_2O per min.

Determination of Glutathione-dependent activity

Whole amount of glutathione, i.e. reduced (GSH) plus oxidized (GSSG) forms, was determined by method suggested by Anderson (Anderson, 1985). The 1 mL assay mixture contained 880 µL of 143 mM sodium phosphate buffer (pH 7.5) and 6.3 mM EDTA, 100 µl of 6 mM DTNB, 10 µL homogenates and 10 µL of 12 mM NADPH that was incubated for 10 min at 30^{o}C. Reaction was started by addition of 5 µL GR enzyme 5 UI/mL and absorbance recorded for 5 min at 412 nm. Enzyme activity was calculated using the extinction coefficient of 14.15 $mM^{-1}cm^{-1}$ for TNB and the amount of GSH was determined by using a standard curve in which the GSH standard equivalents present (5, 10, 15 and 20 nmol) is plotted against the rate of change of absorbance at 412 nm. Activity is reported as nmol per mg total protein.

GPx activity was measured by using a coupled enzyme assay (Flohé and Günzler, 1984). The 1 mL assay mixture contained 770 µL of 50 mM sodium phosphate (pH 7.0), 100 µL 10 mM GSH, 100 µL 2 mM NADPH 10 µL of 1.125 M sodium azide, 10 µL 100 U/mL GR and 10 µL homogenate. The mixture was allowed to equilibrate for 10 min. The reaction was started by adding 50 µL of 5 mM H_2O_2 to the mixture and NADPH oxidation was measured during 5 min at 340 nm. One unit of glutathione peroxidase was defined as the amount of enzyme able to produce 1.0 µmol $NADP^+$ from NADPH per min. GPx activity was determined using the mmolar extinction coefficient 6.22 mM^{-1} for NADPH at 340 nm and reported as units per mg total protein.

GR activity was determined by following the oxidation of NADPH at 340 nm (extinction coefficient 6.2 $mM^{-1}cm^{-1}$) as described by Carlberg and Mannervik (Carlberg and Mannervik, 1985). The 1 mL assay mixture contained 820 µL of 0.2 M potassium phosphate buffer (pH 7.0), 1 mM EDTA, 50 µL of 20 mM GSSG, 50 µL of 2 mM NADPH and 20 µL of homogenate at 30ºC. Assays were initiated by the addition of NADPH. Reaction was monitored for 3 min at 340 nm. A unit of GR activity was defined as the amount of enzyme that catalyzes the reduction of 1 µmol of GSSG per minute (equivalent to oxidation of 1 µmol of NADPH per minute). GR activity was determined using the mmolar extinction coefficient 6.22 mM^{-1} for NADPH at 340 nm and reported as units per mg total protein.

Glutathione-S-transferase (GST) activity was determined by Habig's methods (Habig et al., 1974) based on the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione. The assay mixture of 1 mL contained 890 µL of sodium phosphate buffer 0.1 M, (pH 6.5), 10 µL homogenate and 50 µL of CDNB 20 mM. Reaction was started by addition of 50 µL of 20 mM GSH (Sigma, Spain) and the rate of increase in absorbance was recorded for 2 min at 340 nm.

Enzymatic activity was calculated by using the extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ for CDNB and expressed as nmol/min/mg protein.

Determination of Cytochrome b_5 reductase ($CytB_5Rase$) activity

$CytB_5Rase$ activity was assayed by measuring the rate of potassium ferricyanide reduction spectrophotometrically. The assay mixture contained 1 mM Tris-HCl buffer, pH 7.6 containing 2 mM potassium ferricyanide, 250 nmol NADH and 10 µL of homogenate preparation in a final volume of 1 mL. The reaction was started by addition of cofactor NADH and reduction of ferricyanide was followed for 2 min by recording the absorbance decrease at 340 nm. The enzyme activity was calculated using the extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ for NADH and expressed as nmol/min/mg protein.

Determination of NAD(P)H-quinone oxidoreductase (NQO1) activity

NQO1 activity was determined spectrophotometrically by monitoring the reduction of the standard electron acceptor, 2,6-dichlorophenol-indophenol (DCPIP) at 600 nm (Benson et al., 1980). Reaction was started by the addition of reaction buffer (25 mM Tris.HCl, pH 7.4), containing 0.7 mg/mL bovine serum albumin, 200 µM NADH, and 40 µM DCPIP to 10 µL homogenate in a final volume of 1 mL and the decrease in absorbance at 600 nm was measured for 1 min at 30°C in the presence or absence of 20 µM of NQO1 inhibitor dicoumarol. The dicoumarol-inhibitable part of DCPIP's reduction was calculated using the extinction coefficient of 21.0 $mM^{-1}cm^{-1}$ to calculate NQO1 activity expressed as nmol DCPIP reduced/min/mg protein.

Determination of Thioredoxin reductase (TrxR) activity

TrxR activity was determined by the method of Holmgren and Björnstedt (Holmgren and Bjornstedt, 1995) modified by Hillet al (Hill et al., 1997) and based on the reduction of 5,5'-dithiobis (2- nitrobenzoic acid) (DTNB) determined by the increase in absorbance at 412 nm. The 1 mL assay mixture contained 940 µL of buffer assay (0.1 M potassium phosphate, pH 7.0, 10 mM EDTA, 50 mM KCl, and 0.2 mg/mL bovine serum albumin), 30 µL of 100 mM DTNB, 20 µL of 12 mM NADPH and 10 µL homogenate. Assay was initiated by the addition of NADPH at room temperature and reaction monitored for 5 min and absorbance recorded at 412 nm in the presence or absence of 20 µM of the TrxR inhibitor sodium aurothiomalate (ATM; Sigma). Enzymatic activity was calculated using the extinction coefficient of 14.15 $mM^{-1}cm^{-1}$ for TNB and expressed as nmol/min/mg protein. A unit of activity was defined as 1.0 nmol 5-thio-2-nitrobenzoic acid (TNB) formed /min/mg protein.

Western blot analysis

Equal amounts of protein homogenates were separated on a PAGE-SDS gel and transferred onto a nitrocellulose membrane. Ponceau S staining total protein loading was recorded with the ChemiDoc™ XRS+ System and compiled with Image Lab™ 4.0.1 Software (Bio-Rad Laboratories) to monitor transfer efficiency and quantification of whole protein loading. Then, membranes were blocked with 5% skim milk dissolved in 0.5 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween-20 for 1 h at room temperature. The membranes were subsequently incubated with the primary antibodies anti-Cu,Zn-SOD, anti-CAT, (Santa Cruz Biotecnology), anti-CytB_5Rase (rabbit polyclonal antibody kindly provided by Dr. J. M. Villalba, Universidad de Córdoba, Spain) anti-GPx1, anti-TrxR1, anti-TrxR2 (Acris Antibodies, Germany), anti-NQO1, anti-GR (Abcam, Cambridge, UK), anti-COX-2 (Cell signaling, USA)and anti-CASP-1(Thermo Fisher Scientific, Pierce, USA) (Table 0.1). After three washes with Tris-buffered saline with 0.1% Tween-20 (TBST), blots were incubated with secondary horseradish peroxidase conjugated goat anti-rabbit or anti-sheep antibodies (Calbiochem, Germany) in TBST with 5% skim milk at a 1:10,000 dilution for 1h at room temperature. Blots were then washed three times in TBST and developed using an enhanced chemiluminescence detection substrate Immobilon ^{TM}Western Chemiluminescent HRP Sustratte (Merk Millipore, Germany). Protein levels were visualized by the ChemiDoc™ XRS+ System and compiled with Image Lab™ 4.0.1 Software (Bio-Rad Laboratories, Spain) for quantification. Protein expression levels were corrected for whole protein loading determined by staining membrane with Red Ponceau and further quantification.

Table 0.1. *Antibody using for WB.*

Antibody	2nd Antibody	Size Molecular (kDa)	Dilution	Fabricant
Anti-Cu,Zn-SOD	Anti-sheep	16	1:1000	Santa Cruz Biotecnology
Anti-GPx1	Anti-rabbit	22	1:1000	Acris Antibodies, Germany
Anti-GPx2	Anti-rabbit	22	1:1000	Acris Antibodies, Germany
0Anti-TrxR1	Anti-rabbit	71	1:1000	Acris Antibodies, Germany
Anti-TrxR2	Anti-rabbit	56	1:1000	Acris Antibodies, Germany
Anti-CAT	Anti-rabbit	60	1:1000	Santa Cruz Biotecnology
Anti-GR	Anti-rabbit	56	1:1000	Abcam, Cambridge, UK
Anti-$CytB_5$Rase	Anti-rabbit	34	1:1000	Provided by Dr. J. M. Villalba, Universidad de Córdoba, Spain
Anti-NQO1	Anti-rabbit	31	1:1000	Abcam, Cambridge, UK
Anti-COX-2	Anti-rabbit	74	1:1000	Cell signaling, USA
Anti- CASP-1	Anti-mouse	45	1:1000	Thermo Fisher Scientific, Pierce, USA

Measurement of cytokines

Single-use aliquots of the homogenates liver were used to study. Liver´s IL-1β, IL-6, IL-10, IL-17 and TNF-α were determined with commercially available Enzyme-Linked ImmunoSorbent Assay (ELISA) (Thermo Fisher Scientific, Pierce, USA) kits according to the manufacturers' instructions. Analysis of cytokine IL-1β, IL-6, IL-10, IL-17 and TNF-α were performed using a sandwich ELISA method. Briefly, 96-well plates where coated overnight at 4^{o}C with 100 µl of monoclonal antibody against IL-1β (2,0 µg/ml) or IL-6 (2,01 µg/ml) or IL-10 (2,0 µg/ml) or IL-17 (1,0 µg/ml) or TNF-α (1,0 µg/ml) in PBS 1x buffer (pH 7.2). The plate was then washed four times with wash buffer (PBS 1x + 0.05% Tween-20), blotted dry, and then incubated with blocking solution (PBS 1x + 1% bovine serum albumin) for 1 h. The plate was then washed and 100 µl of each homogenate sample or standard was added. Then the plate was incubated at room temperature for 2 h, followed by washing, and addition of 100 µl of detection antibody IL-1β (0,5 µg/ml) or IL-6 (0,5 µg/ml) or IL-10 (0,5 µg/ml) or IL-17 (0,25 µg/ml) or TNF-α (0,25 µg/ml). The antibody was incubated at room temperature for 2 h. Following additional washing, 100 µl of avidin-HRP conjugated (1:2000) was added to each well, followed by a 30 min incubation. After thorough washing, plate development was performed using ABTS (2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) liquid substrate solution. Then the plate was incubated at room temperature for color

development and the color was monitored using a microplate reader at 405 nm with wavelength correction set at 650 nm. The standard curve for the ELISA was established by using murine standard IL-1β, IL-6, IL-10, IL-17 or TNF-α diluted in PBS 1x buffer. All standard curves obtained an r^2 value between 0.98 and 1. Results were normalized to total protein content in the liver samples, determined by Bradford's method. Data are reported as picogram cytokine per milligram protein. Cytokine standard curves were prepared in ELISA buffer, and samples from the tissue homogenates were calculated from these standard curves.

Real-time PCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and treated with RNase-free DNase (Deoxyribonuclease 1 Amplification Grade, Sigma-Aldrich) to remove genomic DNA. Briefly, about 50 mg tissues were homogenized in one ml TRIzol and then extracted with chloroform by vortexing. A small volume (1.2 ml) of aqueous phase after chloroform extraction of the TRIzol homogenate was adjusted to 35% ethanol and loaded onto an RNeasy column. The column was washed and RNA was eluted. RNA quantity and purity was determined using the UV Spectrophotometer NanoDrop® ND-1000 (Thermo Scientific) and the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

For the synthesis of cDNA, DNase-treated total RNA (0.5 µg) was reverse transcribed using iScriptTM cDNA Synthesis (Bio-Rad) kit. The reaction was realized in the iCycler Thermal Cycler (Bio-Rad), following the protocol: 5 min at 25ºC, 45 min at 42ºC, 5 min at 85ºC and 5 min at 4ºC. Real-time PCR primers were generated using Beacon Designer software (BioRad), purchased from Eurofins MWG Synthesis GmgH and listed as in the table.

Quantitative RT-PCR was conducted in a CFX Connect™ Real-Time PCR Detection System using 1µl of cDNA mix, 500 nM of sense and antisense primers and 5 µl of iTaq™Universal SYBR® Green Supermix (2x) (BioRad) in 10 µl final volumes. PCR program began with 10 min of incubation at 95°C. The reactions consisted of 45 cycles, using a denaturation temperature of 95 °C for 15 s and annealing and extension at 60ºC for 30 s and 72ºC for 30 s to determine the threshold cycle (Ct) value. A melt curve was performed for all reactions to check for product integrity and primer–dimer formation. Standard curves were generated for each gene of interest using dilutions of purified PCR products at known concentrations and all PCR primers had efficiencies of > 95%. Quantitative PCR using primers for β-actin mRNA were conducted in each plate to provide a normalization reference. The Ct for all genes was normalized to the Ct of β-actin. Quantification of

relative gene expression was calculated by the comparative Ct method ($2^{-\Delta\Delta Ct}$), and the data are presented as fold change over the control. All real-time RT-PCR were conducted at least three times from independent RNA preparations. Distilled H_2O served as a negative control.

Table 0.2. *qRT-PCR primer sequences used with SYBRGreen Supermix*

Primers	***Forward Sequence (5'-3')***	***Reverse Sequence (3'-5')***
IL-1β	AGTTGACGGACCCCAAAAG	TTTGAAGCTGGATGCTCTCAT
TNF-α	CTGTAGCCCACGTCGTAGC	TTTGAGATCCATGCCGTTG
IL-6	TGATGGATGCTACCAAACTGG	TTCATGTACTCCAGGTAGCTATGG
NALP-1	CGCACCACAGCTCTACAGAA	AATCCTAGGACTTCCACTTGACA
NALP-3	TGAAACAAAACGTGCCTTAGAA	GCCTACCAGGAAATCTCGAA
CASP-1	TGGTCTTGTGACTTGGAGGAC	AGAAACGTTTTGTCAGGGTCA
ASC	TCCAGGCCTTGAAGGAAATA	TGTAGCTGGAAAAGATTCCTCAG
β-Actin	TGACCGAGCGTGGCTACAG	GGGCAACATAGCACAGCTTCT

CoQ determination

CoQ levels were assessed using a protocol described by Fernandez-Ayala (Fernandez-Ayala et al., 2000). One to 1.5 mg of protein of whole homogenates were resuspended in 500 ml of PBS 1x and incubated with SDS (1% final concentration) for 10 min followed by a vigorous shake with vortex. Briefly, 2 volumes of ethanol:isopropyl alcohol (95:5) were added to SDS solution and mixed with vortex for 1 min. The organic phase was recovered by the addition of 4 volumes of hexane, shaking for 1 min in vortex and centrifugation at 1000 g for 5 min at 4ºC. The higher phase containing Q was recovered and extraction was repeated 2 times more. Hexane phase was dried by using a Rotavapor (Büchi, Switzerland) and residue resuspended in 1 ml of ethanol HPLC grade. Ethanol was dried again by using a Speed Vac and residue was kept at -20ºC until analysis. Samples were suspended in the suitable volume of ethanol prior to HPLC injection. Lipid components were separated by a Beckan 166-126 HPLC system equipped with a 15 cm Kromasil C-18 column in a column oven set to 4ºC, with a flow rate of 1 ml/min and mobile phase containing 65:35 methanol/n-propanol and 1.42 mM lithium perchlorate. CoQ_9 and CoQ_{10} levels were analyzed with

ultraviolet (System Gold 168) based detectors and an electrochemical detector Coulochem III (ESA,USA) with a guard cell 5020 at +500 mV and an analytical cell 5010 with channel one at -500 mV and channel two at +500 mV as necessary. CoQ_6 was used as internal standard. CoQ content was determined as pmol/mg protein

Proteomic analysis by 2D-DIGE

Sample preparation and protein labeling

About 50 mg of frozen tissue was homogenized in 9 volumes of ice-cold tissue lysis buffer containing (2 mM Tris-HCl, 20 mM Hepes, 1 mM EDTA, 70 mM Sacarose and 220 mM Manitol, 1 mM PMSF and protease inhibitors (Sigma). Homogenates were centrifuged at 1,000 × g for 10 min at 4^{o}C. Pellets containing unlysed components and cellular debris were discarded. Protein concentration was determined by the methods Bradford. Then about 30 µL homogenate of every sample was prepared following general guidelines recommended for subsequent DIGE labeling. Briefly proteins were precipitated using the 2D clean-up kit (GE Healthcare) and resuspended in 40 µL of solubilization buffer pH 8.5 (7 M urea, 2 M thiourea, 2% CHAPS, and 30 mM Tris.HCl pH 9.7). Insoluble material was pelleted by centrifugation (12,000 × g, 4^{o}C, and 5 min) and protein concentration in the supernatant was measured using the RC DC protein assay kit (Bio-Rad).

For protein labeling, 400 pmol of CyDye in 1 µl was mixed with 50 µg of protein and incubated on ice for 30 min in the dark. The labeling reaction was stopped by adding 1 µl of 10 mM lysine followed by incubation on ice for 10 min in the dark. Each sample was covalently labeled with a fluorophore either Cy3 or Cy5. A mixture of 25 µg of protein from every sample was labeled with Cy2 and used as internal standard.

The three labeled samples were then combined, and an equal volume of 2x sample buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer (pH 3–10) and 2% (w/v) DTT) was added. Then mixture was centrifuged at 12,000 × g, 4^{o}C, 5 min to eliminate all insoluble material and the supernatant was then supplemented with rehydratation buffer (20 mM DTT, 1% (v/v) IPG buffer (pH 3–10 NL), 7 M urea, 2 M thiourea, 2% CHAPS, and bromophenol blue 0.002 %) to complete a total volume of 450 µL.

2D-DIGE analysis

Samples were subjected to isoelectric focusing (IEF) using an Ettan IPGphor (GE Healthcare) and 24 cm linear, pH 3–10NL Immobiline Dry Strips (GE Healthcare). IEF conditions were: passive sample rehydration for 10 h, 500 v for 1 h, 1000 v for 1 h and 8000 v for 8.36 h.

After IEF, Immobiline Dry Strips were incubated at room temperature for 15 min in equilibration buffer (75 mM Tris.HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS) with 2% (w/v) DTT followed by an additional 15 min incubation in the equilibration buffer, with 4.5% (w/v) iodoacetamide. After equilibration the IPG strips were sealed on the top of 1 mm thick 10% SDS-PAGE (24×20 cm) gels and electrophoresed in an Ettan DALT six system at constant temperature (20^{o}C).

Fluorescence images of the gels were acquired on a Typhoon^{TM}9400 Scanner variable mode imager (GE Healthcare). Cy2, Cy3, and Cy5 images for each gel were scanned at 488/ 510-, 532/530-, and 633/500-nm excitation/emission wavelengths, respectively, at 100 μm resolution. Relative protein quantification across samples was performed according to DeCyder software v 7.0 and multivariate statistical module EDA (Extended Data Analysis) v 7.0 (GE Healthcare) as follows. First, Differential In-gel Analysis (DIA) module co-detected the 3 images of a gel (internal standard and two samples), measured spot abundance in each image and expressed these values as a ratio. The Biological Variation Analysis (BVA) module utilized those images individually processed with the DIA module to match protein spots across gels, using the internal standard for gel to-gel matching. Statistical analysis was then carried out to determine protein expression changes. *P*-values lower than 0.05 as calculated from Student's *t* test were considered significant. Multiple testing was assessed using the False Discovery Rate (FDR) method to obtain the corresponding *q*-values. Multivariate analysis was performed by PCA (Principal Component Analysis) using the algorithm included in the EDA module of the DeCyder software v 7.0 based on the spots matched across all gels.

In-gel digestion of proteins

Gels were stained by Coomassie blue using EZ Blue TM Gel Staining Reagent (Sigma-Aldrich). Protein spots were excised manually from preparative 2D gel and processed to allow subsequent protein identification. Briefly, spots were washed with water, ammonium bicarbonate (25 mM) and acetonitrile. Next, gel plugs were subjected to reduction with 10 mM DTT (GE Healthcare) in 25 mM ammonium bicarbonate (99.5% purity; Sigma Chemical, St. Louis, MO, USA) and alkylation with 55 mM iodoacetamide (Sigma Chemical) in 25 mM ammonium bicarbonate. The gel pieces

were then rinsed with 25 mM ammonium bicarbonate and acetonitrile (gradient grade; Sigma, Darmstadt, Germany) and dried in Savant SpeedVac for 20 min. Bovine trypsin (sequencing grade; Roche Molecular Biochemicals) in a final concentration of 12 ng/ml in 25 mM ammonium bicarbonate (pH 8.5) was added to the dry gel pieces and the digestion proceeded overnight at 37^{o}C. Digestion was stopped by addition of 0.5% TFA (99.5% purity; Sigma Chemical), and tryptic peptides were extracted by incubating the gel pieces with 0.1% TFA/H_2O and 60% ACN for at least 10 min. Extracts were then combined, and peptides were concentrated and passed through ZipTip C18 tips (Millipore) to remove salt and detergent traces following the manufacturer's instructions.

MALDI-MS(/MS) and database searching

For MALDI analysis, 1 μl of the peptides solution was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μl of a 3 mg/ml of α-cyano-4-hydroxy-cinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide digest spots and allowed again to air-dry at room temperature in a MALDI plate. MALDI-MS(/MS) data were obtained using a 4800 Plus Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied Biosystems, MDS Sciex, Toronto, Canada).

The Maldi-TOF/TOF operated in positive reflector mode with an accelerating voltage of 20000 v. and 1000 individual spectra were averaged. All mass spectra were calibrated internally using peptides from the auto digestion of trypsin.

The analysis by MALDI-TOF/TOF mass spectrometry produces peptide mass fingerprints and the peptides observed with a Signal to Noise greater than 10 can be collated and represented as a list of monoisotopic molecular weights.

For protein identification, peptide mass fingerprint searches was performance in non-redundant NCBI database (date: 08/05/2012; 17919084 sequences; 6150218869 residues) without taxonomy restriction using a local license of MASCOT engine *v. 2.3* (Matrix Science, London; http://www.matrixscience.com) through the software Global Protein Server *v.3.6* from ABSciex. Search parameters were:

- Carbamidomethyl Cystein as fixed modification and oxidized methionine as variable modification
- Peptide mass tolerance 50 ppm
- 1 missed trypsin cleavage site allowed

- Peptide charge state +1
- MS-MS fragments tolerance 0.3 Da

In all protein identification, the probability scores were greater than the score fixed by mascot as significant with a p-value minor than 0.05.

Statistical analysis

All results are expressed as mean ± SEM. Serial measurements were analyzed by using Student's t-test or two-way ANOVA with Bonferroni's post hoc test using SigmaStat 3.5 program and figures were performed by using SigmaPlot, 10.0 programs (Systat Software Inc). The critical significance level α was established at 0.050 and, then, statistical significance was defined as $P < 0.05$.

CHAPTER I

CHAPTER I

EFFECTS OF RESVERATROL AND EXERCISE TRAINING ON BODY WEIGHT AND PHYSICAL ACTIVITY

Abstract

In order to determine the effect of RSV and/or exercise on the whole physical capacity of the animals at different ages we performed a simple extenuating test. Neither RSV nor exercise modified the weight of the animals during our study, then, this parameter is not a source of differences in physical performance. Animals reached a stable weight around 30 g at 30 weeks of age and maintained this weight until the end of the study. Regarding physical activity, the capacity of the animals to run until extenuation decreased as they aged. Old animals significantly stayed less time running on a treadmill and covered less distance. The addition of RSV to the diet did not affect the performance of the animals at any age although a trend to increase their capacity was found in mature and old animals. By itself, training only induced significant increase in capacity in mature animals and a small trend, as RSV in old animals. However, in combination, RSV and training significantly increased the capacity of both mature and old animals but not young animals. RSV plus training maintained physical performance of old animals at similar levels then young animals.

Results and discussion

We determine if the same supplementation with RSV and/or the practice of the same training program in animals with different age at the beginning of the procedure influenced physiological parameters affecting weight and physical capacity.

The results of body weight are shown in Figure I.1. Body weights of the young mice group showed similar increments by RSV and/or exercise training as compared with group Control during the study. The final body weight of young mice was higher as compared to that of the control mice at the beginning of the study (8.7%). In mice of mature and old group of age, weight no changes were found for either control or RSV or exercise training-treated animals. Our conclusion is RSV and physical performance are not affecting to the body weight of the animals and then, weight was not a factor to be taken into consideration in this study.

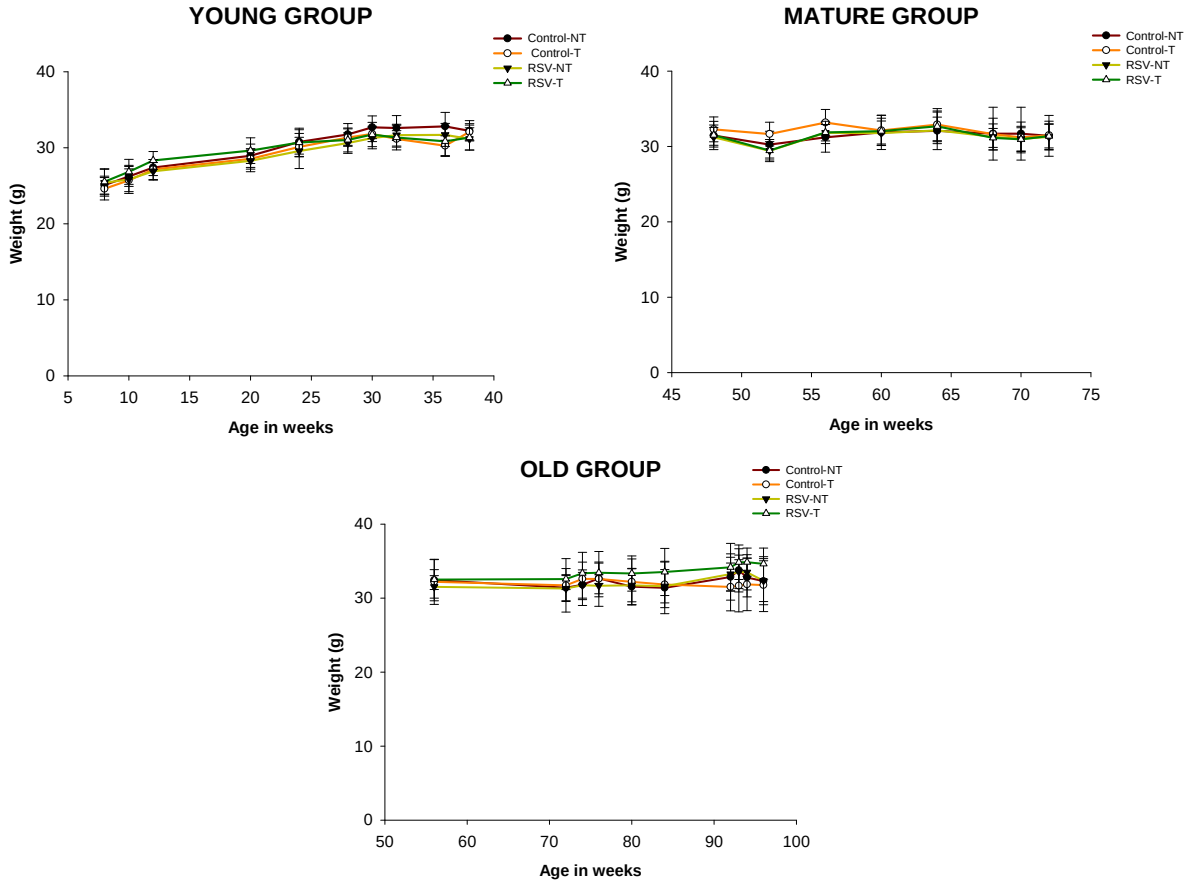


Figure I.1. *Body weight of animals during the experiment - young group, mature group and old group mice (n=4)*

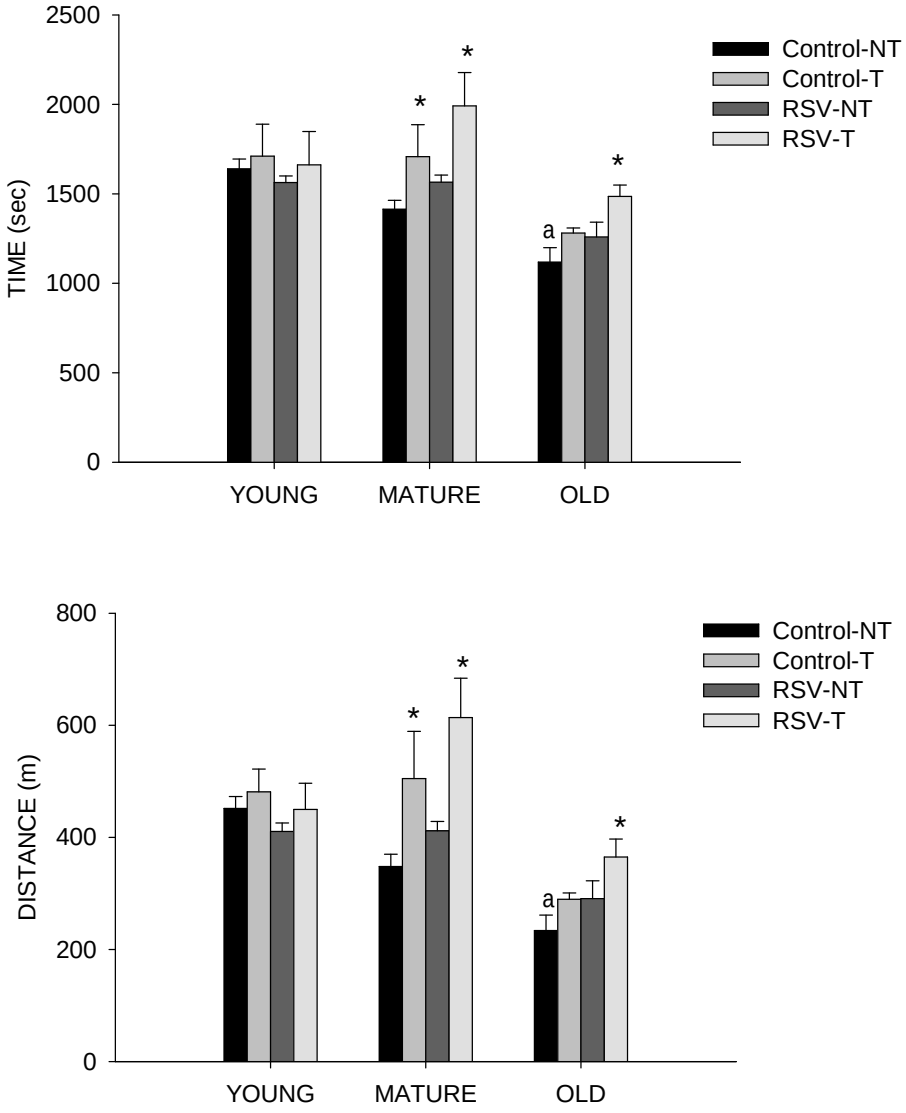


Figure I.2. *Extenuating endurance test. Time (sec) and distance (m) after exhaustion on treadmill of young, mature and old group.*

We carried out an extenuating endurance test. Four animals of each group ran until extenuation. The results are shown in figure I.2. Among all the groups, young group was the group that ran longer and covered more distance. Under the aging, old group showed a significant lower resistance in comparison with young group (1119,0 s vs. 1640,3 s, p= 0.015) and covered less distance (233,8 m vs. 437,9 m, $p < 0.001$). However, in young group, RSV and exercise did not have effect in

resistance and distance on extenuating endurance test. No different significantly was found in time resistance and distance between subgroup by RSV and exercise in this group.

Our results indicate that RSV and exercise training are able to increase muscle resistance in extenuating endurance test and help to maintain higher capacity in mature and old animals. Our findings agree with previous reports showing that RSV supplementation enhanced the effects of exercise on endurance capacity (Hart et al., 2013). RSV also could have potentially beneficial effects which enhance aerobic performance. The mechanism can be explained through the capacity of RSV to enhance mitochondrial biogenesis and induces AMPK in skeletal muscle of mice (Kim et al., 2013). SIRT1 is also an important regulator of metabolism by controlling the activity of key transcription factors such as PGC-1α, FOXO1, and p53, which play a key role in the training response and can be activated by RSV (Baur et al., 2006). Therefore, RSV, activators of SIRT1, could have potentially beneficial effects which enhance aerobic performance, even in mice having a high endurance capacity (Hart et al., 2013). It was suggested that the activity of AMPK, PGC-1α and SIRT1 could play an important role in the exercise-induced adaptive response.

Our results indicate that exercise and RSV produces different effects depending on the age of the animals. In young mice, neither RSV nor exercise increased their capacity in a strenuous test. Training produced higher effect than RSV, however, the combination of RSV and exercise produced a highest effect. In the older group, the effect of exercise or RSV alone was not significant although the combination of both induced a significant increase in the aerobic capacity of mice reaching levels similar to those found in mature and near the scores found in young animals.

CHAPTER II

CHAPTER II

MODULATION OF ENDOGENOUS ANTIOXIDANT ACTIVITY BY RESVERATROL AND EXERCISE IN MOUSE LIVER IS AGE-DEPENDENT

Abstract

Aging is a multifactorial process in which oxidative damage plays an important role. RSV and exercise delay some of the damages occurring during aging and increase lifespan and healthspan. We treated mice at different ages with RSV during 6 months and trained them during the last 6 weeks to determine if RSV and exercise induce changes in endogenous antioxidant activities in liver and if their effect depends on the age of the animal at the beginning of the intervention. Aging was accompanied by the increase in oxidative damage in liver especially affecting the glutathione-dependent system. Both, RSV and exercise reversed the effect of aging and maintained high activities of glutathione, glutathione peroxidase and glutathione-S-transferase activities in old animals. NQO1 activity was also increased. Modulation of antioxidant activities was not completely accompanied by changes at the protein level. Whereas GPx1 protein increased in parallel to the higher activity in old animals, NQO1 protein decreased by RSV although the activity was enhanced. Our results indicate that RSV and exercise revert the effect of aging in liver of old animals maintaining higher antioxidant activities and decreasing oxidative damage. Short-term interventions are enough to produce beneficial effects of RSV or exercise at later ages.

Introduction

Resveratrol (3,5,4'-trihydroxystilbene, RSV), is a stilbene compound produced by different plants with many biologic activities. RSV was hypothesized to be a caloric restriction (CR) mimetic based on its putative activation of SIRT1. As CR, this polyphenol has been shown to increase life span in yeast, nematodes and fruit flies (Howitz et al., 2003; Wood et al., 2004). In vivo, RSV also shows protective activity against age-related deterioration in mice (Baur et al., 2006; Pearson et al., 2008). The antiaging effects of RSV appear to be related to several biologic actions, such as its ability to function as an antioxidant or to modulate different metabolic and signaling pathways (Baur and Sinclair, 2006). Furthermore, RSV attenuates both steady-state and high glucose-induced mitochondrial $O_2^{\bullet-}$ production in various cell types, including primary human coronary arterial endothelial cells (Ungvari et al., 2009). This mitochondrial protective effect of RSV is, at least in part, attributed to the induction of mitochondrial antioxidant systems (Ungvari et al., 2009). In rodents, mitochondrial oxidative stress and activation of redox-sensitive pro-inflammatory pathways (e.g. NF-κB) play a central role in the development of many age-related diseases, which may be prevented or reversed by RSV treatment (Ungvari et al., 2011c). As a polyphenolic compound, RSV has been also shown to be a scavenger of hydroxyl, superoxide, and metal-induced radicals (Bradamante et al., 2004). However, the direct antioxidant effects of RSV are rather poor and the protective effects of RSV against oxidative injury are probably attributable to the upregulation of endogenous cellular antioxidant systems rather than the direct ROS-scavenging activity of the compound. In fact, RSV increases the activity of cellular antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) in rat cardiac cells and human vein endothelial cells (HUVEC) (Spanier et al., 2009; Wallerath et al., 2002).

Aging is accompanied by the accumulation of oxidative damage to cells and tissues associated with a progressive increase in the chance of morbidity and mortality (Beckman and Ames, 1998). Oxidative stress is central to current theories of aging (Beckman and Ames,1998; Harman, 2006) and can affect a number of processes associated with aging including shortening of telomeric DNA, genomic instability, DNA mutations, and increased levels of protein cross linking (Muller et al., 2007). Two major sources of ROS, superoxide anion ($O_2^{\bullet}-$) and nitric oxide ($NO^{\bullet}$) can act individually or together, in the form of peroxynitrate, to damage proteins, lipids and DNA. The antioxidant system is an important mechanism of defense in living organisms because it can accomplish the task of intercepting or degrading ROS (Yu, 1994). Antioxidant enzymes such as SOD, CAT, glutathione reductase (GR), and GPx constitute the major defensive system against

ROS (Sies, 1993). It is well established that SOD detoxifies $O_2^{\bullet-}$, CAT reduces H_2O_2, while GPx reduces both H_2O_2 and organic peroxides. Besides these antioxidant enzymes, glutathione-S-transferase (GST) is a multicomponent enzyme involved in the detoxification of many toxicants and plays an important role in protecting tissues from oxidative stress (Fournier et al., 1992). Otherwise, the enzyme NAD(P)H: quinone acceptor oxidoreductase (NQO1) has role in the protection of cells against cytotoxic effects of electrophiles by catalyzing the two electron reduction of reactive and toxic quinones and quinone epoxides, which results in their detoxification (de Haan et al., 2006). Other enzyme is NADH-cytochrome b_5 reductase ($CytB_5Rase$), which plays a central role in many diverse metabolic reactions in liver such as fatty acid desaturation, fatty acid elongation, cholesterol and plasmalogen biosynthesis and also participates in some cytochrome P-450-dependent reactions (Yildirim et al., 1994). Furthermore, the thioredoxin system is a general protein disulphide reductase system that plays a crucial role in cellular defense against oxidative stress (Björnstedt et al., 1995). Thioredoxin reductases (TrxR) play an important role in redox-regulated cell signaling, also involved in the regeneration of important antioxidants, such as ubiquinone, lipoic acid and ascorbic acid (Arnér et al., 1996; May et al., 1997; Xia et al., 2003).

Physical activity and exercise may also lead to an increase in physiological antioxidant defenses of the organism (Polidori et al., 2000). Antioxidant enzymes are present and active at an intracellular level, and it has been shown that an acute bout of exercise increases their activities in skeletal muscle, heart, and liver with a threshold and magnitude of activation that differs among enzymes and tissues (Ji et al., 1998). Also endurance training has been shown to increase antioxidant enzyme activities in the senescent muscle (Ji et al., 1991). It has been reported that in aged rats under swimming training over 1 year significantly increased SOD in lung and heart, CAT in the liver, and GPx in all three tissues (Gündüz et al., 2004). In mice, low-intensity treadmill training for over 2 months reduces oxidative stress markers such as protein carbonyls and malondialdehyde in skeletal muscle (Kaczor et al., 2007). These findings suggest that aerobic physical activity increases endogenous antioxidant enzyme protection in a variety of cells and tissues.

It is well established that aging is associated with increased oxidative stress, which in turn may be related to decreased antioxidant activities, and that physical exercise and RSV as a CR mimetic can alleviate oxidative damage. Therefore, our main interest in this study is to establish if the practice of exercise and/or RSV affect endogenous antioxidant activities and oxidative damage even starting at old age. Comparison of results obtained in young, mature and old animals will permit us to clarify if there is an age-dependent response to exercise and RSV.

Results

Oxidative damage increased in aging liver.

In order to determine if oxidative stress increases during aging in mouse liver we determined the levels of lipid peroxidation and protein carbonylation in whole homogenates. Significant increases of lipid peroxidation (75%) and protein carbonylation (36%) were found in liver from old animals in comparison with young animals (Table II.1). On the other hand, the levels of sulphydryl groups and glutathione in whole liver homogenate decreased in aging. Sulphydryl groups and glutathione levels decreased a 46% in the old group indicating lower reducing conditions in old-aged organs (Table II.1).

Table II.1. *Oxidative stress markers during liver aging.*

	Groups		
Markers	Young	Mature	Old
MDA	0.259 ± 0.044	0.341 ± 0.018	0.481 ± 0.97[a]
Protein carbonylation	0.052 ± 0.005	0.062 ± 0.010	0.071 ± 0.004[a]
Sulphydryl groups	16.17 ± 3.97	9.23 ± 0.72	8.85 ± 1.74[a]
Glutathione	37.86 ± 3.81	33.79 ± 2.96	21.43 ± 5.84[a]

MDA levels, sulphydryl groups and glutathione are indicated as nmol/mg protein. Protein carbonylation are indicated in arbitrary units determined as indicated in methods section. [a]*Significant differences vs. young group, p < 0.05.*

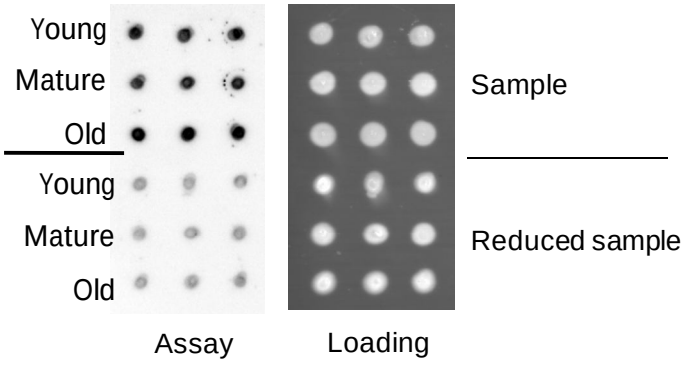


Figure II.1. *Slot blotting for detection of protein carbonyl groups. Slot blot detection was developed using an enhanced chemiluminescence detection substrate Immobilon ™Western Chemiluminescent HRP Substrate (Millipore, USA) for detection of protein carbonyl groups.*

Endogenous antioxidant activities are affected in aging.

In order to determine the cause of the higher oxidative damage found in old liver, we determined the activity of endogenous antioxidant enzymes (Table II.2). In general, aging decreased the endogenous antioxidant capacity in liver cells by affecting key enzymes such as CAT, SOD and GPx. Old liver showed a 20% less CAT activity and a 30% less SOD activity in comparison with young liver. On the other hand, the glutathione-dependent activities such as GPx (70% less activity) and GST (30% less activity) suffered a higher decrease in aging. This decrease will affect the whole quenching capacity of the cell against ROS since these activities are involved in protection against superoxide and hydrogen peroxides.

Other activities such as GR, TrxR and the Q-dependent NQO1 and $CytB_5$Rase did not show any change during aging. Even more, TrxR showed a tendency to increase during aging (Table II.2).

Table II.2. *Antioxidant activities in mice liver.*

	Groups		
Activity	Young	Mature	Old
Catalase	95.55 ± 4.81	88.16 ± 9.53	77.31 ± 2.55[a]
Superoxide dismutase	2163.0 ± 399.1	1642.9 ± 370.9	1542.7 ± 386.4[a]
Glutathione peroxidase	105.3 ± 13.9	68.6 ± 5.5	35.1 ± 12.8[a]
Glutathione reductase	24.72 ± 0.48	23.77 ± 2.80	28.24 ± 1.30
Glutathione -S- transferase	1113.1 ± 153.6	926.1 ± 114.3	751.4 ± 212.2[a]
Thioredoxin reductase	4.01 ± 0.09	4.50 ± 1.57	5.24 ± 0.66
NQO1	5.76 ± 0.20	6.27 ± 1.26	5.44 ± 0.94
$CytB_5$ reductase	398.9 ± 32.0	228.8 ± 16.4	327.5 ± 32.8

Activities are indicated as nmol/min/mg protein. [a]Significant differences vs. young group, $p < 0.05$.

Exercise and RSV partially prevent oxidative damage in liver.

The main task of our study was to determine whether RSV intake alone or combined with physical activity at different ages can affect oxidative damage and modify endogenous antioxidant profile in liver. For this purpose, we measured the levels of oxidative markers such as MDA or protein carbonylation in liver after RSV treatment for 6 months and/or training during 1.5 months. Interestingly, independently of the presence of RSV in the diet, exercise significantly decreased the levels of lipid peroxidation in young and mature animals whereas in old animals only showed a

tendency to decrease (Figure II.2A). RSV did not show any effect on lipid peroxidation levels. Regarding protein oxidation, was the addition of RSV intake reduced the levels of protein carbonyls again in young and mature animals but not in old animals. This decrease was around a 20% in young group and a 60% in mature animals (Figure II.2B).

We could not find significant changes related to free protein sulphydryls, in our studies, although a tendency to increase these levels was found in the mature group with exercise (Figure II.2C). On the other hand, a clear increase in the levels of glutathione was found in old livers with all the interventions, RSV, exercise and their combination reaching around 80% more glutathione in all the cases (Figure II.2D).

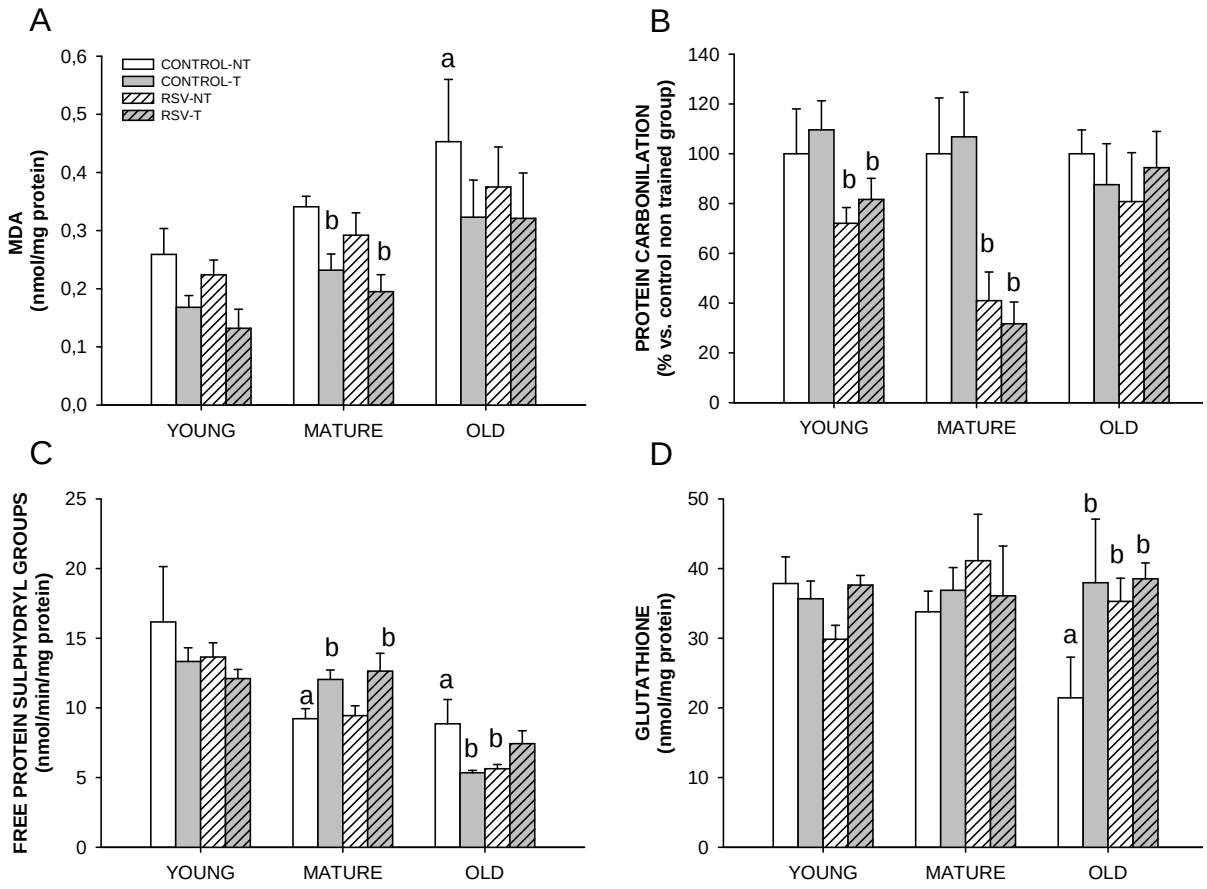


Figure II.2. *Oxidative damage in mice liver. Animals were maintained as indicated in figure 0.10, liver dissected and processed as indicated in Methods section. A) Lipid peroxidation in young, mature and old mice liver membranes and effect of exercise and/or RSV treatment. B) Protein carbonylation in the three groups of age. C) Free protein sulphydryl groups in whole mice liver samples. D) Glutathione level in mouse liver in all the groups. Exercise is indicated as NT: non-trained or T: trained. Data are indicated as nmol/mg protein except for protein carbonylation (B) where data is indicated as the percentage of signal obtained by densitometry respecting with age-related respective control-NT which is considered as 100%. Data represent the mean ± SEM (n=4).* [a] *Significant difference vs Control-NT levels in young group;* [b] *Significant difference vs. Control-NT levels in the respective aged group, $p < 0.05$.*

Antioxidant activities are differentially affected by RSV and/or exercise.

In order to determine if RSV and/or exercise can modify the activity of endogenous antioxidant enzyme in liver and if its effect is influenced by the age of the animal we measured all the above-indicated activities in liver from animals after RSV administration, training or the combination of both. CAT and SOD activities were not affected by these factors although a tendency to increase in old group after training was found (Figure II.3A-B). Interestingly, both, GPx and GST activities were substantially affected in old but neither in young nor in mature livers (Figures II.3C-D). These

activities were severely lower in these livers and both, RSV and exercise were able to restore activity levels near those found in young animals (Figures II.3C-D).

Regarding the activities of enzymes that were not affected in aging, no changes were also found after RSV or exercise in GR (Figure II.3E) or $CytB_5$Rase activities (Figure II.3G). However, in the case of TrxR activity, a decrease of this activity was found in RSV+training group independently of the age of the animal (Figure II.3F). And more interestingly, NQO1 activity was importantly increased by exercise and also by RSV in both, young and old animals but not in the mature group (Figure II.3H).

Taken together, all these results indicate that in general, aging severely affect the glutathione-dependent antioxidant system in liver reducing both glutathione levels and both GPx and GST activities. We demonstrated that exercise and RSV either by separate or together can significantly restore the observed defects in the glutathione-dependent antioxidant system especially in old animals. With regard to all the other antioxidant activities, the effect of RSV or exercise will depend on the specific enzyme and animal age being NQO1 an interesting activity to be considered in the old group.

Antioxidant protein levels in liver are affected by RSV and exercise in old group.

Most of the studies performed on the role of antioxidants in aging have been based on the levels of antioxidant enzymes determined by western blotting. To determine if enzymatic activity is directly related to protein levels of these enzymes we also determined the changes at the protein level induced by exercise or RSV in the old group which showed the most significant changes in enzyme activities (Figure II.4). In agreement with their respective activities, no significant changes in protein levels were found for CAT, SOD1, or $CytB_5$Rase were found (Figure II.4A). In the case of TrxR activity that showed a lower activity in the group of RSV plus exercise, no differences at the protein levels were found neither with TrxR1 nor with TrxR2 (Figure 4A). Interestingly, the levels of GPx1 were significantly increased in the old group of animals by both physical activity and/or RSV (Figure II.4B) in agreement with the increase in activity found in this group (Figure II.3C). However, in the case of NQO1, the increase in activity found in old livers was not accompanied by an increase at the protein level. On the contrary, RSV significantly decreased NQO1 protein levels independently of the exercise (RSV+RSV-T group vs. Control+Control-T group, $p<0.03$).

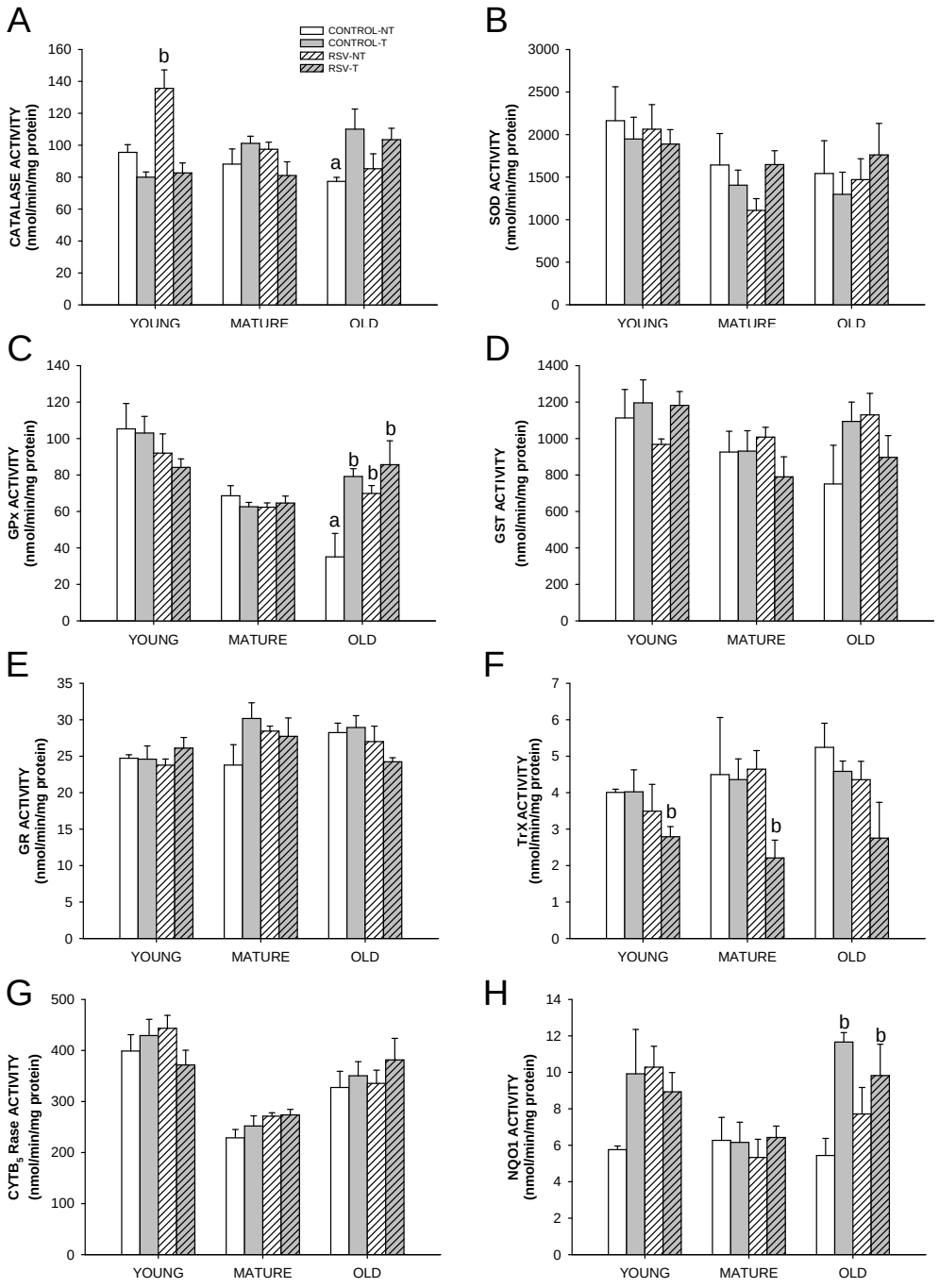


Figure II.3. *Endogenous antioxidant enzymatic activities in mouse liver. Animals were maintained as indicated in figure 0.10, liver dissected, processed and whole antioxidant activities determined as indicated in Methods section. A) CAT activity. B) SOD activity. C) GPx activity. D) GST activity. E) GR activity. F) TrX activity. G) CytB_5Rase activity. H) NQO1 activity. Data are indicated as nmol/min mg protein. Data represent the mean ± SEM (n=4).* [a] *Significant difference vs Control-NT levels in young group;* [b] *Significant difference vs. Control-NT levels in the respective aged group, p < 0.05.*

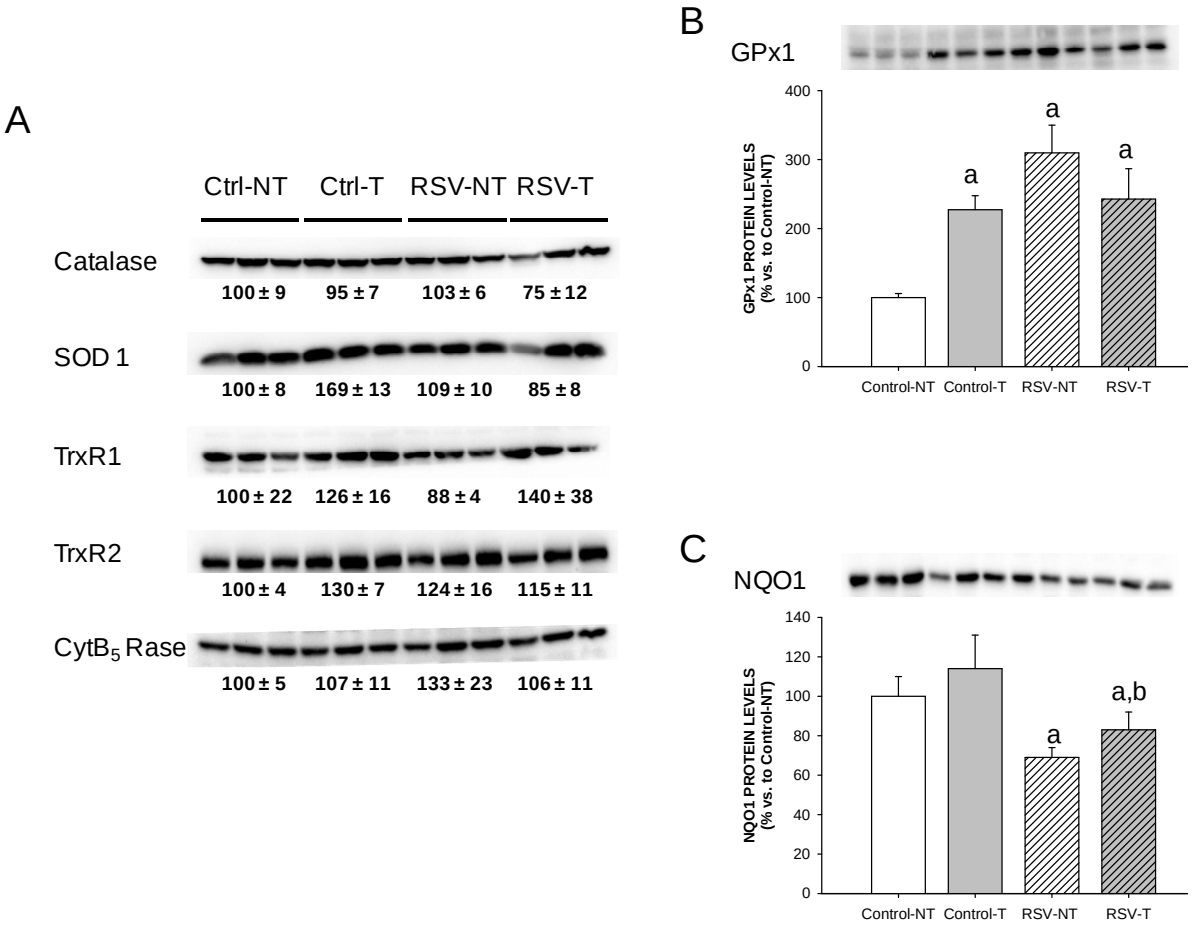


Figure II.4. *Antioxidant protein levels in old mice liver. Protein levels of antioxidant enzymes were determined by WB as indicated in Method section. A) WB analysis of CAT, SOD1, Thioredoxin reductases 1 and 2 and $CytB_5Rase$. Numbers under blots indicate the mean of density related to protein loading measured by densitometry of ponceau red staining of whole proteins. B) WB and densitometric quantification of GPx in whole mice liver homogenate. C) WB and densitometric quantification of NQO1 in whole mice liver homogenate.* [a] *Significant difference vs Control-NT levels;* [b] *Significant difference vs. Control-T levels, $p < 0.05$ (n=3).*

Discussion

Many theories have been proposed to explain aging (Bengtson et al., 2009). Among other possibilities, it has been suggested that aged tissues become more susceptible to oxidative damage produced by free radicals because an unbalance between ROS production and the activity of endogenous antioxidant mechanisms (Junqueira et al., 2004; Lu and Finkel, 2008). In fact, oxidative stress is a central mechanism in current theories of aging (Beckman and Ames, 1998; Harman, 2006). In agreement with this hypothesis, we found higher rates of oxidative damage and lower levels of free protein sulphydryls and glutathione in liver from aged animals.

CR, exercise and RSV exert anti-aging effects reducing the progression of age-associated damage with concomitant increased healthspan and in some case such as CR, longer median and maximum lifespan (Mercken et al., 2012). Furthermore, CR and exercise increase physical capacity of mice at the same time that decrease muscle and oxidative damage induced by extenuating exercise indicating not only more efficient bioenergetics but probably also high endogenous antioxidant capacity (Rodríguez-Bies et al., 2010). In fact, CR, exercise and also RSV share common pathways influencing cell metabolism and antioxidant mechanisms (López-Lluch et al., 2008).

Long-term RSV treatment has shown a CR-mimic capacity extending health during aging in mice. This effect permits to mice to live healthier showing less aging-dependent deleterious effects on cardiovascular function, motor coordination and insulin response (Pearson et al., 2008). However, RSV has been unable to increase lifespan although it enhances health in aged animals (Miller et al., 2011; Pearson et al., 2008). On the contrary, another interesting compound in aging studies, the inhibitor of mTOR pathway, rapamycin, shares common molecular mechanisms with RSV but also increases lifespan in mice (Miller et al., 2011), suggesting the existence of separated mechanisms for health and lifespan extension. Furthermore, a recent report showing the effect of life-long dietary supplementation with different age-related compounds such as green tea extract, curcumin, oxaloacetic acid, medium-chain triglyceride oil or also RSV have demonstrated no effect on life-span in mice (Strong et al., 2013)

In this work we have used RSV, a polyphenol found in grapes, red wine and nuts, formerly proposed as CR mimetic that is able to increase bioenergetic efficiency and decrease oxidative damage in animals fed with a high fat rich diet (Baur et al., 2006). We have used a procedure in which animal nutrition was supplemented with RSV and further trained with a regular aerobic exercise. Both, RSV treatment and exercise started at different ages in order to determine whether these interventions either by separate or in combination were effective independently of the animal

age. It has been previously shown that RSV is able to modify the whole physiology of the animal affecting different organs (Baur et al., 2006; Lagouge et al., 2006). However, compared to the studies of Baur et al (Baur et al., 2006) and Lagouge et al (Lagouge et al., 2006) our mice received a diary RSV dose between 4 to 40 lower respectively, indicating that RSV is able to affect the organism also at lower doses.

We have used liver samples to determine if a putative hormetic effect of both, RSV and exercise can also influence antioxidant protection in this organ, which is essential in metabolic and detoxifying processes. In general, it seems that both interventions are more effective in mature and old animals indicating that physical activity and RSV or their combination are effective even when starting at a later age in the life of the animal. Our results agree with previous works that indicate that long-term RSV intake attenuates oxidative damage in tissues specially affected during aging such as liver, heart or kidney (Wong et al., 2009).

Higher levels of oxidative damage in liver can be due to a lower antioxidant activity. Our results indicate that aging is accompanied by a decrease of most of the antioxidant enzymes and in the level of non-enzymatic antioxidants such as glutathione in liver. This can be part of a vicious cycle since ROS can themselves reduce the activity of antioxidant enzymes, such as CAT (Datta et al., 2000). In general, RSV and/or exercise increased the activity of several of the enzymes involved in endogenous antioxidant activities in liver in the old group of animals without affecting young or mature groups. Our findings agree with other studies in mice showing training-associated increases in CAT (Bronikowski et al., 2002; Gündüz et al., 2004) and SOD2 (Bronikowski et al., 2002) activities in liver, SOD2 in lung and heart muscle (Gündüz et al., 2004). However, other studies have shown that long-term training effect depends on the age of the animal being almost null in senescent mice after 50 weeks of exercise (Navarro et al., 2004). In our work, GPx and NQO1 activities increased in old group and other activities showed a tendency to augment and reach the levels of young animals enhancing then the general endogenous antioxidant capacity of liver.

Very interestingly, aging especially affected the glutathione-dependent system since total glutathione, GPx and GST activities significantly decreased in the old group. A similar effect was found only in the oldest rats in a study by Rikans and Hornbrook (Rikans and Hornbrook, 1997). Interestingly, both RSV and exercise were able to revert the drop of glutathione and the decrease in the activity of this family of enzymes in old animals reaching similar levels than those found in young animals. It is most likely that this age-dependent effect depends on the extent of damage suffered by the cells, being more susceptible to be positively affected by RSV and exercise those

organs showing a higher rate of oxidative damage. In agreement with this assumption, the decrease of activity of these enzymes found after ethanol-induced damage was reverted by exercise in both old and young rats and not only in old animals (Mallikarjuna et al., 2010), showing that when young animals suffer oxidative damage, exercise also induce the activity of these enzymes. Surprisingly, neither aging nor RSV or exercise modified GR activity in our extracts indicating that aging affect the expression of the most effective enzymes against oxidized compounds, GPx and GST. Similar results were found in a previous study about the effect of exercise on glutathione-dependent activities (Thirunavukkarasu et al., 2003). Very interestingly, both RSV and exercise affected in the same way GPx and GST activity although exercise was more effective than RSV in increasing glutathione levels in old animals indicating a similar mechanism.

Another interesting effect is based on NAD(P)H-dependent oxidoreductases such as NQO1 and $CytB_5Rase$. Only NQO1 was sensible to both, exercise and RSV. NQO1 is a flavoprotein that catalyzes the two-electron reduction and detoxification of quinones and their derivatives (Talalay et al., 1995) and is a component of the plasma membrane redox system, which provides electrons for energy metabolism and recycling of antioxidants (Hyun et al., 2010). In fact, NQO1 maintains cellular levels of ubiquinol and vitamin E, two important biological antioxidants involved in the detoxification of ROS (Ross, 2004). The decrease of NQO1 activity found in aged livers agrees with previous finding showing decrease of this activity in plasma membrane of aged rats and mice (De Cabo et al., 2004; López-Lluch et al., 2005). This activity was further increased by CR but, in agreement with our results, only in aged animals (De Cabo et al., 2004; López-Lluch et al., 2005), probably indicating an important function of Q-dependent oxidoreductases in the prevention of lipid peroxidation in cell membranes during aging (López-Lluch et al., 2010). The RSV and exercise-dependent increase in the activity of NQO1 probably explain the reduction of lipid peroxidation found in aged animals. On the other hand, $CytB_5Rase$ is also involved in the recycling of quinones in membranes and maintenance of plasma membrane redox system (Navarro et al., 1998). Our results indicate that aging did not affect $CytB_5Rase$ levels in agreement with previous reports showing that the levels of this enzyme were not altered significantly in rat liver during aging (Bello et al., 2005; Plewka et al., 1998). However, our results report the whole activity in liver but a higher activity located specifically at the plasma membrane cannot be discarded (Navarro et al., 1998). Furthermore, activity of NQO1 and $CytB_5Rase$ at the plasma membrane have been recently proposed as regulators of the activity of the sirtuins, known regulators of longevity, to regulate metabolic, antioxidant and expression mechanisms necessary for cell maintenance and survival

(Crane et al., 2012). For these reasons, activation of these enzymes by CR, exercise or RSV in aged animals can be important in longevity and protection against oxidative damage.

The effect of RSV on antioxidant activities agrees with previous reports (Pearson et al., 2008). RSV has also shown anti-inflammatory properties in studies performed in monkeys (Csiszar et al., 2012) or even in humans (Agarwal et al., 2013). RSV is also able to decrease oxidative damage produced by exercise such as isometric contraction, in mice muscle increasing glutathione and affecting GPx activity (Ryan et al., 2010). However, some age-related dysfunctions such as sarcopenia seem to be independent of oxidative damage and antioxidant protection produced by RSV reduces oxidative damage without attenuation of sarcopenia (Jackson et al., 2011). Furthermore, RSV seems to protect more lipids than proteins against oxidation (Figure II.2). In agreement with our findings, Jackson and coworkers (Jackson et al., 2011), demonstrated that RSV is able to decrease lipid peroxidation without affecting protein carbonilation.

Our study describes the physiological situation in liver after 6 months of RSV treatment and/or 1.5 months of exercise. This is a limit of our study since no mechanistical evidences are shown. We think that is very complex to determine the mechanisms involved in how liver reached a new equilibrium in RSV-supplemented diet or after a regular training process. Furthermore, animals responded in different way to RSV or exercise stimulus depending on the age at the beginning of procedures. Then, we think that many factors have been involved in the age-dependent response to these stimuli. Probably, among these factors Nrf2-dependent mechanisms can be involved. In fact, it has been suggested that a different response to oxidative stress in aging can be due to decay in the activation of Nrf2 factor in tissues. Recently, Ungvary group has shown that the deficient response of cardiovascular vessels such as aorta in aged animals is due to deficient response of Nrf2 factor and activation of the NF-κB system (Ungvari et al., 2011a; Ungvari et al., 2011b). This age-dependent Nrf2 decrease has been also reported in liver together with the concomitant increase of oxidative damage and decrease in GPx, GR, and NQO1 activities (Shih and Yen, 2007). This dysregulations of Nrf2-dependent antioxidant response in the vasculature has been associated to the decrease in IGF1 levels during aging (Valcarcel-Ares et al., 2012). Probably, RSV and exercise are able to increase the activity of Nrf2 in tissues where it is dysregulated. In fact, in aged vasculature, RSV attenuated ROS production and increased the transcriptional activity of Nrf2 (Csiszar et al., 2012). Mechanisms involved in this effect will be studied in depth. Aging is a multifactorial process therefore any thorough investigation cannot be limited to a unique factor. Nutrition and life style importantly affect aging process. We show here that both RSV and exercise act as hormetic

compounds likely sharing mechanisms that activate antioxidant mechanisms especially in aged animals. Very importantly, the highest induction levels of antioxidant mechanism are found in aged animals in which oxidative damage increases and antioxidant activity decreases. Then, it seems that CR, physical activity and RSV activate protective mechanisms against oxidative damage especially in those situations where the balance between oxidative damage and antioxidant defenses has been shifted towards higher damage and no high changes are found when the normal antioxidant balance is maintained at normal levels (Figure II.5). In view of our results we can assert that any study focusing on the effects of CR, RSV and exercise on longevity and/or healthspan in animals should be conducted mainly in those situations were an oxidative imbalance is produced, such as in aged animals. Our results also suggest that long-term interventions are not required in order to improve quality of life at later ages. Thus, changes in lifestyle and nutrition in aged animals can achieve significant improvements in antioxidant defenses leading to a reduction of oxidative damage.

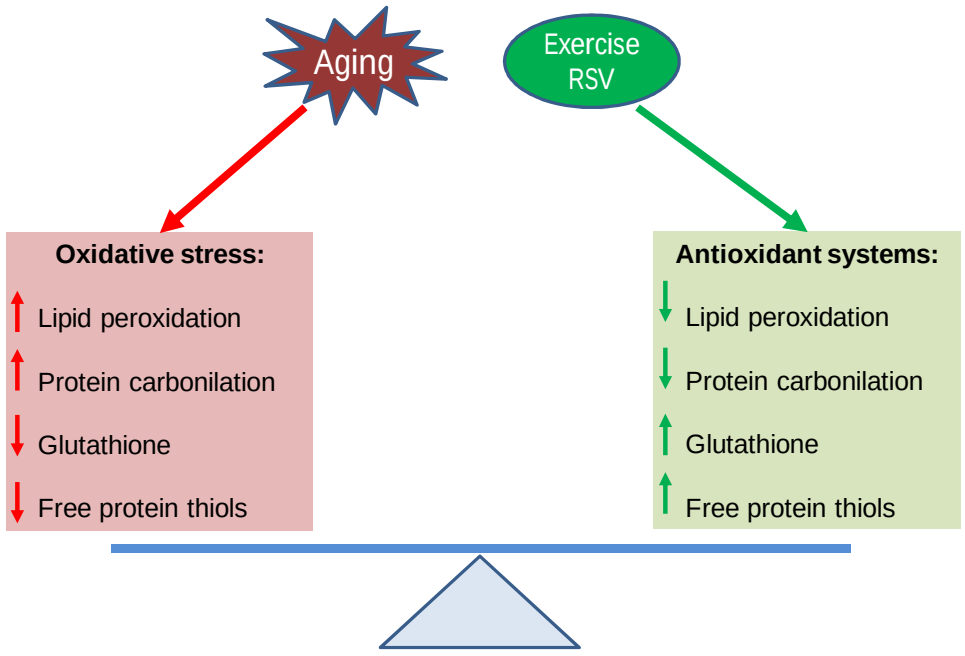


Figure II.5. *Schematization of the effect of RSV and/or exercise on endogenous antioxidant system and oxidative damage in mouse liver. RSV and/or exercise decrease lipid peroxidation and protein carbonylation in mouse liver by affecting especially glutathione-dependent and NQO1 antioxidant systems (Tung et al., 2013).*

CHAPTER III

CHAPTER III

ANTI-INFLAMMATION EFFECT OF RESVERATROL AND/OR EXERCISE ON AGING

Abstract

Aging is associated with higher oxidative stress and inflammation. The aim of this study was to investigate the effect of RSV and/or physical activity on aging inflammation livers from male C57BL/6J mice. Forty-eight mice in 3 group of age (young, mature and old) were used. Levels of IL-1β, IL-6, IL-10, IL-17 and TNF-α in liver were evaluated by ELISA analysis. Protein expression of cyclooxygenase (COX-2) and CASP-1 were determined by Western blot analysis. The mRNA expression of the components of NALP-3 inflammasome included TNF-α, IL-1β, ASC, CASP-1, NALP-1 and NALP-3 were also analyzed. We found that aging increased inflammation C57BL/6J mice liver. Levels of pro-inflammatory cytokines IL-1β, IL-6, IL-17 and TNF-α increased in old mice. Furthermore, mRNA expression of TNF-α, IL-1β, ASC, CASP-1, NALP-1 and NALP-3 also increased significantly as compared with young and mature animals. Interestingly, RSV and/or exercise decreased them in old mice group. COX-2 protein levels also increased in aged mice group as compared with other group and reduced by RSV and/or exercise. More interestingly, we found that the anti-inflammatory IL-10 also increased during aging. It could be explained by a protective mechanism by the organism itself during the process of aging. The present study showed that aging is accompanied by the increase of proinflammatory factors in liver that can be reduced or modulated by RSV and/or physical activity.

Introduction

The free radical theory of aging proposes that free radicals, generated during endogenous metabolism, are responsible for the declining function and efficiency of physiological systems in aging (Harman, 1956). Aging is a biological process influenced by changes in redox status and by the oxidative stress-induced inflammatory reactions (Chung et al., 2011). Then, there are strong evidences strongly supportive of molecular inflammation as a major biological alteration underpinning the aging process and age-related diseases. Inflammation is known to be extremely complex and the inflammatory response is depended on free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Chung et al., 2001). The key players in the inflammatory reaction are the age-related upregulation of nuclear factor-κB (NF-κB), interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS). Then, gene expression of pro-inflammatory IL-1β, IL-6, TNF-α, COX-2, iNOS is enhanced by redox-sensitive transcription factor NF-κB during aging (Chung et al., 2006). Transcriptional regulation by NF-kB in many inflammatory pathways suggests an important role in chronic inflammation during aging (Jung et al., 2009). Chronic low-grade systemic inflammation is a common manifestation of aging. Previous studies have shown that higher elevations in circulating levels of pro-inflammatory cytokines such as IL-6 and TNF-α, and acute phase proteins such as C-reactive protein and serum amyloid A, are typical in the elder when compared to the young, even in the absence of chronic disease (Brüünsgaard and Pedersen, 2003). Increases in oxidative stress with aging may also contribute to the development of chronic inflammation and disease. It is well established that aging is associated with increases in tissue and circulating levels of ROS, as well as declines in antioxidant capacity (Kregel and Zhang, 2007).

Proinflammatory cytokines that are involved in chronic inflammation are TNF-α and members of its superfamily: IL-1β, IL-6, IL-17, IL-18. Cyclooxygenases (COX) are enzymes responsible for the formation of prostanoids which are involved in inflammation process (Wu et al., 2010). COX-2 catalyzes the production of prostaglandins (PGs) from arachidonic acid in a variety of tissues, including the liver. COX-2 is overexpressed in chronic liver inflammation and cirrhosis. IL-6 is a cytokine with pleiotropic effects. Overproduction of IL-6 has been shown to induce chronic inflammations, autoimmune diseases, and hematopoietic disorders (Wunderlich et al., 2010). TNF-α is a pro-inflammatory multifunctional cytokine predominantly produced in macrophages, CD4+ and also CD8+ T cells, and in activated NK cells and neutrophils in humans. TNF-α also contributes to the production of IL-6 through activation of several pathways (Williams et al., 2008). IL-17 is a potent proinflammatory cytokine produced by activated memory T cells (Aggarwal and Gurney,

2002). Interleukin-1 (IL-1α and IL-1β) plays a critical role in acute and chronic inflammation. IL-1β, a pro-inflammatory cytokine, is released by activated macrophages and neutrophils and contributes to systemic inflammatory manifestations (Di Iorio et al., 2003).

The inflammasomes are cytoplasmic multiprotein complexes that have recently been identified in immune cells as an important sensor of signals released by cellular injury and death (Boaru et al., 2012). The NALP-3 inflammasome is composed of the NALP-3 (NACHT, leucin-rich-repeat (LRR), and pyrin domain-containing protein 3), apoptosis-associated speck-like protein (ASC) and caspase-1 (CASP-1) (Martinon et al., 2006; Schroder et al., 2010). The NALP-3 interacts with ASC to activate CASP-1, subsequently leads to maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18, which are involved inflammation response (Agostini et al., 2004). CASP-1 is responsible for the conversion of pro-IL-1β to mature IL-1β. In addition, two other related cytokine precursors, pro-IL-18 and pro-IL-33, are also cleaved by CASP-1 (Ye and Ting, 2008). A recent study has shown that NALP-3 inflammasome is upregulated and activated in the liver in response to lipopolysaccharide (LPS) stimulation (Ganz et al., 2011).

Resveratrol (RSV) (3,5,4′-trihydoxy-*trans*-stilbene) is a polyphenol found in a large number of plant species such as mulberries, peanuts, grapes, and is present in red wine (Rivera et al., 2009). This polyphenol has been reported to exert multiple health-promoting benefits such as anti-inflammatory, anti-oxidant, anti-tumor, anti-platelet aggregation, anti-aging and anti-atherogenic effects (Baur et al., 2006; Saiko et al., 2008; Szkudelska and Szkudelski, 2010). This polyphenol modulates the expression of many genes and modifies the activity of many molecules, including NF-κB (Shakibaei et al., 2009). RSV is hypothesized to accomplish these beneficial effects by reducing expression of several cytokines including IL-6 (Zhong et al., 1999), IL-12, IL-2, and IFN-γ (Gao et al., 2001). RSV inhibits the expression of TNF-α in many types of cells including stimulated human macrophages (Feng et al., 2004), rat macrophages (Ma et al., 2005). In fact, RSV elicits inhibitory effects at all stages of the inflammatory response: it modifies the activity of inflammatory cells by inhibiting production of reactive oxygen and nitrogen species; it regulates enzyme expression, and it decreases the activity of several enzymes involved in the synthesis of pro-inflammatory mediators (De La Lastra and Villegas, 2005). This suggests that RSV has many targets and also explains why the mechanisms involved in the anti-inflammatory activity of this polyphenol are not yet completely understood.

On the other hand physical activity can both cause and attenuate inflammation. Many studies have demonstrated that regularly performed cardiovascular exercise training may reduce markers of

systemic inflammation (Beavers et al., 2010; Woods et al., 2006). Exercise has anti-inflammatory effects, and therefore, in the long term, regular physical activity can protect against the development of chronic diseases and aging (Pedersen and Saltin, 2006). Regular exercise results in higher circulating levels of adiponectin and lower levels of several circulating pro-inflammatory adipokines, including IL-6, TNF-α, retinol-binding protein 4 and leptin (Ben Ounis et al., 2009; Lim et al., 2008; Mujumdar et al., 2011). So, increased physical activity can bring about a reduction in systemic inflammation via a decrease in pro-inflammatory adipokine secretion (Yudkin, 2007). Furthermore, aerobic exercise reduces age-related increase in oxidative stress and inflammation and increases anti-oxidant defenses in mice and rats (Kalani et al., 2006; Keller et al., 2004; Navarro et al., 2004).

Liver is being recognized as playing a central role in mediating systemic inflammation. The liver not only contains the greatest concentration of the body's resident tissue macrophages (e.g., the Kupffer cells), but hepatocytes themselves can also elaborate a variety of proinflammatory cytokines (Glasgow et al., 2007).

Although the anti-inflammatory activity of RSV and/or exercise has been well documented in many studies, its effect on the liver of aging has not been established. In the present work, we study the effect of RSV and/or exercise in systemic inflammation as a function of age in mouse liver and assess whether RSV and physical activity modulate these effects. The level of IL-1β, IL-6, IL-10, IL-17, TNF-α and their mRNA expression were evaluated in this study. The level of expression of COX-2 also was determined to estimate it’s modulated by RSV and/or exercise. Furthermore, we study also the regulation of liver NALP-3 inflammasome components assess the change by aging and the effect of RSV and/or exercise in old group mice.

Results

Proinflammatory cytokines increased in old liver.

In order to determine if inflammation increases during aging in mouse liver we studied the levels of proinflammatory cytokines in whole homogenates (Table III.1). Elisa determination of cytokine levels in whole homogenated demonstrated that levels of IL-1β, IL-6 and TNF-α were significantly higher in livers from old mice. In these mice IL-1β, TNF-α and IL-6 nearly doubled the levels found in young animals whereas mature mice showed intermediate levels. IL-17 levels also showed a trend to increase as the age of the animals rise (Table III.1). On the contrary, the levels of the anti-inflammatory cytokine IL-10 also increased nearly twice in old animals (Table III.1).

Table III.1. *Level of cytokine in mice liver during aging.*

	TNF-α	**IL-17**	**IL-10**	**IL-1β**	**IL-6**
Young	13,74 ± 2,71	7,05 ± 0,11	14,37 ± 0,94	101,29 ± 16,58	14,75 ± 2,28
Mature	14,26 ± 0,92	9,06 ± 0,45	25,98 ± 1,32	80,90 ±14,41	19,06 ± 2,21
Old	25,02 ± 2,94$^{*\#}$	9,99 ± 1,38	31,72 ± 4,77*	249,47 ± 38,98$^{*\#}$	32,54 ± 1,82$^{*\#}$

Data are indicated as pg/mg protein. Data represent the mean ± SEM (n=4) *Significant differences vs. young group or their group control, $p < 0.05$. $^{\#}$Significant differences vs. mature group, $p < 0.05$

The increase of proinflammatory cytokines was also accompanied by a rise in the levels of mRNA of IL-6, IL-1β and TNF-α in mouse liver. This increase was significant in the case of TNF-α and IL-1β and nearly significant in the case of IL-6 (Figure III.1).

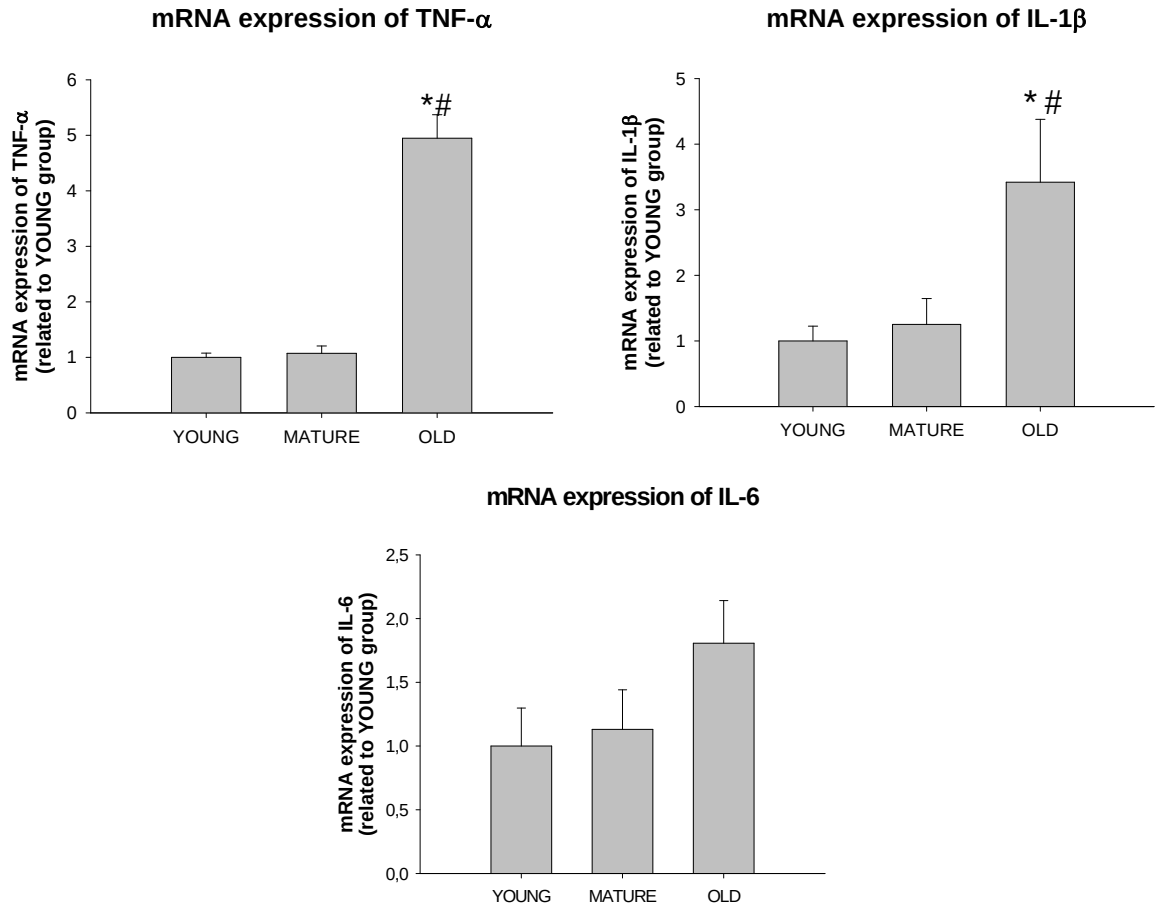


***Figure III.1**. Level mRNA expression of IL-6, IL-1β and TNF-α in mice liver during aging.* Significant differences vs. young group, # Significant differences vs. mature group, $p < 0.05$*

Resveratrol and of exercise modulates cytokine levels in mouse liver.

In order to determine if RSV and/or exercise can modulate the proinflammatory frame found in old mice liver, we determined the levels of these cytokines in mice fed with RSV and/or practicing exercise (Figure III.2). In general, we found a decrease of proinflammatory cytokine levels produced by both, RSV and exercise independently of each other. However, only in the case of IL-1β, we found a significant decrease of cytokine levels due to RSV alone or in combination with training. Levels reached in RSV-treated animals were similar to those found in young animals. In the case of IL-17 and TNF-α, a trend to decrease was found whereas in the case of IL-6 no changes were found (Figure III.2). A similar response was found with IL-10, with a trend to decrease due to RSV plus exercise (Figure III.2).

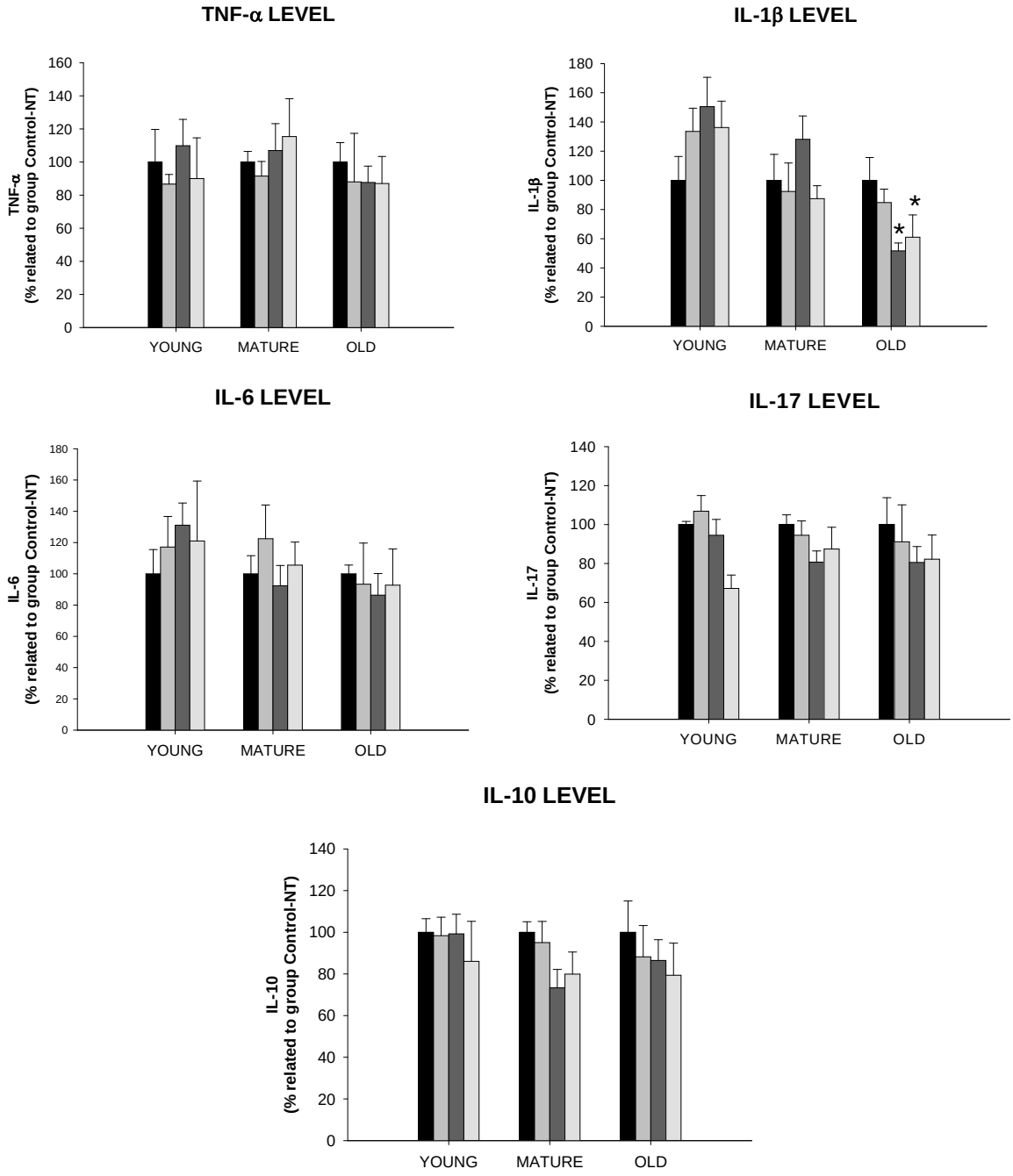


***Figure III.2**. Protein level of TNF-α, IL-1β, IL-6, IL-17, IL-10 in three young, mature and old group animal experimental.* *Significant differences vs. control group, $p < 0.05$*

Interestingly, the levels of mRNA did not follow a similar pattern since we found a clear decrease in IL-1β and also TNF-α levels in liver in old group induced by either exercise or RSV alone or in combination (Figure III.3).

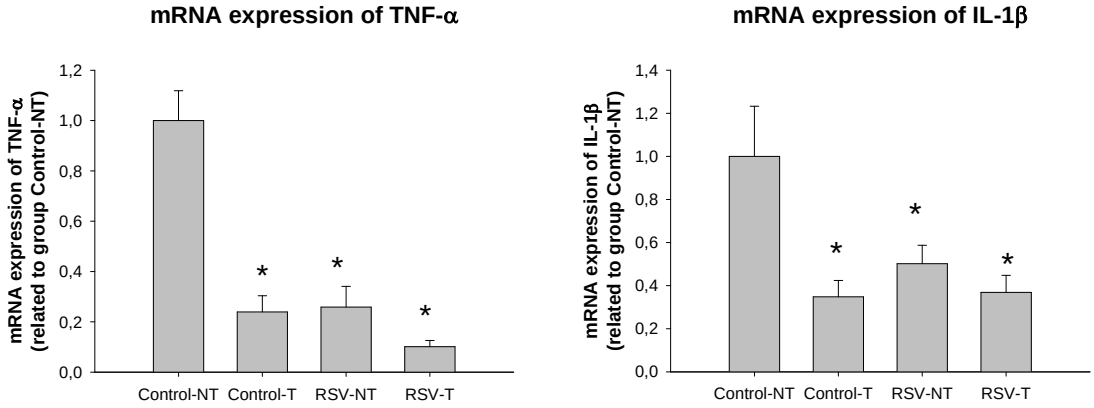


Figure III.3*. mRNA expression of TNF-α and IL-1β in old group under effects of RSV and exercise.* *Significant differences vs. group control, $p < 0.05$*

RSV and exercise also modulate COX-2 levels in old mice.

We also determined the regulation of other factors in inflammation such as COX-2 levels. As in the case of cytokines, COX-2 levels in mice liver increased as animal age and a significant increase of around 60% was found in old animals vs. young or mature animals (Figure III.4). In this case, exercise and/or RSV induced a decrease in COX-2 levels in liver at all ages. Exercise decreases these levels in around a 40% whereas RSV alone produced a similar effect. Combination of both, exercise and RSV, reduced yet more COX-2 levels in young and especially in mature animals (Figure III.4).

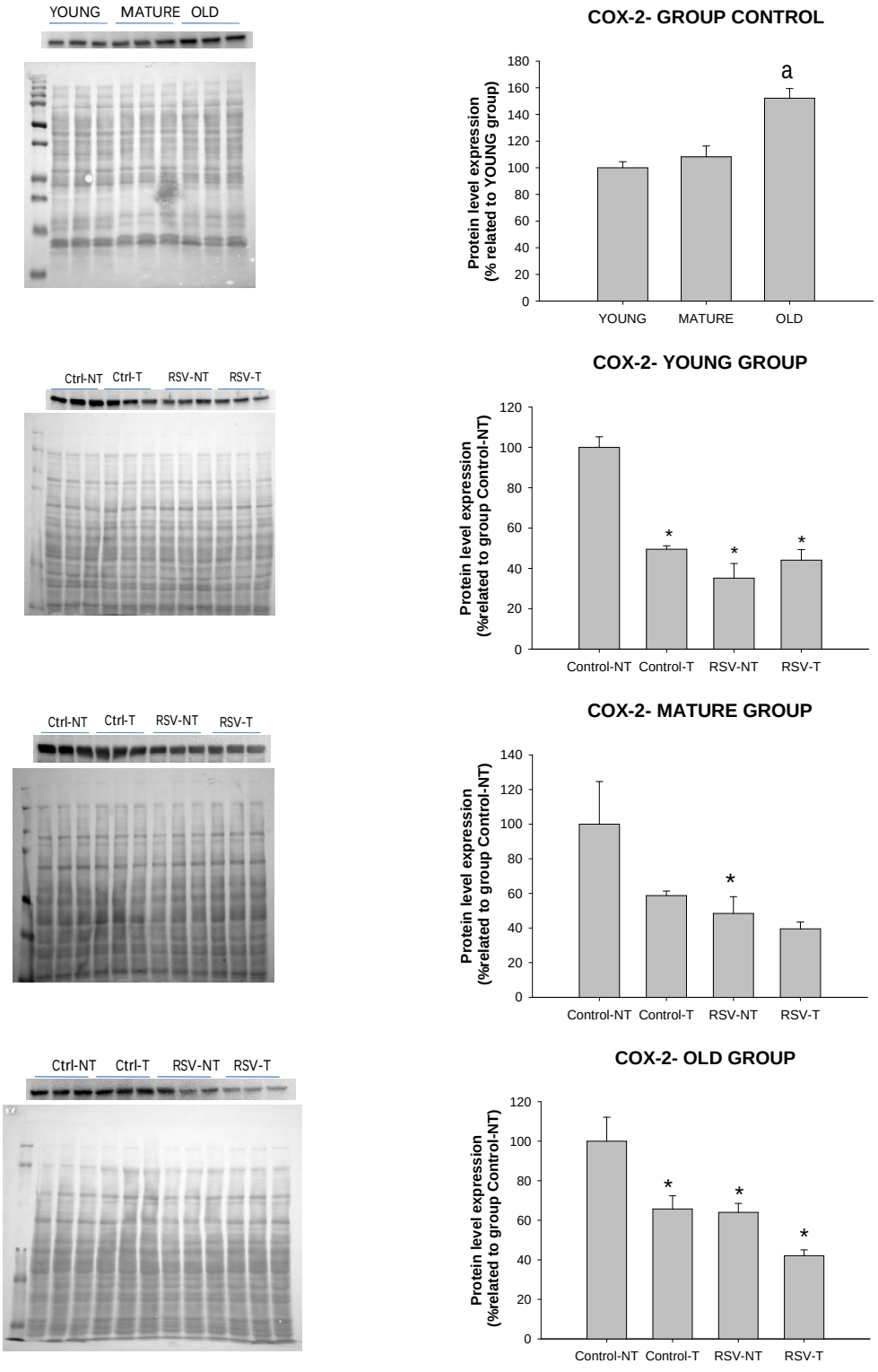


Figure III.4. *COX-2 protein levels in mice liver. WB analysis of COX-2 during aging and in young, mature and old group animal. [a]Significant differences vs. young group, *Significant differences vs. control group, $p < 0.05$.*

Inflammasome is also affected by age and modulated by exercise and or RSV.

We also determined the levels of inflammasome components. Inflammasome components ASC, CASP-1, NALP-1 and NALP-3 increased their respective expression in aging (Figure III.5). In aged animals, RSV, exercise and their combination reduced significantly the expression of these components (Figure III.6).

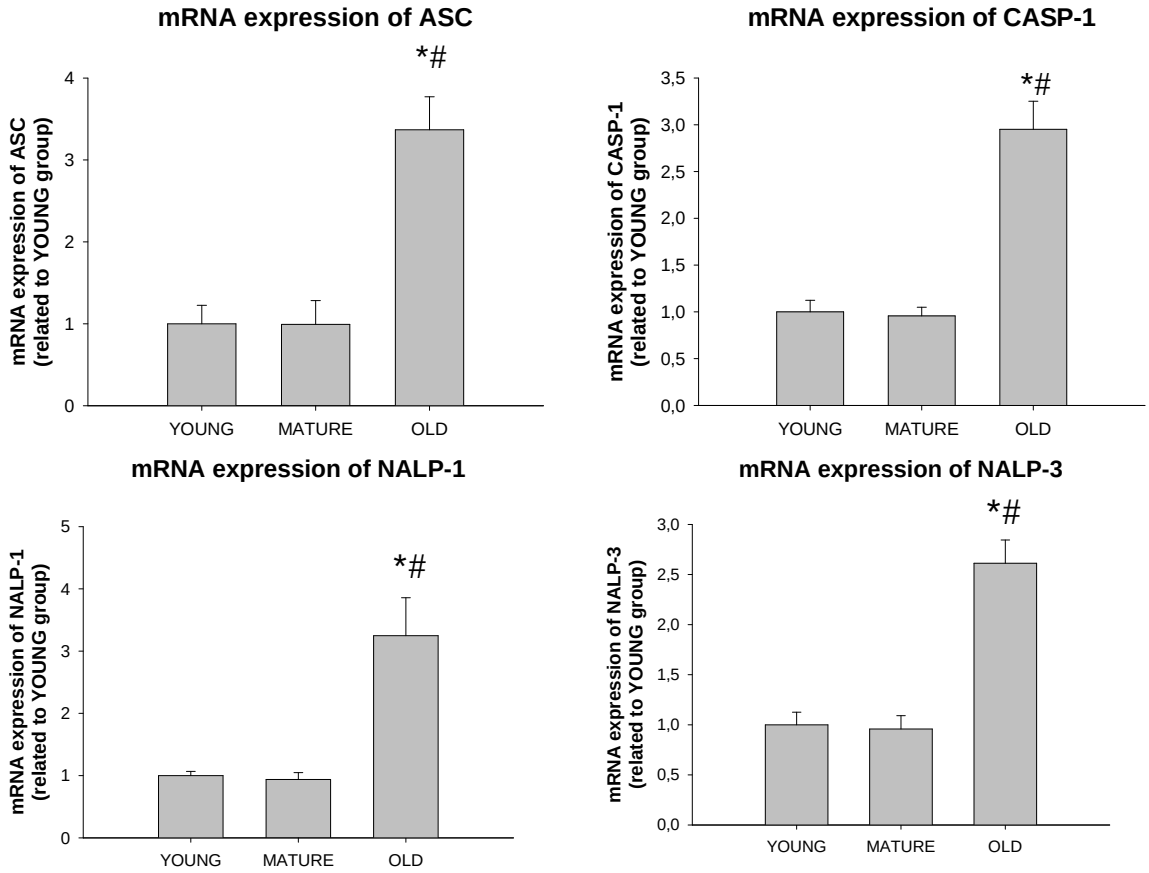


Figure III.5*. mRNA expression of ASC, CASP-1, NALP-1 and NALP-3 during aging. *Significant differences vs. young group, p < 0.05. #Significant differences vs. mature group, p < 0.05.*

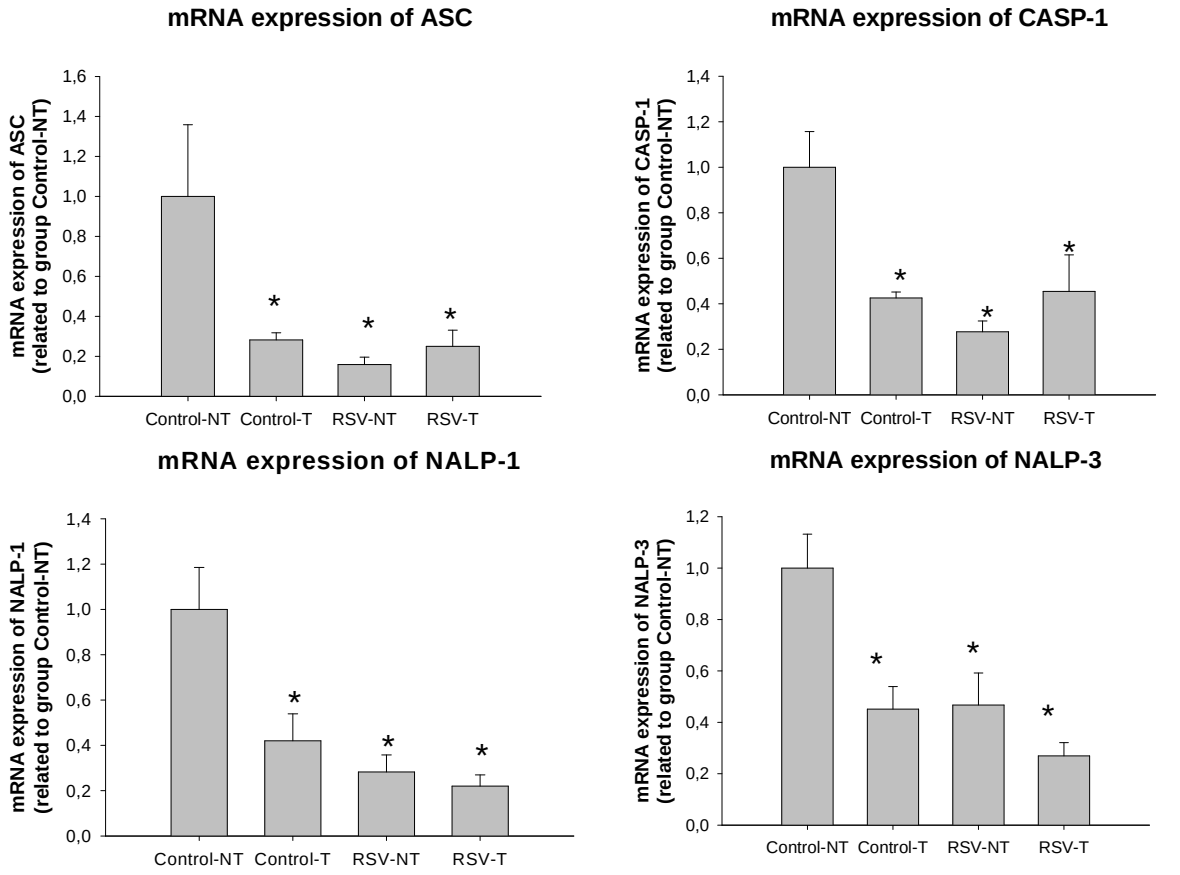


Figure III.6. *mRNA expression of ASC, CASP-1, NALP-1 and NALP-3 in old group under effects of RSV and exercise. *Significant differences vs. group control, $p < 0.05$.*

Protein levels of CASP-1 in liver.

We determined the changes of expression protein level CASP-1 by aging and induced by exercise or RSV in old animal group. We found that by effect of age, the level of protein expression of CASP-1 was increased significantly in old group as compared with young group while there is no significantly increased between young and mature group. In aged animals, protein level expression of CASP-1 was decreased significantly by effect of physical activity, RSV and its combination (Figure III.7).

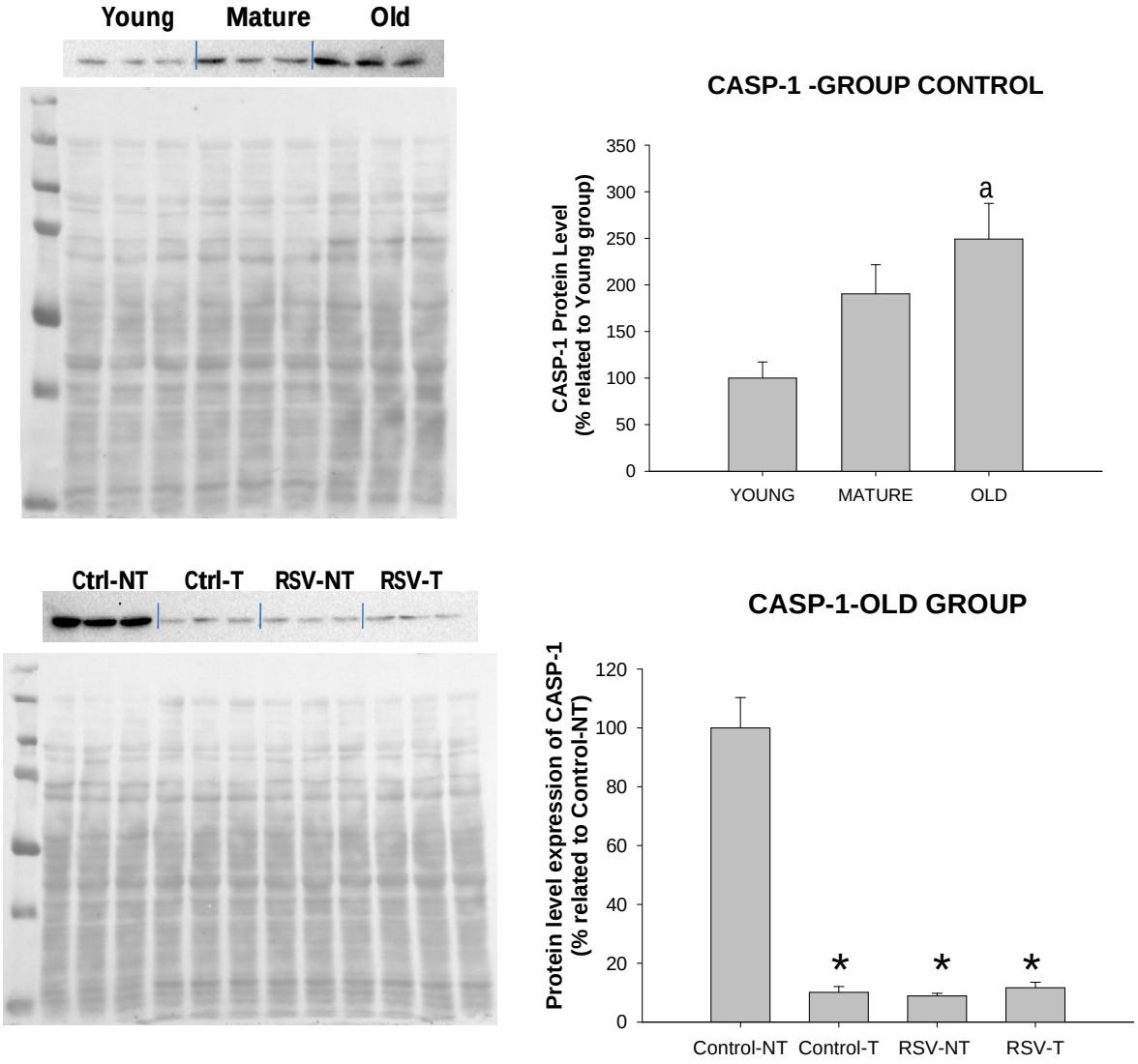


Figure III.7. *CASP-1 protein levels in mice liver. WB analysis of CASP-1 during aging and in old group.* [a]*Significant differences vs young group, *Significant differences vs control group* $p < 0.05$.

Discussion

Aging of liver is associated with a variety of functional alterations although mechanisms of liver degeneration are still not completely defined (Schmucker, 1998). Inflammatory activity is frequently proposed as a contributor to biological aging. An increase in inflammatory signaling has been often observed during aging, with inflammation potentially mediating age-related changes in the liver (Gee et al., 2005). Chronic low-grade inflammation accompanies aging as well as some chronic medical disorders. Thus, during aging, increased plasma levels of TNF-α, IL-6 has been found (Bruunsgaard et al., 2000; Dobbs et al., 1999). Our data have shown the level of pro-inflammatory IL-1β, IL-6, IL-17 and TNF-α increased in old liver in comparison with young and mature liver. This means that during aging the levels of pro-inflammatory cytokines increase, confirming its important role on the chronic inflammation. Interestingly, we have also found that cytokine IL-10 also augmented in old group mice compared with other group. IL-10 is well known for its immunosuppressive activity reducing the extent of hepatic damage caused by aging (de Vries, 1995). That could be the mechanism protective by organism itself in process of aging.

IL-6 is a pleiotropic cytokine that exerts multiple biological activities on several target cells including hepatocytes. IL-6 mediates many of the hepatic aspects of the acute phase response, as well as the induction of acute phase proteins by cultured hepatocytes or cells lines derived from hepatocytes (Perlmutter et al., 1989). Expression of IL-6 can be induced in various cell types including fibroblasts, monocytes/macrophages, T cells, B cells, endothelial, epidermal, synovial, and diverse tumor cells (Le and Vilček, 1990). Blood levels of the acute phase responses CRP, IL-6, and TNF-α tend to increase during aging in humans (Ferrucci et al., 2005). Blood IL-6 also tends to increase during aging in rodent models (Longo and Finch, 2003; Panda et al., 2009). As a key cytokine, TNF-α is a potent mediator of inflammation and carcinogenesis, and induces the inflammatory effects of IL-6 and increases production of IL-1β by up-regulating the transcription factor NF-κB (Wilson, 2008). In the liver, TNF-α is not only a mediator of hepatotoxicity but also contributes to the restoration of functional liver mass by driving hepatocyte proliferation and liver regeneration (Schwabe and Brenner, 2006). IL-17 is also an important cytokine involved in inflammatory conditions such as autoimmune diseases, psoriasis, arthritis, and inflammatory bowel diseases (Afzali et al., 2010; Leung et al., 2010; Mai et al., 2010).

Our study indicates that both RSV and exercise decrease the proinflammatory profile found in aged liver. Although liver is no directly related with exercise, our results agree with previous reports indicating that exercise training reduced IL-1β in rat skeletal muscle (Lira et al., 2009) but also in

adipose tissue (Gomez-Merino et al., 2007). In humans, previous studies have shown that long-term resistance training reduces circulating IL-6, IFN-γ and TNF-α in healthy older people (Córdova et al., 2011). A recent study has shown that long-term exercise may protect the bowel by reducing expression inflammatory cytokine TNF-α, IL-1β, IL-6, and apoptotic protein caspase-3, caspase-7 (Packer and Hoffman-Goetz, 2012). On the other hand, exercise also increased the levels of anti-inflammatory cytokine, IL-10, and decreased inflammatory marker, CRP, in plasma of old rats (Asghar et al., 2007). However, we did not found the increase the level IL-10 by RSV and/or exercise, that could be explained by we measured the IL-10 in homogenates of liver, and other reports measured IL-10 in plasma (Asghar et al., 2007).

Regarding to RSV, we have found the RSV decreased TNF-α in old group mice. The anti-inflammatory properties of RSV have been demonstrated in many studies. Fulgenziet et al. (Fulgenzi et al., 2001) showed that RSV reduced TNF-α-mediated vascular leakage in a mouse liver perfusion model (1μM). Our data also agree with recent study showing that RSV induces a reduction of IL-17 production in a concentration-dependent manner in a model of inflammation in vitro (Lanzilli et al., 2012).

The anti-inflammatory activity of RSV may be explained by the inhibition of COX-2 as well as its antioxidant effect (Martinez and Moreno, 2000; Subbaramaiah et al., 1998). Inflammation plays a major role in the pathophysiology of several diseases. RSV has been shown to inhibit inflammatory responses through the inhibition of synthesis of various pro-inflammatory mediators, modulation of prostaglandin synthesis, and through the inhibition of factors such as NF-κB, COX-2 and iNOS (Shakibaei et al., 2009). In a colitis model, RSV reduced COX-2 and NF-κB p65 protein expression, alleviated oxidative events, and returned prostaglandin E_2 production to basal levels (Martín et al., 2006). RSV is also able to inhibit NF-κB, and AP-1 activation supplying an additional mechanism for inactivation of COX-2 by the polyphenol, because COX-2 transcription can be stimulated by both transcription factors (Subbaramaiah and Dannenberg, 2003). Our data show the capacity of RSV and/or exercise to reduce the level expression of COX-2 in aging liver, confirmed again the capacity of exercise and RSV to inhibit COX-2 in liver mice.

The oxidative damage produced by free radicals promotes the activation of NALP-3 inflammasome (Martinon, 2010). The inflammasome is a cytosolic multiprotein complex that, upon assembly with caspase 1, has the enzymatic ability to cleave pro-IL-1 and pro IL-18 into active cytokines. Several inflammasome complexes exist and among them the NALP-3 inflammasome has been shown recently to be directly activated by the presence of sustained amounts of ROS (Zhou et al., 2010).

Bauernfeind et al. (2009) (Bauernfeind et al., 2009) revealed that NF-κB dependent signaling was clearly able to upregulate the expression of NALP-3 and thus permitted the activation of NALP-3 inflammasome. The NALP-3 inflammasome is implicated in recognizing certain no microbiological danger signals leading to CASP-1 activation and subsequent IL-1β and IL-18 secretion (Vandanmagsar et al., 2011). Consequently, these cytokines provoke inflammatory responses and accelerate the aging process (Salminen et al., 2012). NALP-3 activation is also enhanced in many age-related diseases, e.g. atherosclerosis, obesity and type 2 diabetes. We have shown that the expression of mRNA of genes that are directly linked to the activity of the inflammasome, NALP-3, NALP-1, ASC, CASP-1, IL-1β were augmented by aging and reduced by RSV, exercise or combination of both in old group. This increase can be related to a chronic damage of liver, since a previous study has shown that the expression of inflammasome genes (including NALP-1, NALP-3, NLRC4/NALP4, AIM2 (absent in melanoma 2), IL-1β, IL-18, ASC, and TNF-α) during the process of acute and chronic liver insult by LPS increased (Boaru et al., 2012). NALP-3 activation is also enhanced in many age-related diseases, e.g. atherosclerosis, obesity and type 2 diabetes. Furthermore, NF-κB signaling is a vital inducer of NALP-3 expression and the aging process can stimulate NF-κB signaling, thus probably enhance the priming and potentiation of the inflammasome activation (Salminen et al., 2012). Vandanmagsar et al. (2011) revealed that obesity was associated with the activation of NALP-3 in adipose tissue and calorie restriction and exercise-mediated weight loss in obese individuals with type 2 diabetes is associated with a reduction in adipose tissue expression of NALP-3 as well as with decreased inflammation and improved insulin sensitivity (Vandanmagsar et al., 2011). Higher levels of inflammasome can be related to higher ROS levels found in aged animals since the ROS-induced activation of NALP-3 has been clearly attributable to a priming process, since ROS inhibitors blocked the priming step in NALP-3 activation (Bauernfeind et al., 2009).

In conclusion, our findings show that the pro-inflammatory cytokine (TNF-α, IL-1β, IL-6, IL-17), COX-2 augmented and NALP-3 inflammasome was activated during aging. The treatment of RSV and/or exercise training can reduce this increase. Therefore, taking RSV and practicing exercise should be strongly encouraged in older people, for not only improves physical function and the maintenance of independence, but may also attenuate chronic low-level systemic inflammation.

CHAPTER IV

CHAPTER IV

MODULATION OF ANTIOXIDANT DEFENCES BY RESVERATROL AND EXERCISE ON OLD MICE IN DIFFERENT ORGANS AND TISSUES

Abstract

Aging is associated with increased oxidative stress. A regimen of resveratrol and exercise was administered to old mice during 6 months to determine putative anti-aging effects. Both regimen had improved antioxidant activity in the major organs of the mice, as indicated by increased in different manner of superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, glutathione-S-transferase, thioredoxin reductase, NADH-cytochrome b_5 reductase, and NAD(P)H-quinone acceptor oxidoreductase activities. In addition, a decreased amount of malondialdehyde was observed in the liver, heart, and muscle of mice exposed to the treatment regimen. Our data demonstrate that a regimen of resveratrol treatment and exercise enhances endogenous antioxidant enzyme systems in old mice. These results suggest that physical activity and resveratrol may be of great importance for the prevention of diseases related to aging.

Introduction

Aging is usually associated with an increase in free radical concentrations, mainly because of a decline in antioxidant mechanisms and a rise in pro-oxidant factors (Mezzetti et al., 1996). The reactive oxygen species (ROS) have become active factor in aging research because of their potential involvement in many degenerative diseases. These ROS are highly reactive and damage many biological macromolecules such as DNA, RNA, protein and lipids (Feuers et al., 1993). Antioxidant enzymes constitute an important defense system to clear up the harmful ROS in vivo.

Resveratrol (*trans*-3,4',5-trihydroxystilbene) (RSV) a naturally occurring phytoalexin that can be found in red wine, berries, and peanuts. The positive effects of RSV in biological systems are wide-ranging as a cancer chemoprevention agent (Wolter et al., 2004), a powerful anti-inflammatory factor (Donnelly et al., 2004) and an antioxidant agent (Cai et al., 2003). Several investigations have cited the possible role and protective effects of RSV against certain forms of oxidant damage, through a hydrogen-electron donation from its hydroxyl groups (Lopez et al., 2003). Therefore, RSV may improve the health and prolong the average lifespan of mammals that suffer from age.

It has been known for some time that physical activity is associated with a better health by benefiting the cardiovascular system. Physical activity may lead to an increase in physiological antioxidant defenses of the organism in old subjects. It reduces the production of oxidants and oxidative damage, improve antioxidant defense system, and increase the resistance of organs and tissues against the deleterious action of free radicals (Polidori et al., 2000). Furthermore, physical activity level correlates closely with antioxidant enzymatic activities, especially on glutathione in liver and brain (Yamamoto et al., 2003).

The aim of the present study was to evaluate the anti-aging properties of RSV and/or physical activity in old mice by determining superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), NAD(P)H-quinone acceptor oxidoreductase (NQO1), NADH-cytochrome b_5 reductase ($CytB_5Rase$) and thioredoxin reductase (TrxR) activities in different tissues mouse as well as its levels of protein´s expression. Furthermore, the content of glutathione, sulphydryl group and lipid damage through malondialdehyde (MDA) was also determined.

Results

RSV and exercise decreased oxidative damage in old mice.

In order to value the levels of some oxidant marker we determined the level of glutathione, sulphydryl group and MDA in different tissue. The result was showed in Table IV.1.

The sulphydryl groups were very high in brain in comparison with other tissue whereas liver showed lower the level of the studied organs. No intervention produces effect in brain. Exercise increased levels in heart and muscle whereas decreased them in kidney and liver. On the other hand, RSV only or its combination with exercise also increased levels in heart and muscle whereas decreased them in kidney and liver.

Regarding glutathione, the highest levels per protein were found in liver whereas other tissue showed similar level around 6 nmol/mg proteins. In this case, exercise increased level in heart, muscle and liver without affecting brain and kidney. On the other hand, RSV increased the level in brain, muscle and liver without affecting kidney. Combined RSV with exercise only affected liver and heart levels.

Level of MDA indicates lipid peroxidation where higher in brain and lower in heart, kidney, and liver. Exercise decreased level MDA in liver whereas RSV decreased MDA only in muscle. Combination of both produced clear decreased in heart, muscle and liver.

Table IV.1. *Oxidative stress markers in old mice.*

	Control-NT	**Control-T**	**RSV-NT**	**RSV-T**
		Sulphydryl group		
Brain	272,62 ± 18,83	253,59 ± 16,6	283,94 ± 41,93	266,52 ± 12,18
Heart	52,3 ± 6,49	91,14 ± 9,89*	66,18 ± 8,49*	65,43 ± 11,52*
Kidney	58,47 ± 8,71	26,83 ± 6,75*	37,2 2 ± 8,1*	37 ± 2,57*
Muscle	51,34±21,84	81,33±20,37*	66,31±14,69*	69,17±4,79*
Liver	8,86 ± 1,74	5,35 ± 0,17*	5,63 ± 0,30*	7,44 ± 0,93
		Glutathione		
Brain	5,67 ± 0,14	5,60 ±0,46	6,97 ± 0,5*	6,16 ± 0,54
Heart	6,19 ± 0,22	8,44 ± 0,91*	7,49 ± 0,65	7,69 ± 0,39*
Kidney	5,85 ± 0,69	6,45 ± 0,43	6,34 ± 0,69	6,52 ± 0,55
Muscle	6,52±0,44	10,27±0,52*	8,70±0,65*	7,75±0,72
Liver	21,43 ± 5,85	37,97 ± ,13*	35,28 ± ,33*	38,54 ± 2,27*
		MDA		
Brain	4,66 ± 0,24	4,68 ± 0,53	4,82 ± 0,38	4,71 ± 0,29
Heart	0,47 ± 0,06	0,31 ± 0,11	0,34 ± 0,05	0,29 ± 0,06*
Kidney	0,51 ± 0,12	0,55 ± 0,03	0,5 ± 0,07	0,52 ± 0,03
Muscle	1,07±0,05	1,14±0,02	0,69±0,06*	0,85±0,05*
Liver	0,45 ± 0,11	0,32 ± 0,06*	0,38 ± 0,07	0,32 ± 0,07*

*Values are the mean ± SE. * Significant difference vs. Control-NT levels, $p<0.05$*
Sulphydryl group, glutathione and MDA levels are indicated as nmol/mg protein

Antioxidant activities are improved by RSV and exercise in old mice

We determined the activity of several antioxidant enzymes in the different tissue. The antioxidant activities are summarized in table IV.2, 3, 4.

Catalase activity was very higher in kidney and liver and lower in heart. Interestingly, in high activity organ, CAT was induced by exercise. RSV only induced activity in kidney whereas the combination of both increased the activity in both organs. A trend to increased in muscle were found but without significance.

In the case of SOD the response was different. Liver showed highest activity but exercise or RSV decreased this activity although its combination increased it. Heart activity was induced by both also its combination but without significance. Exercise combined with RSV slight increased its activity in brain. A trend to increase was also found in muscle but without significance.

Glutathione peroxidase activity was higher in kidney, muscle and liver and lower in heart and brain. Interestingly, GPx was increased significantly by exercise and RSV in all organs. The combination of both increased significantly the activity in both organs kidney and liver and tend to be increased in other organs but without significance.

Glutathione reductase activity was found higher in kidney, liver and lower in heart, muscle and lowest in brain. Exercise, RSV and its combination do not produce effect on this activity of GR in all organs studied.

Glutathione-S-transferase activity was found highest in liver and lowest in heart. Exercise, RSV and its combination increased significantly the GST activity in liver but reduced significantly in heart. A trend to increase was found in brain but without significance.

In the case of cytochrome b_5 reductase, the highest activity was found in heart and lowest in muscle. Exercise increased significantly the CytB_5Rase activity in kidney, also trend to increase in brain, liver and muscle although no significance. RSV tends to increase the activity of CytB_5Rase in liver, brain.

The NAD(P)H-quinone acceptor oxidoreductase activity was found highest in heart and lowest in muscle. Exercise significantly increased NQO1 activity in heart, liver and kidney. RSV also increased significantly NQO1 activity in heart, liver and brain. Its combination increased significantly in almost organs studied.

The thioredoxin reductase activity was only determined in kidney and liver. Exercise, RSV and its combination increased significantly TrxR activity in kidney. However, in liver, a decrease of TrxR activity tends to be decreased by exercise, RSV and significantly decreased by its combination.

Table IV.2. *Antioxidant CAT and SOD activities in old mice.*

	Control-NT	**Control-T**	**RSV-NT**	**RSV-T**
		CAT		
Brain	2,07 ± 0,09	2,04 ± 0,05	2,11 ± 0,02	2,21 ± 0,07
Heart	3,37 ± 0,43	3,14 ± 0,39	3,35 ± 0,29	2,88 ± 0,34
Kidney	194,62 ± 22	236,37 ± 20,86*	249,77 ± 12,58*	275,75 ± 18,2*
Muscle	44,36 ± 5,37	57,95 ± 5,20	56,29 ± 5,06	58,06 ± 2,56
Liver	77,32 ± 2,55	110,1± 12,6*	85,3 ± 9,35	103,46 ± 7,14*
		SOD		
Brain	2,34 ± 0,12	2,39 ±0,08	2,55 ± 0,1	2,77 ± 0,09*
Heart	7,51 ± 0,35	10 ± 0,35*	9,25 ± 0,34*	8,62 ± 0,21*
Kidney	2,21 ± 0,25	2,35 ± 0,21	2,41 ± 0,14	2,5 ± 0,08
Muscle	19,34 ± 4,82	26,66 ± 4,19	27,70 ± 1,75	32,45 ± 1,33
Liver	1542,68 ± 386,45	1298,06 ± 260,39*	1470,57 ± 246,51	1761,37 ± 370,18*

*Values are the mean ± SE. *Significant difference vs. Control-NT levels, p<0.05*
Activities are indicated as nmol/min/mg protein

Table IV.3. *Antioxidant GPx, GR, GST activities in old mice.*

	Control-NT	**Control-T**	**RSV-NT**	**RSV-T**
		GPx		
Brain	17,39 ± 0,49	19,73 ± 0,59	21,47 ± 1,46*	19,76 ± 0,56
Heart	13,6 ± 2,41	16,09 ± 2,09*	16,14 ± 1,42*	14,76 ± 0,74
Kidney	30,3 ± 10,49	39,83 ± 3,84*	36,54 ± 1,42	39,52 ± 1,75*
Muscle	28,71 ± 0,49	30,50 ± 0,75	30,09 ± 1,34	28,54 ± 1,09
Liver	35,11 ± 12,86	79,19 ± 4,27*	69,89 ± 4,34*	85,68 ± 13,11*
		GR		
Brain	2,30 ± 0,18	2,09 ± 0,08	2,19 ± 0,09	1,99 ± 0,14
Heart	4,84 ± 1,09	5,39 ± 0,61	5,3 ± 0,28	4,09 ± 0,55
Kidney	24,13 ± 0,90	22,46 ± 0,25	22,54 ± 1,17	26,62 ± 1,44
Muscle	8,36 ± 1,12	5,84 ± 1,1	9,42 ± 1,15	8,15 ± 0,89
Liver	28,24 ± 1,3	28,94 ± 1,62	27 ± 2,12	24,24 ± 0,55
		GST		
Brain	90,67 ± 2,39	92,4 ± 1,39	97,43 ± 2,69	99,93 ± 4,67
Heart	18,36 ± 0,61	12,35 ± 0,58*	13,62 ± 0,51*	12,81 ± 0,77*
Kidney	35,15 ± 7,18	28,39 ± 0,99	31,45 ± 1,82	32,87 ± 1,85
Muscle	50,39 ± 7,64	54,22 ± 1,63	51,91 ± 3,69	54,5 ± 2,01
Liver	751,39 ± 212,18	1094,43 ± 106,56*	1130,53 ± 118,03*	897,28 ± 119,87*

*Values are the mean ± SE. *Significant difference vs. Control-NT levels, $p<0.05$*
Activities are indicated as nmol/min/mg protein

Table IV.4. *Antioxidant $CytB_5Rase$, NQO1 and TrxR activities in old mice.*

	Control-NT	Control-T	RSV-NT	RSV-T
		$CytB_5Rase$		
Brain	245,35 ± 2,12	253,75 ± 3,73	257,87 ± 5,49	227,67 ± 0,78
Heart	771,51 ± 90,96	729,55 ± 72,32	714,8 ± 63,33	889,47 ± 69,22*
Kidney	148,12 ± 15,06	164,69 ± 12,95*	150,38 ± 7,67	197,04 ± 21,28*
Muscle	4,09 ± 0,51	5,26 ± 0,22	4,41 ± 1,05	5,70 ± 0,83
Liver	327,46 ± 32,78	350,52 ± 27,54	335,54 ± 25,78	381,46 ± 42,32*
		NQO1		
Brain	5,33 ± 0,1	5,65 ± 0,26	7,35 ± 0,4*	6,81 ± 0,66*
Heart	16,26 ± 3,47	19 ± 2,94*	12,32 ± 2,65*	13,45 ± 2,45*
Kidney	1,13 ± 0,16	2,36 ± 0,18*	1,61 ± 0,4	3,05 ± 0,9*
Muscle	0,48 ± 0,01	0,54 ± 0,04	0,65 ± 0,07	0,58 ± 0,07
Liver	5,44 ± 0,94	11,66 ± 0,52*	7,71 ± 1,46*	9,82 ± 1,72*
		TrxR		
Brain		No determined		
Heart		No determined		
Muscle		No determined		
Kidney	1,55 ± 0,36	2,32 ± 0,34*	2,95 ± 0,54*	3,05 ± 0,33*
Liver	5,24 ± 0,66	4,58 ± 0,28	4,36 ± 0,51	2,75 ± 0,98*

*Values are the mean ± SE. *Significant difference vs. Control-NT levels, $p<0.05$*
Activities are indicated as nmol/min/mg protein

Antioxidant protein levels are differentially affected by RSV and/or exercise in old mice.

In brain, no significant changes in protein levels were found for CAT (Figure IV.1.A). Interestingly, the protein levels of GPx1 showed a higher expression in the group of RSV plus exercise in compare with group control (Figure IV.1.C). More interestingly, the levels of SOD1 and $CytB_5Rase$ were significantly increased in the old group of animals by physical activity and RSV (Figure IV.1B, E). Moreover, the combination of both interventions significantly increased NQO1 protein (Figure IV.1.F). Also the level of TrxR1 was significantly increased in the group of RSV plus exercise (Figure IV.1.G).

In heart, there are no significantly changes significant changes in protein levels were found for CAT (Figure IV.1.A). In the case of GR at the protein levels that showed a higher expression in the group of RSV plus exercise (Figure IV.1.D). Interestingly, the levels of SOD1 were significantly increased in the old group of animals by RSV (Figure IV.1.B). GPx1, TrxR1 and TrxR2 were not affected by these interventions (Figure IV.1.C, G). Additionally, the combination of both interventions significantly decreased NQO1 protein (Figure IV.1.F).

In kidney, expression of protein level of CAT and NQO1 were decreased by exercise and RSV (Figure IV.1.A, F). The level of GPx1, and TrxR1 was decreased by exercise and RSV but increased by its combinations (Figure IV.1.C, G). TrxR2 was increased by exercise but not affected by RSV (Figure IV.1.G). The level of protein GR was practically not changed by these(Figure IV.1.D). Interestingly, protein of SOD1 and $CytB_5Rase$ were significantly increased by RSV and exercise (Figure IV.1.B, E).

In muscle, we have found the RSV has increased significantly the expression of CAT in old animals (Figure IV.1.A). Exercise also augmented significantly the CAT in old group. In case of protein $CytB_5Rase$, RSV and exercise do not affected the expression of this protein in old mice (Figure IV.1.E). Interestingly, the levels of NQO1 were significantly increased by RSV in agreement with the increase of activity (Figure IV.1.F). More interestingly, the levels of SOD1 were significantly increased in the old group of animals by both physical activity and/or RSV (Figure IV.1.B). Also RSV increased the expression of GR, while physical activity do not have influenced in old group (Figure IV.1.D).

In liver, no significant changes in protein levels were found for CAT or $CytB_5Rase$ (Figure IV.1.A, E). In the case of TrxR, no differences at the protein levels were found either with TrxR1 or with TrxR2 (Figure IV.1.G). Interestingly, the levels of GPx1 were significantly increased in the old animals by both physical activity and/or RSV (Figure IV.1.C). Moreover, SOD1 was increased by

exercise (Figure IV.1.B). However, in the case of NQO1, RSV significantly decreased NQO1 protein levels independently of the exercise (Figure IV.1.F).

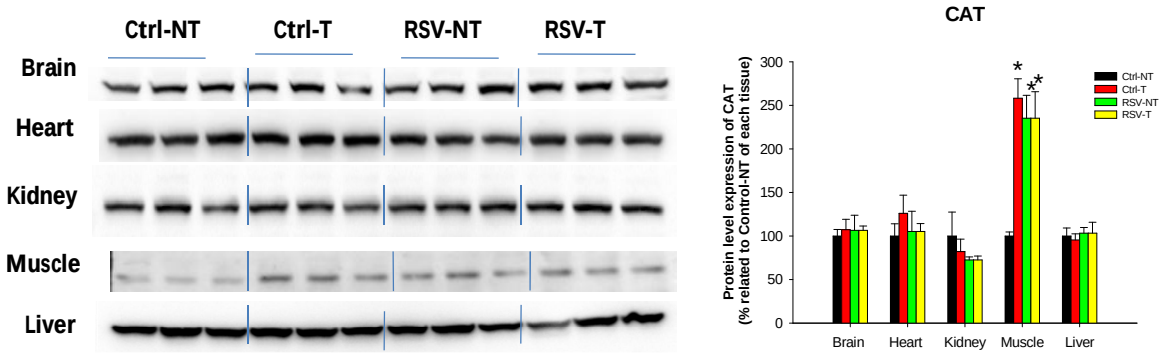


Figure IV.1.A *- Protein expression of Catalase (CAT) in difference tissue in old mice.*

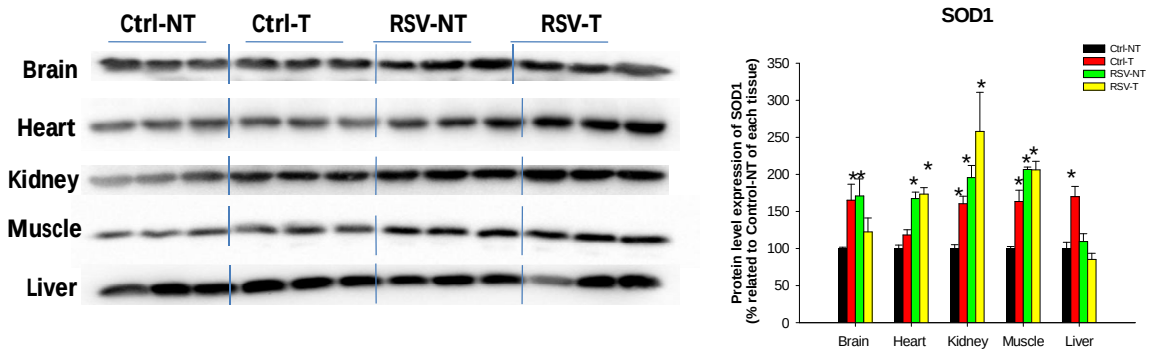


Figure IV.1.B *- Protein expression of Superoxide dismutase 1 (SOD1) in difference tissue in old mice.*

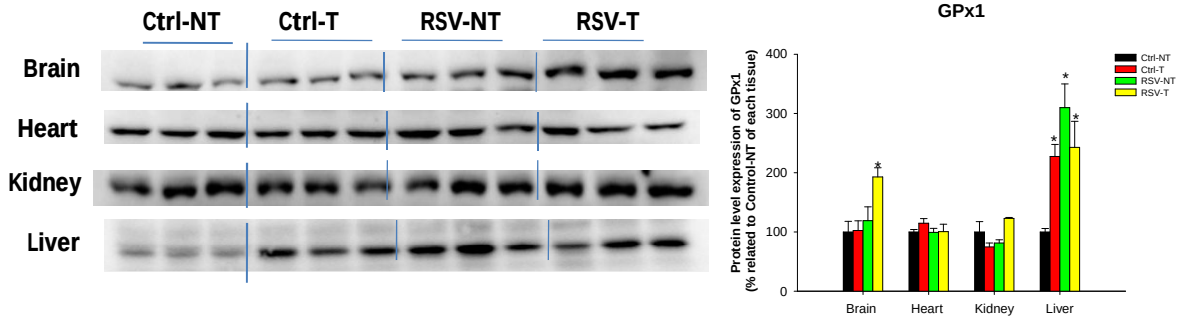


Figure IV.1.C *- Protein expression of Glutathione peroxide 1 (GPx1) in difference tissue in old mice.*

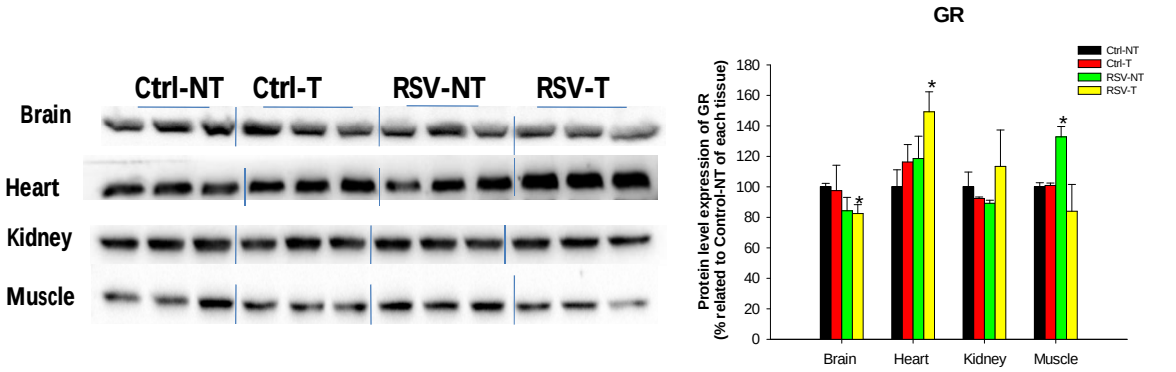


Figure IV.1.D *- Protein expression of Glutathione reductase (GR) in difference tissue in old mice.*

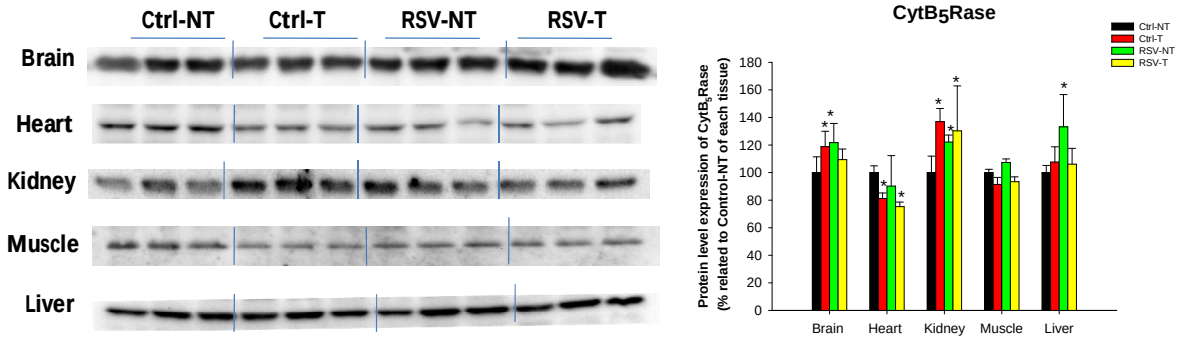


Figure IV.1.E *- Protein expression of Cytochrome b_5 reductase ($CytB_5Rase$) in difference tissue in old mice.*

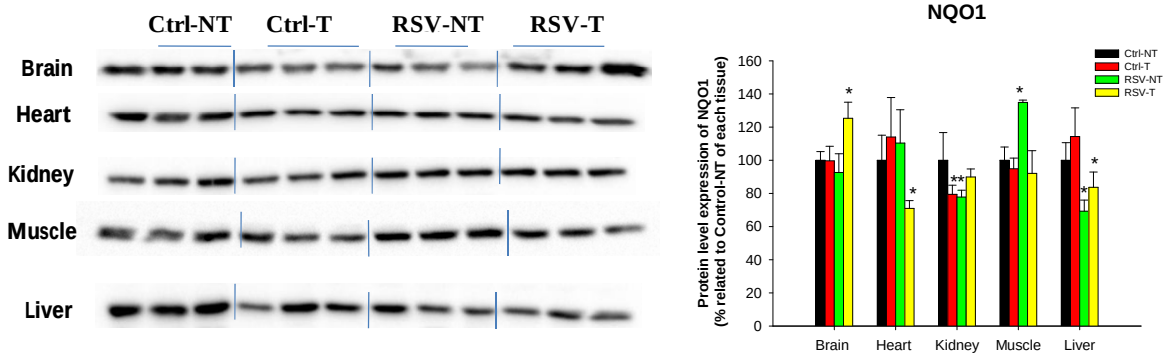


Figure IV.1.F *- Protein expression of NAD(P)H: Quinone Oxidoreductase 1 (NQO1) in difference tissue in old mice.*

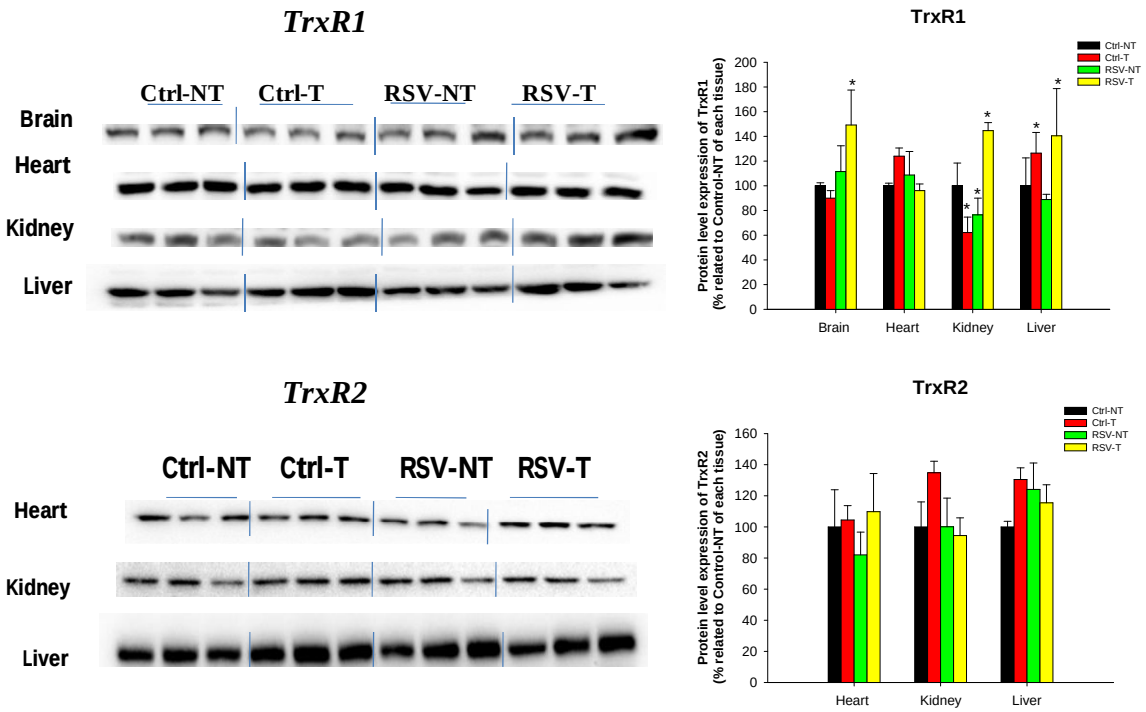


Figure IV.1.G *- Protein expression of Thioredoxin reductase (TrxR1 and TrxR2) in difference tissue in old mice.*

*Protein levels of antioxidant enzymes were determined by WB. Numbers under blots indicate the mean of density related to protein loading measured by densitometry of ponceau red staining of whole proteins. *Significant difference vs. Control-NT levels, $p < 0.05$ (n=3).*

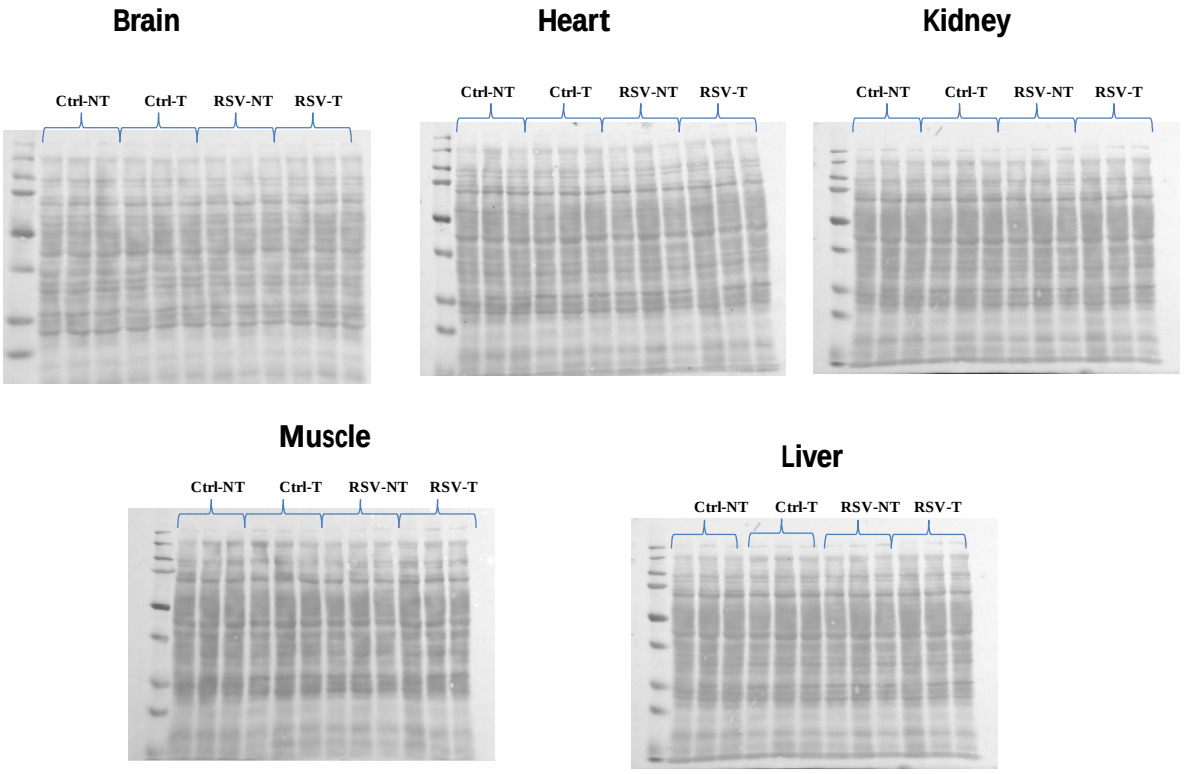


Figure IV.2. *Representative of ponceau red staining of whole proteins from brain, heart, kidney muscle and liver of old mice.*

Discussion

It is considered that the deleterious and irreversible changes produced by free radicals throughout the life of the organism are the main factors involved in aging. Thus, free oxygen radicals have been proposed as important causative agents of aging. In fact, the theory of aging by Harman in 1956 postulates that aging changes are caused by reactions caused by free radical. Abundant evidences show that a variety of ROS and other free radical are truly involved in the occurrence of molecular damage, which can lead to structural and functional disorders, diseases and death.

RSV has been shown to have potent antiaging and health-promoting activities. Furthermore, physical activity ameliorates age-related impairments by reducing the oxidative damage and improving antioxidant defense systems. Many antioxidants and antioxidant activities are involved in the protection against oxidative damage. These endogenous enzymatic antioxidant defenses include CAT, SOD, $CytB_5Rase$, NQO1, glutathione, GPx, GR, GST and TrxR.

Lipid peroxidation is one of the main events induced by oxidative stress and is particularly active in biomembranes like mitochondria. Polyunsaturated fatty acids (PUFAs) are one family of the most important components of cell membranes in living systems. Free radicals attack PUFAs leading to the formation of highly reactive electrophilic aldehydes, including MDA, 4-hydroxy-2-nonenal (HNE), and the most abundant products. The Nohl´s study has reported accumulation of lipid peroxidation products during aging (Nohl, 1993). Accordingly with this study, we have found that MDA levels increase along aging in mice liver. In this chapter we also found that RSV and/or exercise can protect these membranes in muscle, heart and liver in old mice indicating the protective activity of RSV and/or exercise against lipid peroxidation.

On the other hand, glutathione, which is the most important low molecular antioxidant, is a tripeptide antioxidant used by a variety of enzymes in xenobiotics' detoxification. It conjugates to toxic electrophilic substrates to render them less harmful and to facilitate their removal from cells. Furthermore, it covalently binds to proteins in response to stress or in the regulation of normal cellular processes (Dalle-Donne et al., 2009). In our experiments, RSV significantly incremented glutathione levels all five studied organ and furthermore in heart, muscle and liver by exercise.

Regarding enzymatic antioxidant, we have found that RSV and/or exercise can affect them in different way depending on the organ affected. Taken into consideration the different roles and locations of each enzyme, this effect can reflect the adaptative mechanisms of these organs against the mild oxidative stress induced by exercise or to the regulation by RSV. Our results indicate that the activity and also the level protein´s expression of a series of enzyme antioxidant CAT, NQO1, GPx, GR and SOD1 were increased in old mice by RSV, exercise and its combination. Our results

agree with previous work by Wong et al. which showed that long-term RSV intake attenuates oxidative damage in tissues specially affected during aging such as liver, heart or kidney (Wong et al., 2009). Moreover, the previous study of Thirunavukkarasu and coworkers showed that exercise increases glutathione-dependent activities (Thirunavukkarasu et al., 2003). Similarly, in the present study, administration of RSV and practicing exercise appears to have improved the activity of GPx in old mice.

In conclusion, our results indicate that both, RSV and exercise improve in different manner of activities of endogenous antioxidant enzymes such as CAT, SOD1, GPx, GR, GST, NQO1 in old mice, preventing the decrease of these activities associated with aging. Consequently, taking RSV and practicing exercise should be strongly encouraged in older people, for not only improves physical function and the maintenance of independence, but may also attenuate damage oxidative cause by aging.

CHAPTER V

CHAPTER V

ANALYSIS OF COENZYME Q LEVEL AS AN EFFECT OF AGING AND MODULATION BY RESVERATROL AND EXERCISE IN MICE

Abstract

Coenzyme Q (CoQ) is a ubiquitous and endogenous lipid-soluble antioxidant, which is found in all organisms and plays a crucial role in the cellular redox state. Particularly, CoQ may play a role in the aging process, and its levels may decrease with age. The natural polyphenol resveratrol (RSV) may also increase CoQ expression in skeletal muscle, liver, heart, and kidney mitochondria. As RSV, exercise training also elicits a broad range of effects, including increased anti-oxidant capacity, and may have anti-inflammatory effects. In this study, we examine whether exercise training and/or RSV therapy can increase tissue-specific levels of CoQ in mice. We allocated mice to 3 different age groups (young, mature, and old) and treated them with RSV over the course of 6 months, with administration of exercise training during the last 6 weeks. We determined the effects of RSV and exercise on the levels of CoQ_9 and CoQ_{10} in brain, liver, and muscle tissue. Our results demonstrated that, in response to aging, levels of CoQ_9 and CoQ_{10} decreased in brain but increased in liver and muscle. Treatment with RSV increased the levels of both, CoQ_9 and CoQ_{10} in brain in the three age groups. This effect was also found in young and old animals after exercise. Levels of CoQ_9 and CoQ_{10} in liver increased in the young and old age groups following RSV treatment. In muscle, CoQ_9 increased in the old group resulted from both RSV and exercise training, while CoQ_{10} increased only in the RSV-treated group. Moreover, we have found that the CoQ_9/CoQ_{10} ratio in brain tissue was stable, and was not affected by age or the administration of RSV or exercise training. In liver and muscle, the CoQ_9/CoQ_{10} ratio was lower in the young group than the old group. The liver-specific CoQ_9/CoQ_{10} ratio was not affected by RSV or exercise training in the old age group; however, in this old mice group, the combination of RSV and exercise, or RSV alone, reduced the muscle-specific CoQ_9/CoQ_{10} ratio compared with the control group. Our data demonstrate that a regimen of RSV treatment and exercise enhances the levels of CoQ but their affect is organ-dependent and may have relevant implications in their anti-aging abilities in mice.

Introduction

Aging is commonly defined as progressive deleterious alterations in various organs and tissues. According to the theory of free radicals in aging, first introduced by Harman in 1956, the biological systems involve oxidative stress originating as a result of an imbalance between the generation of oxidizing species and cellular antioxidant defense (Harman, 1956). This can cause damage to all cellular macromolecules, including proteins, DNA, and lipids, thus leading to the cellular degeneration and damage related to aging. This theory has led to the suggestion that antioxidants such as Coenzyme Q (CoQ) may play a role in the prevention of the aging process. However, it has been reported that CoQ may be decreased by aging, at least in some tissue (Bentinger et al., 2010).

CoQ (2,3-dimethoxy-5-methyl-6-multiprenyl-1,4-benzoquinone) is composed of a tyrosine-derived quinone ring, linked to a polyisoprenoid side chain, consisting of 9 or 10 subunits in higher invertebrates and mammals (Crane, 2001; Hargreaves, 2003). CoQ is an endogenous enzyme cofactor that is produced in most living cells in humans, is distributed in cellular membranes, and is an essential component of the mitochondrial respiratory chain. It is only lipid-soluble antioxidant that animal cells synthesize de novo. CoQ is a redox molecule and then, can exist in reduced CoQ and oxidized CoQ forms in the biological tissues. The major form of CoQ found in the living organism is the reduced form, ubiquinol ($CoQH_2$), which is primarily responsible for the antioxidant properties of CoQ. This molecule also plays a crucial role in cellular metabolism, acting as the electron carrier between complexes I and II and the complex III of the mitochondrial respiratory chain; regulating uncoupling proteins, the transition pore, β-oxidation of fatty acids, and nucleotide synthesis pathway. CoQ is considered as a central molecule in the maintenance of an antioxidant system for protecting membranes from peroxidation (Ebadi et al., 2001). It occupies a privileged position because it links basic aspects of cell physiology such as energy metabolism, antioxidant protection, and the regulation of cell growth and death (Bentinger et al., 2010; Turunen et al., 2004). CoQ can inhibit lipid peroxidation by preventing the production of lipid peroxyl radicals ($LOO^{\cdot}$) and, moreover, $CoQH_2$ reduces the initial perferryl radical, with concomitant formation of ubisemiquinone and H_2O_2. This quenching of the initiating perferryl radicals, which prevent propagation of lipid peroxidation, protects not only lipids, but also proteins from oxidation. CoQ is also involved in inflammation via its antioxidant/radical-scavenging activity. CoQ also exerts anti-inflammatory properties via NFκB-dependent gene expression. CoQ_{10} reduces the production of proinflammatory mediators (IL-6 and prostaglandin E2, TNF-α).

The involvement of mitochondria both as producers and as targets of cellular free radicals has been the basis for the mitochondrial theory of aging. This theory of aging considers somatic mutations of mitochondrial DNA induced by oxygen radicals as the primary cause of energy decline (Lenaz et al., 2000). The knowledge of important role of CoQ in mitochondrial function has led others to propose that CoQ may play a role in the aging process (Lenaz et al., 1999). Many studies have shown that CoQ levels generally decline with aging (Ernster and Dallner, 1995). However, CoQ distribution is not uniform among the various tissues and organs, indicating that Q levels are adapted to the particular physiology of the tissue. In mice, rats, and humans, maximal CoQ concentrations are present in kidney and heart, whereas lower amounts can be detected in liver, brain, and skeletal muscle (Aberg et al., 1992; Lass et al., 1999). It has been shown that during aging, decreases in CoQ content may occur in mitochondria of liver, heart, kidney, skeletal muscle and brain tissues in mice and rats.

A series of studies showed that RSV has anti-oxidant properties, anti-inflammatory properties, and anticancer activity. RSV induces multiple genes expression mimicking caloric restriction, which can extend the lifespan of model organisms and protect against aging-related diseases (Chung et al., 2012). Previous investigations have also studied putative changes in Q levels with CR. It has been reported that long-term CR increases total CoQ in mitochondria from skeletal muscle, liver, heart, and kidney (Lass et al., 1999). So, in this study we hipothetize that RSV also could increase CoQ in some tissue.

Numerous studies have shown that performing a minimum amount of exercise decreases the risk of death, prevents the development of certain cancers, lowers the risk of osteoporosis, increases cognition, improves blood sugar regulation and increases longevity. Exercise training prevents and restores age-related impairment. Exercise also significantly diminished the risk of death in elderly people (Buchman et al., 2012). In addition to its general effect on the physiology of the organism, exercise can also affect the levels of CoQ (Bentinger et al., 2010). Furthermore, in rats exercised until exhaustion, the concentration of Q and α-tocopherol increase in skeletal muscle mitochondria (Quiles et al., 1999).

Several studies have indicated that the beneficial effects of exercise may be further enhanced with RSV supplementation, specifically in skeletal muscle. The combination of exercise and RSV has been shown to attenuate age-related impairment. In a model of aging, senescence accelerated prone mice placed on a 12-week exercise regime with RSV supplementation exhibited increases in running endurance capacity, oxygen consumption, and mitochondrial function compared to those on

exercise alone (Murase et al., 2009). Then, the objective of the present study was to evaluate the effects of age on CoQ content in various tissues of the mouse, also the effect of RSV and/or exercise on the content of CoQ.

Results

We have quantified both CoQ_9 and CoQ_{10} levels in whole homogenates of liver, brain, and muscle of three groups of aged experimental animals by method HPLC using detector UV and electrochemical (Figure V.1). The CoQ_9/CoQ_{10} ratio has been reported to correlate negatively with life expectancy in mammals, although its exact role has not been determined (Lass et al., 1999). Interestingly, calorie restriction, the most prominent experimental manipulation that increases lifespan, results in a decrease in the CoQ_9/CoQ_{10} ratio in liver plasma membrane of both rats and mice (De Cabo et al., 2004; López-Lluch et al., 2005). Thus, we calculated CoQ_9/CoQ_{10} ratios in different mouse tissues. The total level of CoQ (reduced and oxidized forms of both CoQ_9 and CoQ_{10}) was also calculated.

CoQ_9 and CoQ_{10} levels in brain.

Our data showed that with age, the level of CoQ_9 decreased in brain as expected. A significant decrease of CoQ_9 in old mice was found with respect to young mice animals. Furthermore, in young mice, CoQ_9 levels increased significantly with exercise and RSV. In addition, CoQ_9 levels increased significantly by RSV in mature group, while there were no effects of exercise. In old mice, we found that both exercise and RSV can increase CoQ_9 levels.

With increasing age, CoQ_{10} levels do not change significantly. In the young group, CoQ_{10} levels increased with exercise and RSV. In the mature group, CoQ_{10} levels increased significantly with RSV, but not with exercise. In old mice, we found that exercise and RSV can increase CoQ_{10} levels.

We found that the ratios of CoQ_9/CoQ_{10} are quite constant with total CoQ_9 approximately five fold higher than total CoQ_{10} in brain (Figure V.2).

CoQ_9 and CoQ_{10} levels in liver.

The liver is on a central position in the regulation of mammalian metabolism. Liver tissues involved in detoxification have extraordinarily high concentrations of ubiquinol, may protect them from radicals escaping from p450.

We found surprisingly that with age, the level of CoQ_9 in mature group and old group is higher significantly as compare with young group. In young group, the CoQ_9 level has increased significantly by exercise, RSV and its combination. Nevertheless, the CoQ_9 level has decreased

significantly by exercise, RSV and its combination in mature group. In old group, we have found that exercise reduced the CoQ_9, but RSV increased it.

We also found surprisingly that by effect of age, the level of CoQ_{10} in old group is higher significantly as compare with mature group and young group. In young group, the CoQ_{10} level tends to increase, even not significantly, by exercise and RSV. However, the CoQ_{10} level has decreased significantly by exercise, RSV in mature group. In old group, we have found that exercise reduced the CoQ_{10}, but RSV tends to increase it.

We also have found the ratios of CoQ_9/CoQ_{10} are decreased in the young group as compared with old group. However, the ratio CoQ_9/CoQ_{10} seems to be not changed by RSV or exercise in old group (Figure V.3).

CoQ_9 and CoQ_{10} levels in muscle.

We found surprisingly that by effect of age, the level of CoQ_9 in old group higher significantly as compare with mature and young group. In young group, the CoQ_9 level has not affected significantly by exercise, RSV and its combination. Interestingly, the CoQ_9 level has increased significantly by exercise, RSV in mature group. In old group, we also have found that exercise and increased the level of CoQ_9, but not by RSV or combination of both.

Interestingly the level of CoQ_{10} is not affected by effect of age. The level of CoQ_{10} in old group is higher as compare with mature group, but similar in young group. In young group, the CoQ_{10} is slight decreased by RSV but not by exercise. And in mature group, the CoQ_{10} is increased by exercise and/or by RSV. The CoQ_{10} level has increased significantly by RSV but not by exercise in old group.

We also have found the ratios of CoQ_9/CoQ_{10} are decreased in the young group as compared with old group. Especially in old group, the combinations of RSV and exercise or RSV alone have reduced the ratio CoQ_9/CoQ_{10} as compared with group Control (Figure V.4).

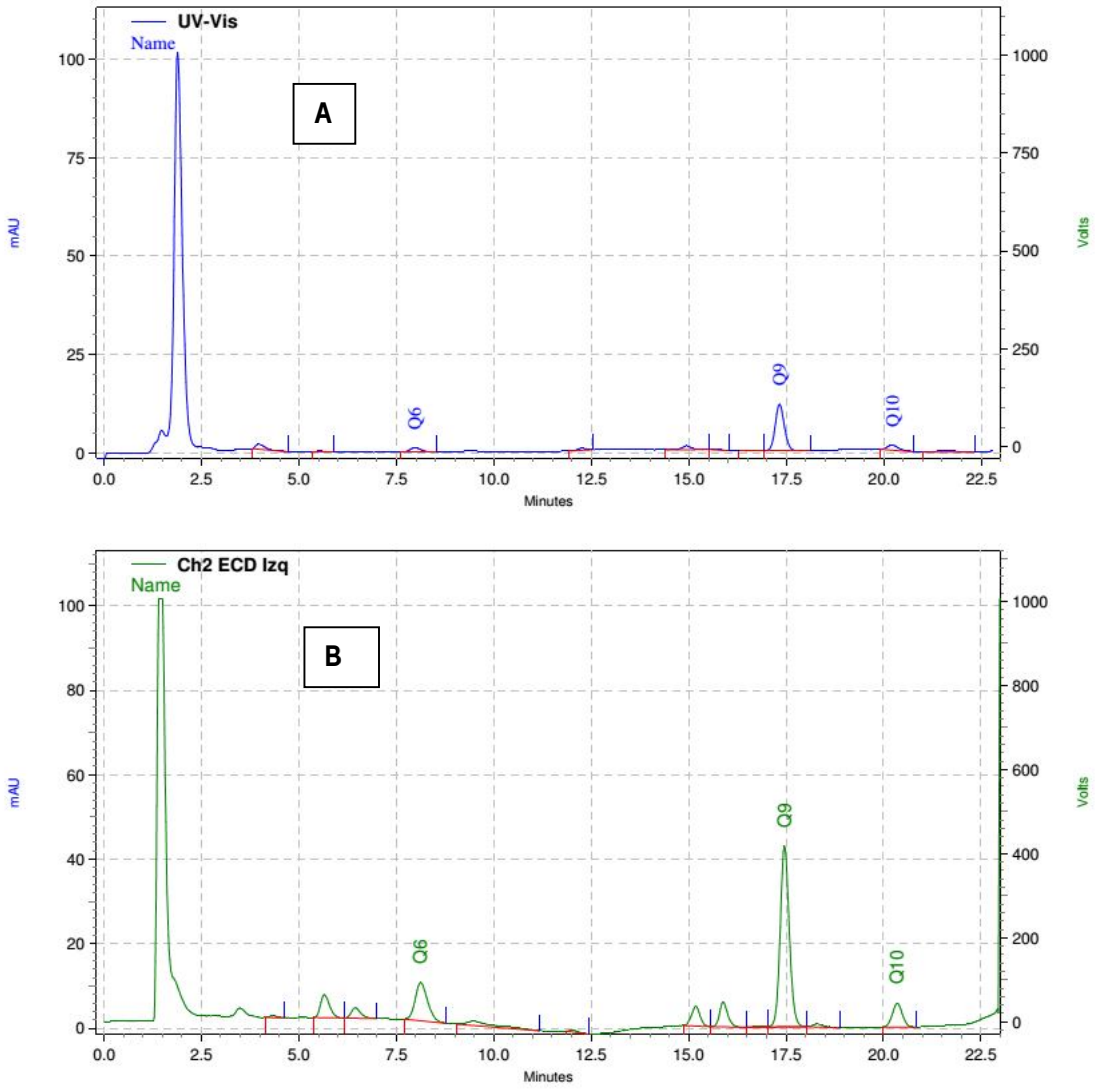


Figure V.1. *Representative diagrams from CoQ analysis performed by HPLC. A. Using ultraviolet (System Gold 168, Beckman) detectors; B. Using electrochemical (Coulochem III ESA; Esainc, Chelmsford, MA, USA) detectors.*

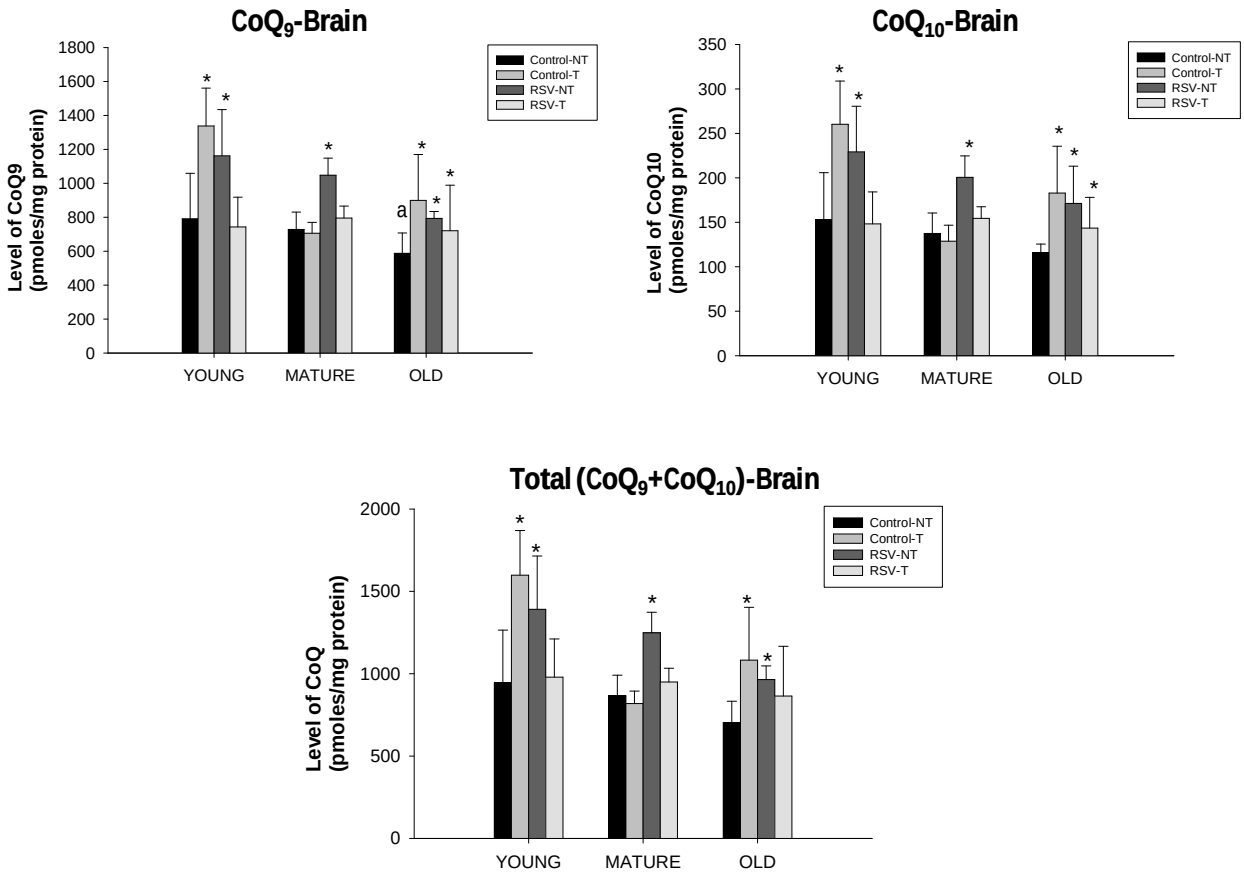


Figure V.2. *Tissue distribution of CoQ homologues in brain. Data are means ± SEM (n=4). [a]Significant difference vs. Control-NT level in young group [*]Significant difference vs. Control group in the respective aged group*

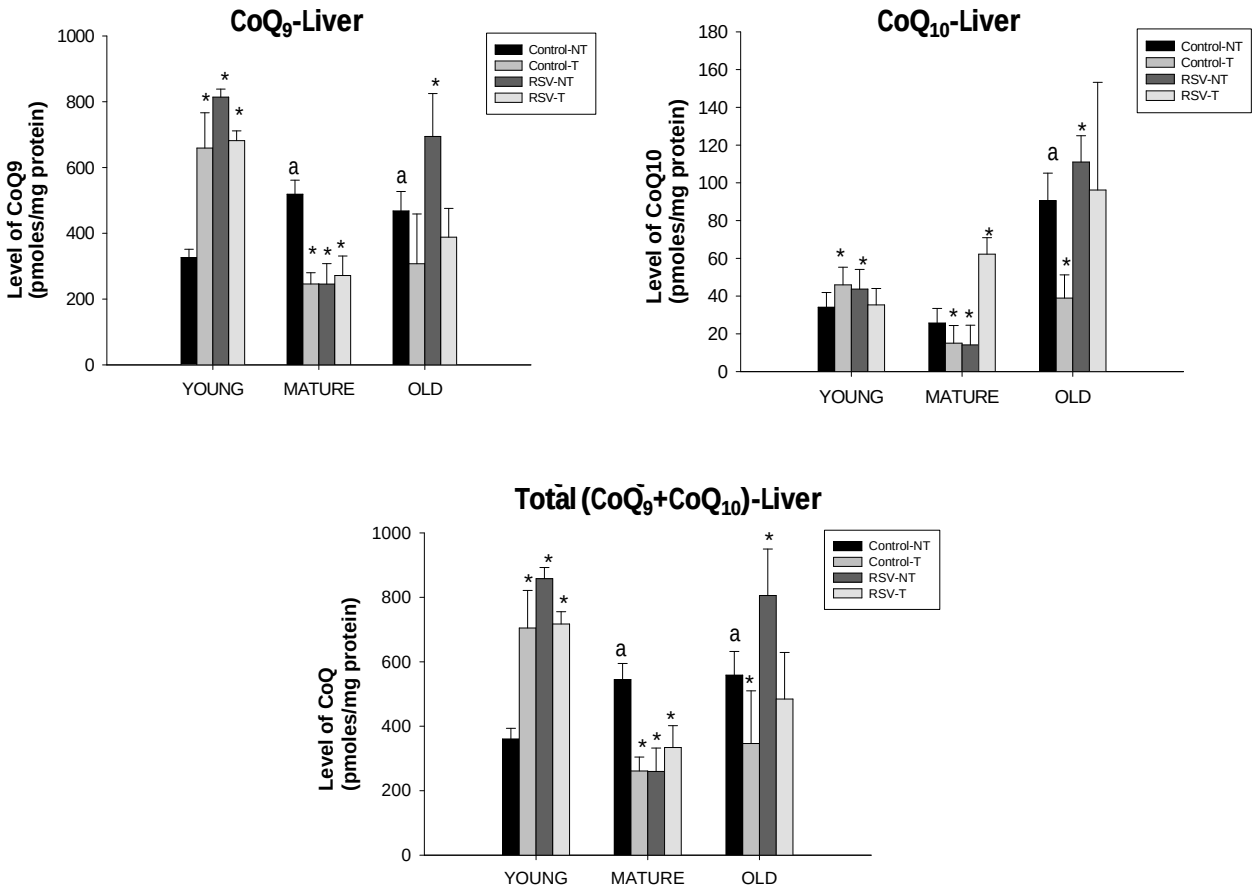


Figure V.3. *Tissue distribution of CoQ homologues in liver. Data are means ± SEM (n=4).* [a]*Significant difference vs. Control-NT level in young group* [*]*Significant difference vs. Control group in the respective aged group*

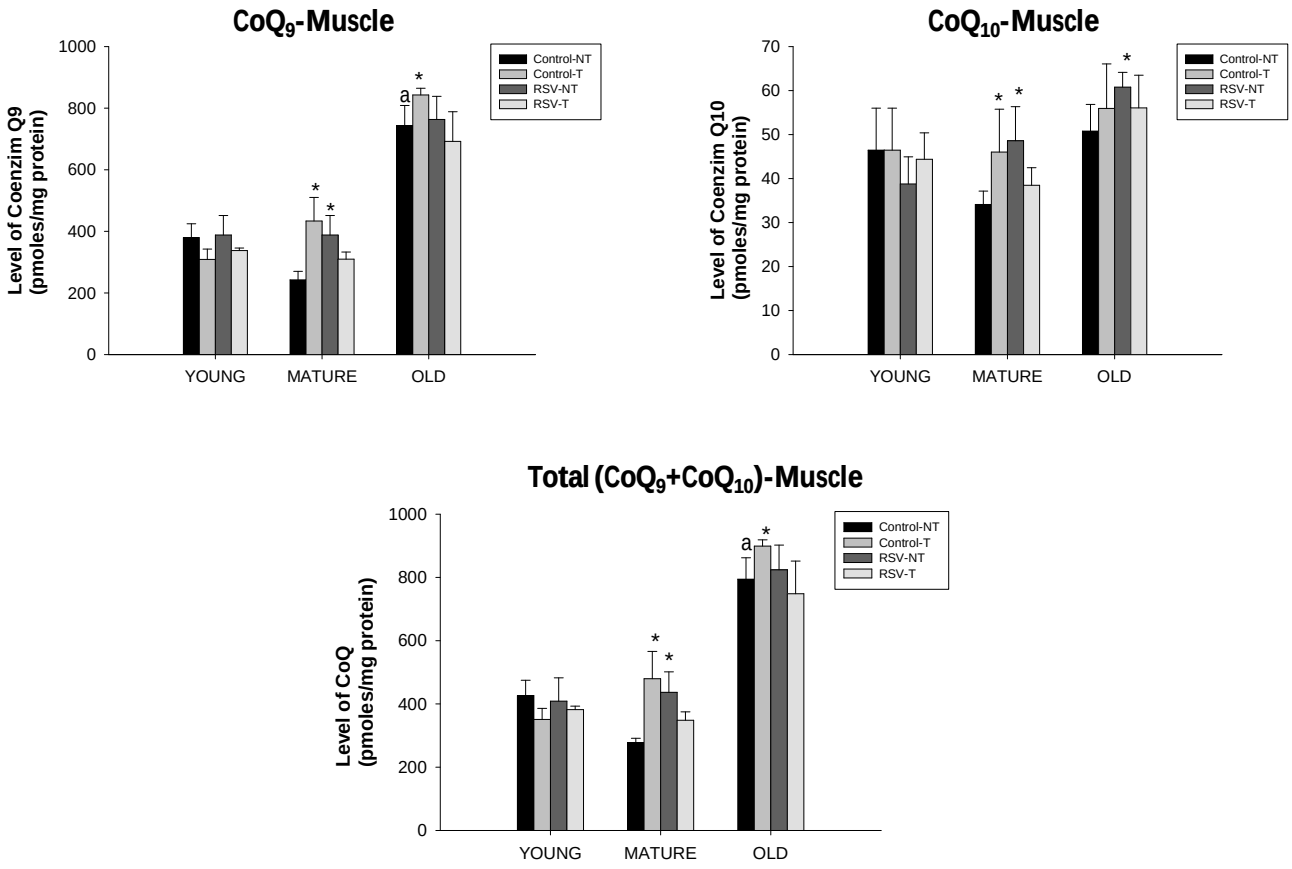


Figure V.4. *Tissue distribution of CoQ homologues in muscle. Data are means ± SEM (n=4). [a]Significant difference vs. Control-NT level in young group [*]Significant difference vs. control group in the respective aged group*

Discussion

CoQ is an endogenous compound that coexists in oxidized (ubiquinone) and reduced (ubiquinol) form. CoQ has important function in the mitochondrial electron transport chain as an electron carrier (Ernster and Dallner, 1995). Mice can synthesize both CoQ_9 and CoQ_{10}, which are differentiated by the length of their isoprenoid side chain. While the CoQ_9 is the major form in mouse, an appreciable amount of CoQ_{10} is present. CoQ distribution is not uniform in various tissues and organs, demonstrating that CoQ levels are adapted to the particular physiology of the tissue or organ. CoQ is rapidly broken down, due to its short half-life, allowing levels of Q in tissues to be determined by a coordinated balance between synthesis and degradation, both of which occur in all tissues. The results of this study have shown that the CoQ_9 and CoQ_{10} levels vary in different tissues and organs, whereby the highest concentration are found in brain, followed by muscle, with the lowest measured in liver. In the present study, decreases in CoQ levels in brain in old group were compared to young and mature group. The findings were in line with those reported by Kalen et al (1989) indicating that the tissue content of CoQ decreases during aging (Kalen et al., 1989), possibly due to the decline of energy metabolism. However, the present results have shown that in muscle and liver, CoQ levels increased in old and mature group as compared with young group of animals, indicating that CoQ levels are adapted to the particular physiology of the tissue. CoQ levels play a basic role in enzyme function and coupling (Boitier et al., 1998). We consider that the main function CoQ_9 is bioenergetics while CoQ_{10} act principally as an antioxidant against oxidative damage. CoQ_{10} can provide rapid protective effects against lipid peroxides and anti-oxidative effects and anti-aging properties at the skin level (Blatt and Littarru, 2011).

Moreover, the present study has revealed that RSV and/or physical activity produces different effects on CoQ content depending on the age and tissue type. It is worth noting that, in the present study, the concentration of CoQ was affected positively by training in the group of young mice, and less significantly in the mature group. However, the effect on the liver in old mice was negative.

The present study findings have also revealed that the CoQ_9 levels in muscle in the old group were significantly higher than those measured in the mature and young group. This data may be contrasted with earlier reports, which had shown that the level of CoQ_9 in mitochondria in skeletal muscle decreases during aging, whereas it is not affected in kidney, brain or heart in mice (Bentinger et al., 2010; Lass et al., 1999). This difference can be attributed to the fact that, in the present study, the whole tissue homogeneous muscle was used in measurements, while the previous studies focused on mitochondria in the different organs.

Reports that CoQ supplementation reduces oxidative stress and increases antioxidant enzyme activity in patients with coronary artery disease (Lee et al., 2012), protect mitochondria from damage during neurodegenerative diseases (Henchcliffe and Beal, 2008; Shults et al., 2002), in human CoQ_{10} deficiencies (Quinzii et al., 2008). CoQ_{10} administration resulted in an increase in its amount only in the serum, liver homogenate, liver mitochondria, and kidney mitochondria, but not in the homogenates or mitochondria of brain, heart or skeletal muscle and also caused an elevation of the CoQ_9 in nearly all the tissues (Lass et al., 1999). Previous reports have shown that long-term CR increased the CoQ_9 content of mitochondria isolated from skeletal muscle (Lass et al., 1999), liver, heart, and kidney (Kamzalov and Sohal, 2004), although another study reported that CR induced a decrease in CoQ_9 and CoQ_{10} in liver mitochondria (Armeni et al., 2003; Parrado-Fernandez et al., 2011). CoQ also is increased under cold adaptation and with exercise (Bentinger et al., 2010). Also CoQ is an effective therapeutic agent in clinical congestive heart failure (Kumar et al., 2009). CoQ_{10} is also a critical adjuvant therapy for patients with cardiac diseases due to its beneficial effects on cellular bioenergetics, regulation of cell membrane channels and its antioxidant effect (Kumar et al., 2009). It is well known that the reactions of the electron transport chain of mitochondria produce damaging free radicals. Increases the level of CoQ with RSV and exercise may have a function in mitochondrial electron transport and oxidative phosphorylation. The increased levels of CoQ may act as an antioxidant and thus protect the inner mitochondrial membrane from damage inflicted by lipid peroxidation and free radical formation.

In conclusion, our study shows that levels of CoQ_9 and CoQ_{10} in tissue homogenates can be increased by RSV and exercise training in different manner. This data maybe support the benefits of RSV and exercise training in health in older people.

CHAPTER VI

CHAPTER VI

PROTEOMIC ANALYSIS OF THE EFFECT OF AGING AND MODULATION BY RESVERATROL AND EXERCISE ON MOUSE LIVER

Abstract

Aging process is consequence of the deleterious and irreversible change produced by free radicals throughout the life. Resveratrol (RSV), a naturally occurring phytoalexin, has been shown to have potent anti-aging and health-promoting capacity. It is also well known that physical activity ameliorates age-related impairments. In the present study, male C57BL/6J mice (n=48) allocated to 3 age groups (young, mature and old) were used to investigate the effects of RSV and physical activity on protein expression in the liver during aging. Each age group was further divided into 4 subgroups (control no-trained, control trained, RSV no-trained and RSV trained). RSV dose was 16.5 mg/kg/animal/day during 6 months and training was performed 6 weeks before sacrifice. The liver was collected and analyzed by proteomic 2D-DIGE and protein identification by MALDI-TOF/TOF mass spectrometry. We have detected intensity changes in several hundreds of spots and identified several spots from the gels. Our results showed that most of the differentially expressed proteins are associated with the electron transport chain, urea cycle, enzymes oxidoreductases, regulatory factors in mitochondrial protein synthesis, the aldehyde dehydrogenase family and antioxidant capacity. These results support the use of physical activity and/or RSV can be of great importance for the prevention of metabolic diseases and related to aging.

Introduction

Aging is usually associated with an increase in plasma free radical concentrations, mainly due to a decline in antioxidant mechanisms and a rise in pro-oxidant factors such as glucose and insulin concentrations (Mezzetti et al., 1996). It has been suggested that senescence may result from the accumulation of unrepaired structural damage to cells, which disrupts the cellular functions when the organism enters into contact with different endogenous and exogenous agents (Harman, 1984). The reactive oxygen species (ROS) have become an active field in aging research because of their potential involvement in many degenerative diseases. These ROS are highly reactive and capable of damaging many biological macromolecules such as DNA, RNA, protein and lipids (Feuers et al., 1993). Antioxidant enzymes constitute an important defense system to clear up the detrimental ROS in vivo.

Resveratrol (*trans*-3,4',5-trihydroxystilbene) (RSV) a naturally occurring phytoalexin that can be found in red wine, berries, and peanuts, has been shown to extend both mean and maximum life span in model organisms such as yeast and metazoans (Howitz et al., 2003; Wood et al., 2004). It has been shown to have a number of beneficial effects on cardiovascular diseases, including promotion of vasodilation, and prevention of oxidative damage and platelet aggregation (De La Lastra and Villegas, 2005). Recently, RSV has attracted great attention due to its anti-inflammatory, cell growth-modulatory and anticarcinogenic effects, and especially its potentially mimic caloric restriction in *Caenorhabditis elegans* (Collins et al., 2006; Viswanathan et al., 2005), *Drosophila melanogaster* (Wood et al., 2004) and mice (Baur et al., 2006). This molecule produces changes associated with longer lifespan, including increased insulin sensitivity, reduced insulin-like growth factor-1 levels, increased AMP-activated protein kinase and peroxisome proliferator-activated receptor-γ coactivator 1α activity, increased mitochondrial number, and improved motor function (Baur et al., 2006). Moreover, RSV is a potent activator of sirtuin 1 in vitro (Borra et al., 2005; Howitz et al., 2003), and also regulates the expression of various genes involved in inflammation, cytoprotection, and carcinogenesis (Holmes-McNary and Baldwin Jr, 2000; Manna et al., 2000). Therefore, RSV may improve the health and prolong the average lifespan of mammals that suffer from age.

It has been known for some time that physical activity (i.e., exercise) is associated with a better health by benefiting the cardiovascular system. Exercise may lead to an increase in physiological antioxidant defenses of the organism in old subjects. It reduces the production of oxidants and oxidative damage, improve antioxidant defense system, and increase the resistance of organs and

tissues against the deleterious action of free radicals (Polidori et al., 2000). Furthermore, the physical activity level correlates closely with antioxidant enzymatic activities, especially on GSH in liver and brain (Yamamoto et al., 2003). However, some studies (Reid, 2008; Sureda et al., 2009) have showed evidence that physical exercise - particularly if too intense - is associated both to muscular damage and high formation of free radicals.

Proteomic approach is an efficient method to screen for differences in protein expression as well as to identify new proteins in a particular tissue, which can be associated with specific conditions such as drug treatment, diseases or nutritional status. The main objective of this study was to investigate the effects of RSV and physical activity on protein expression in the liver during aging progress. The liver was investigated as the principal target due to the role of this organ in energetic and xenobiotic metabolism of drugs. To this end 2D differential gel electrophoresis (2D-DIGE) analysis coupled with mass spectrometry protein identification was performed in order to identify the hepatic proteins differentially expressed.

Results

We used homogenate liver for comparison by 2D-DIGE. Three biological replica of the three group of age were run in 24 independent gels, each containing two experimental samples labeled with Cy3 and Cy5 and an internal standard comprised of pooled samples from all strains labeled with Cy2. Presence of the internal standard allowed normalization of individual protein abundance between samples from different gels. Using DeCyder v 7.0 software we could detect 954 protein spots across 75% of gels images. Gel images of liver protein extracts are shown in Figure VI.1.

Figure VI.2, 3, 4, and 5 are shown the protein expression statistically significant differences in intensity between the group control by aging, the group young, mature and old animals by RSV treatment and exercise training.

Table VI.1 lists the DIGE-identified proteins that exhibited 1.3 or more change significantly (Student'st-test, $p \leq 0.05$) of expression in the mice liver by aging.

Table VI.2, 3 and 4 lists the DIGE-identified proteins that exhibited 1.3 or more change of expression in the mice liver by effect of physical activity and/or RSV in young, mature and old group, respectively.

By aging, we found 16 proteins showed a significantly difference with p-value less than 0.05, identification was succesfully obtained for 11 proteins, some protein decreased significantly: dimethylglycine dehydrogenase, phosphoglucomutase-1, selenium-binding protein 2, aldehyde dehydrogenase, ornitine aminotransferase, arginase-1, dihydrodiol dehydrogenase, 3-hydroxibutyrate dehydrogenase, and peroxiredoxin 1 whereas other increased significantly by age: serum albumin and peroxiredoxin 4.

In young group, by effect of physical activity, we found 10 proteins showed significantly difference and we were able to identify 2 down-regulated: Arginase-1 and aldehyde dehydrogenase X and whereas 2 up-regulated: phosphoglucomutase-1 and cysteine sulfinic acid decarboxylase. By effect of RSV treatment, we found 12 proteins showed significantly difference and 11 proteins were identifided. Some of them were increased by RSV: the protein mitochondrial inner membrane protein (mitofilin), NADH-ubiquinone oxidoreductase 75 kDa subunit, cytochrome b-c1 complex subunit 1, ATP synthase subunit beta; 3-mercaptopyruvate sulfurtransferase, 3-hydroxyanthranilate 3; 4-dioxygenase whereas glycogen phosphorylase, dimethylglycine dehydrogenase, selenium-

binding protein 2, selenium-binding protein 1, aldehyde dehydrogenase X were down-regulated. Furthermore, cysteine sulfinic acid decarboxylase was also increased by combination of both RSV and exercise.

In mature group, by effect of exercise, we found 10 proteins showed significantly difference and we were able to identify 2 of them. By effect of RSV, we found 9 proteins showed significantly difference and we were able to identify 2 of them. And by combination of RSV and exercise, we identified 1 protein from 3 proteins showed significantly difference. We have identified some protein was decreased by exercise: aldo-keto reductase family 1 member C13. The succinate dehydrogenase flavoprotein subunit was decreased by both exercise and RSV. Aldo-keto reductase family 1 member C13 was increased by RSV. And the protein expressed sequence AU018778 (protein Ces1f) was decreased by combination of both.

In old group of mice, by effect of exercise, we found 6 proteins showed significantly difference and we were able to identify 1 of them. By effect of RSV, we found 9 proteins showed significantly difference and we were able to identify 3 of them. And by combination of RSV and exercise, we identified 1 protein from 4 proteins showed significantly difference. We were able to identify 1 up-regulated protein: epoxide hydrolase 2. By effect of RSV treatment, we found epoxide hydrolase 2, ketohexokinase, Rho GDP-dissociation inhibitor were up-regulated. Also by effect of combination of both RSV with exercise, we found protein ketohexokinase was up-regulated.

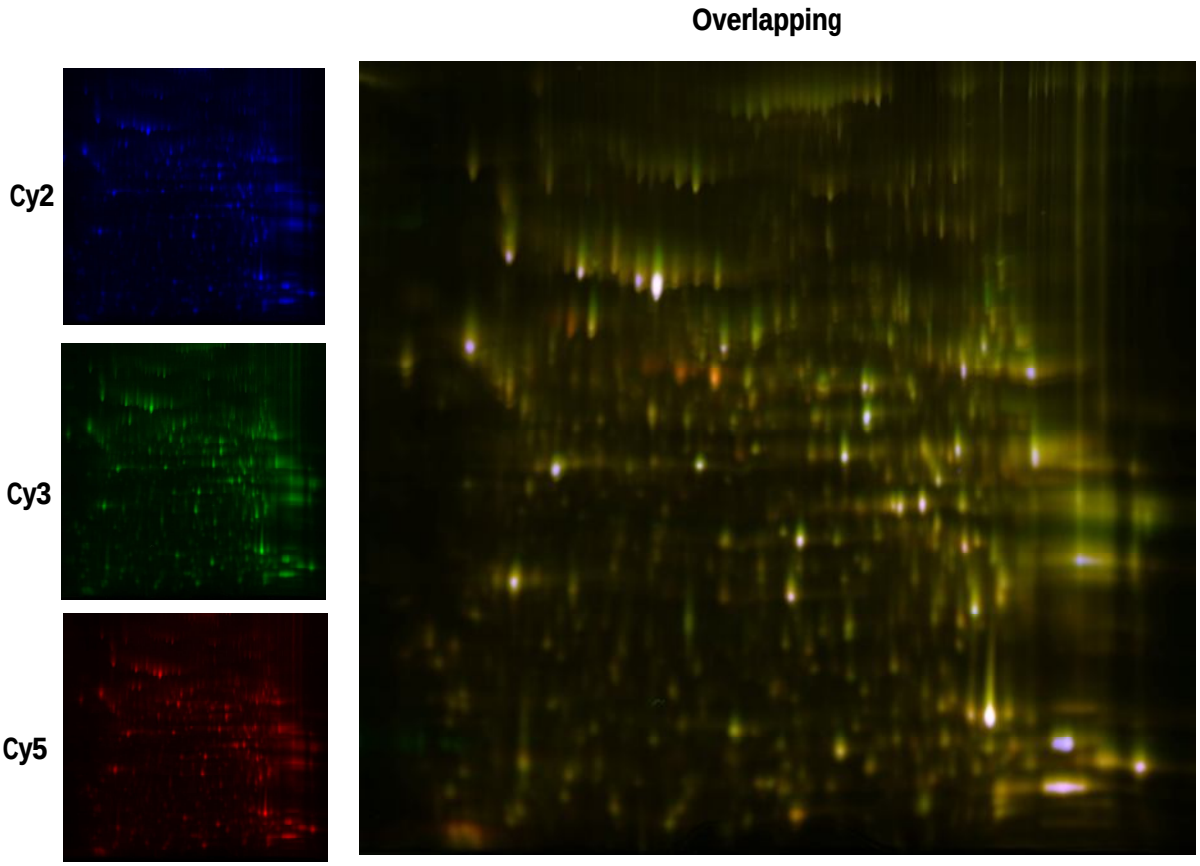


Figure VI.1. *Proteome analyses of mice liver. Representative images of Cy2, Cy3, Cy5 and the overlap of the 3 dyes of 2-D gels from mice liver.*

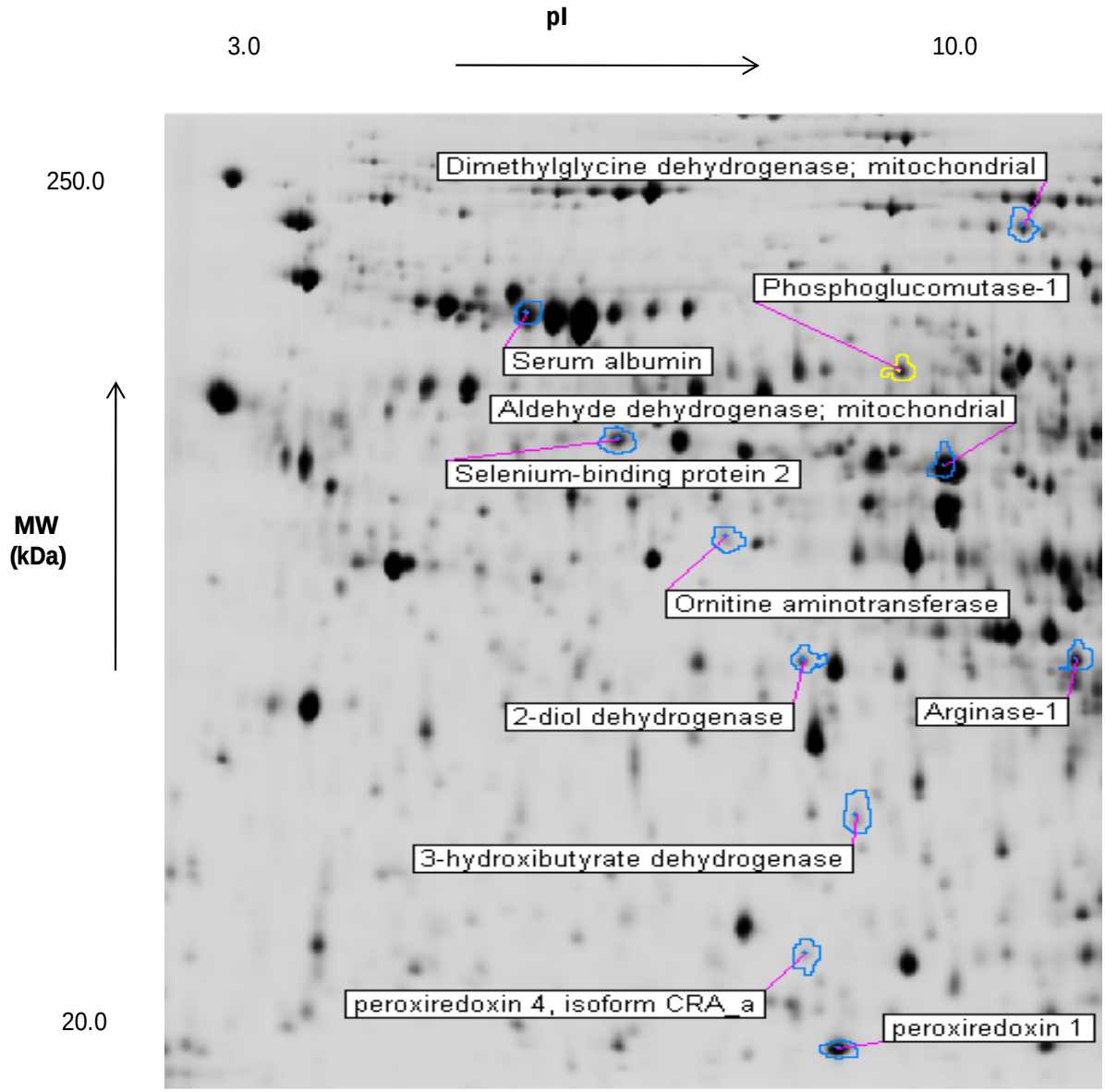


Figure VI.2. *A typical 2D-pattern (Cy2-labelled) gel image of a 50 µg protein extract separated in a 24 cm, pH 3–10 NL IPG strip in the first dimension and 10% polyacrylamide gel in the second. Spots that showed statistically significant differences in intensity between the control group animals by age are marked.*

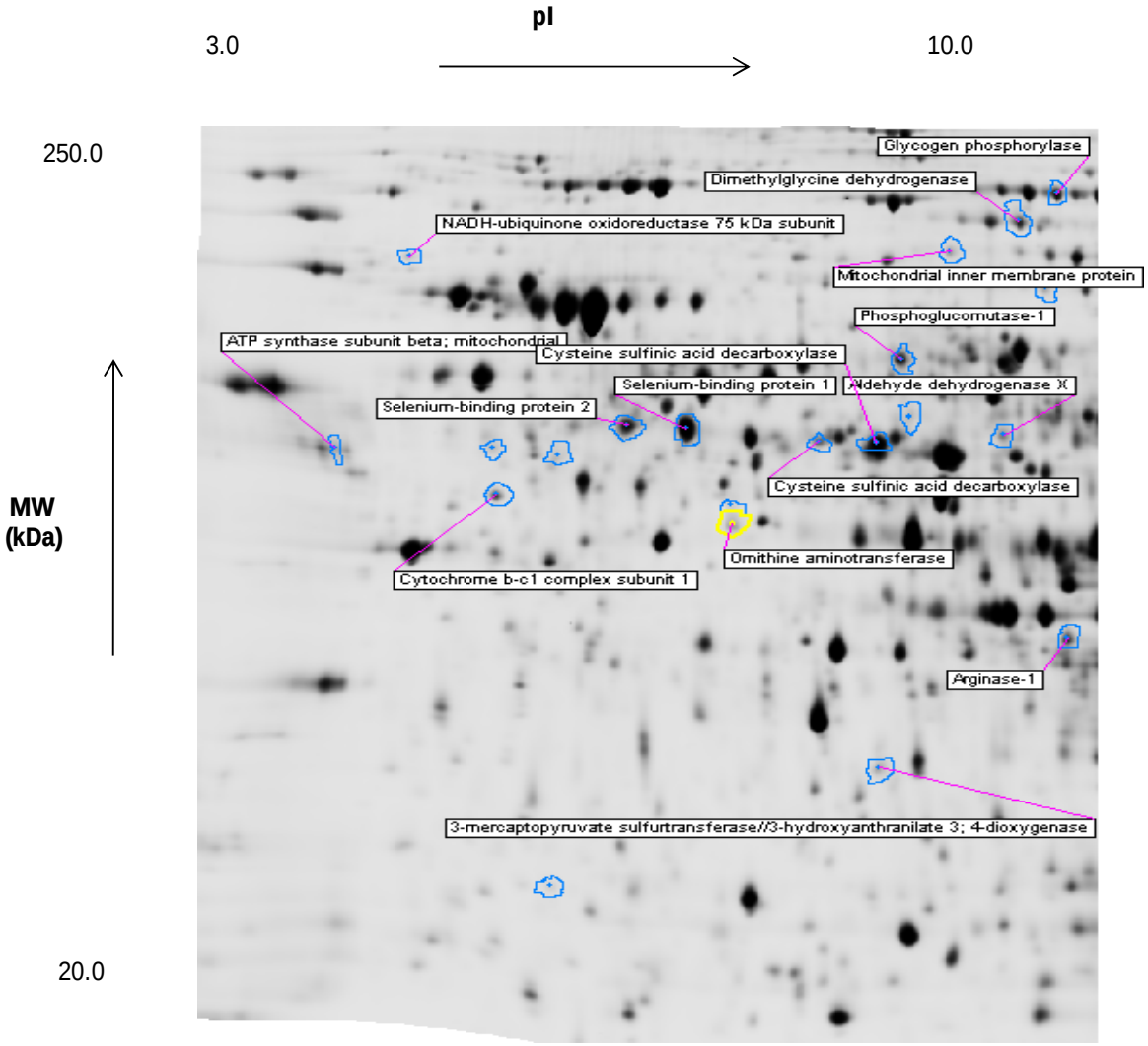


Figure VI.3. *A typical 2D-pattern (Cy2-labelled) gel image of a 50 µg protein extract separated in a 24 cm, pH 3–10 NL IPG strip in the first dimension and 10% polyacrylamide gel in the second. Spots that showed statistically significant differences in intensity between the young group animals by RSV treatment and exercise training are marked.*

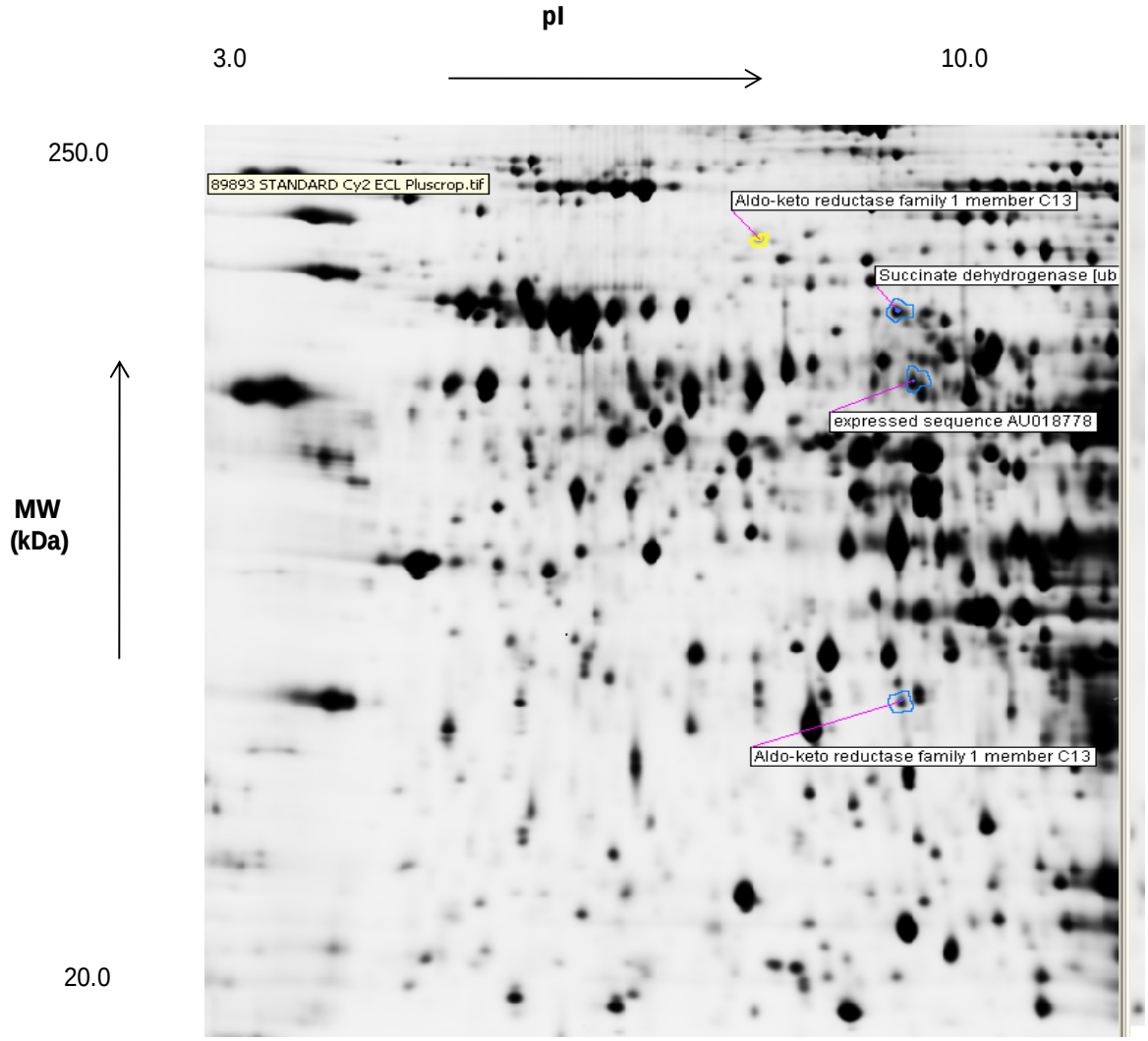


Figure VI.4. *A typical 2D-pattern (Cy2-labelled) gel image of a 50 µg protein extract separated in a 24 cm, pH 3–10 NL IPG strip in the first dimension and 10% polyacrylamide gel in the second. Spots that showed statistically significant differences in intensity between the mature group animals by RSV treatment and exercise training are marked.*

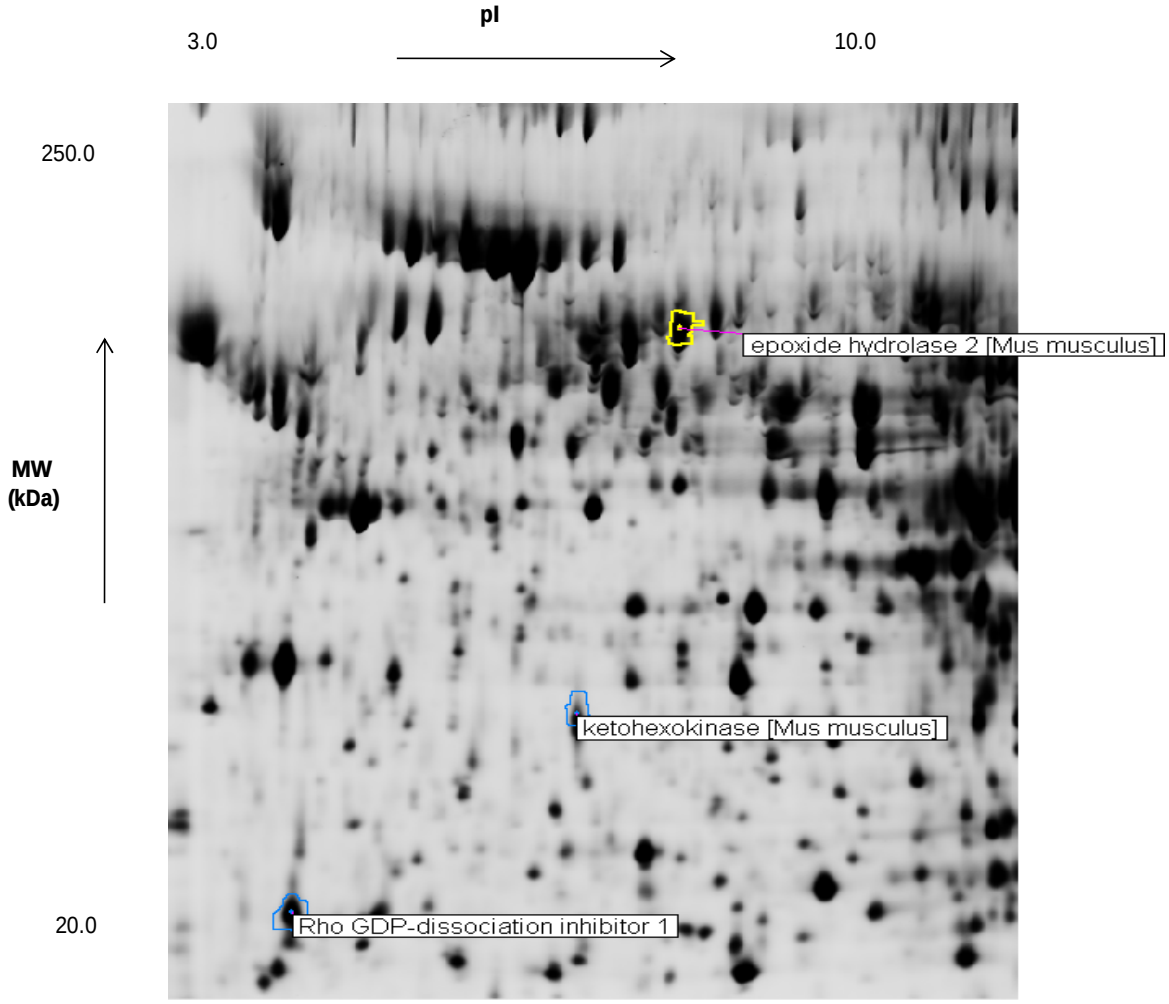


Figure VI.5. *A typical 2D-pattern (Cy2-labelled) gel image of a 50 µg protein extract separated in a 24 cm, pH 3–10 NL IPG strip in the first dimension and 10% polyacrylamide gel in the second. Spots that showed statistically significant differences in intensity between the old group animals by RSV treatment and exercise training are marked.*

TableVI.1. *Lists of protein identified that exhibited 1.3 or more change of expression in the mouse liver by aging process.*

Master No.	***Protein AC***	***Name***	***T-test***	***Av. Ratio***	***1-ANOVA***	***pI***	***Mw (kDa)***
Down regulated proteins by aging							
564	*Q63836*	*Selenium-binding protein 2*	*0.0060*	*-2.96*	*0.015*	*5.7*	*53*
758	*P29758*	*Ornitine aminotransferase*	*0.0041*	*-2.22*	*0.00039*		
926	*Q61176*	*Arginase-1*	*0.017*	*-1.85*	*0.00057*	*6.5*	*35*
143	*Q9DBT9*	*Dimethylglycine dehydrogenase; mitocondrial*	*0.0065*	*-1.75*	*0.0061*	*7.69*	*97*
930	*Q9DBB8*	*2-Diol dehydrogenase (Trans-1,2-dihydrobenzene-1,2-diol dehydrogenase)*	*0.037*	*-1.41*	*0.0078*	*6.03*	*36.6*
1147	*Q99L13*	*3-Hydroxibutyrate dehydrogenase*	*0.003*	*-1.39*	*0.0077*	*8.3*	*35*
425	*Q9D0F9*	*Phosphoglucomutase-1*	*0.018*	*-1.34*	*0.0075*	*6.1*	*61*
617	*P47738*	*Aldehyde dehydrogenase; mitochondrial*	*0.0021*	*-1.14*	*0.0054*	*7.5*	*57*
1428	*P35700*	*Peroxiredoxin 1*	*0.39*	*-1.1*	*0.0082*	*6.8*	*19*
Up regulated proteins by aging							
328	*P07724*	*Serum albumin*	*0.047*	*1.88*	*0.052*	*5.7*	*70*
1316	*O08807*	*Peroxiredoxin 4, isoform CRA_a*	*0.022*	*1.51*	*0.0076*	*8.1*	*32*

Protein abundance mapping by aging process

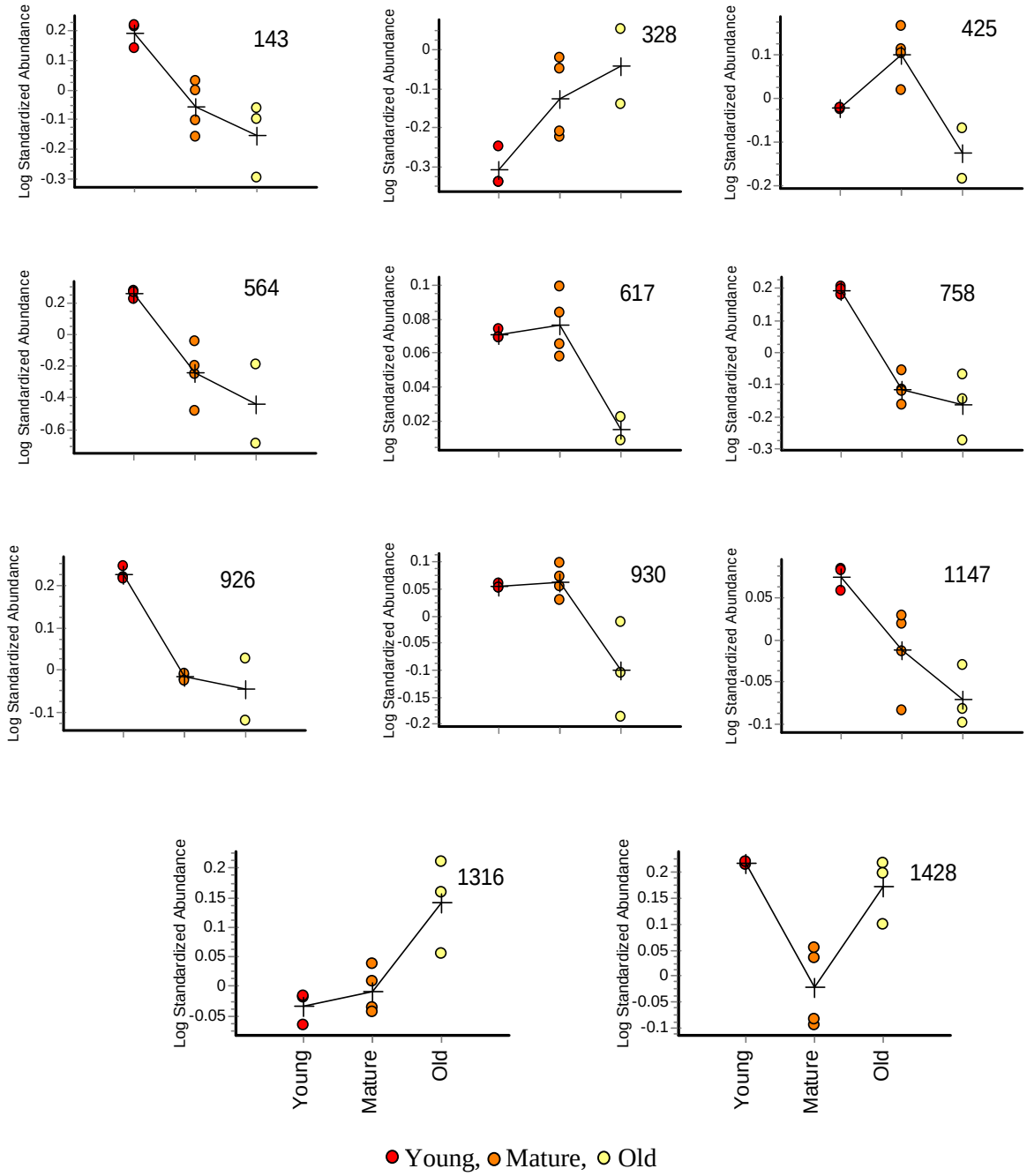


Table VI.2. *Lists protein identified that exhibited 1.3 or more change of expression in the mouse liver by effect of physical activity and/or RSV in young group*

Master No	***Protein AC***	***Name***	***T-test***	***Av. Ratio***	***1-ANOVA***	***pI***	***Mw (kDa)***
Down regulated proteins by exercise							
1156	*Q61176*	*Arginase-1*	*0.00033*	*-1.73*	*0.0036*	*6.5*	*34*
758	*Q9CZS1*	*Aldehyde dehydrogenase X*	*0.0085*	*-1.53*	*0.00059*	*6.5*	*58*
Up regulated proteins by exercise							
768	*Q9DBE0*	*Cysteine sulfinic acid decarboxylase*	*0.031*	*1.55*	*0.011*	*6.1*	*55*
774	*Q9DBE0*	*Cysteine sulfinic acid decarboxylase*	*0.019*	*1.45*	*0.0092*	*6.1*	*55*
602	*Q9D0F9*	*Phosphoglucomutase-1*	*0.00018*	*1.4*	*0.0066*	*6.1*	*61*
Up regulated proteins by combination of exercise with RSV							
774	*Q9DBE0*	*Cysteine sulfinic acid decarboxylase*	*0.033*	*1.42*	*0.0092*	*6.1*	*55*
Down regulated proteins by RSV							
742	*P17563*	*Selenium-binding protein 1*	*0.00013*	*-2.7*	*0.00096*	*5.8*	*53*
739	*Q63836*	*Selenium-binding protein 2*	*0.00012*	*-1.91*	*0.0027*	*5.7*	*53*
758	*Q9CZS1*	*Aldehyde dehydrogenase X*	*0.0062*	*-1.51*	*0.00059*	*6.5*	*58*
288	*Q9DBT9*	*Dimethylglycine dehydrogenase*	*0.0072*	*-1.43*	*0.0011*	*7.69*	*97*
226	*Q9ET01*	*Glycogen phosphorylase*	*0.0046*	*-1.42*	*0.0016*	*6.63*	*97*
Up regulated proteins by RSV							
895	*Q9CZ13*	*Cytochrome b-c1 complex subunit 1*	*0.0016*	*2.01*	*0.0046*	*5.8*	*53*
373	*Q91VD9*	*NADH-ubiquinone oxidoreductase 75 kDa subunit*	*0.002*	*1.76*	*0.0087*	*5.51*	*80*
1380	*Q99J99 Q78JT3*	*3-mercaptopyruvate sulfurtransferase 3-hydroxyanthranilate 3; 4-dioxygenase*	*0.0056*	*1.68*	*0.0012*		
782	*P56480*	*ATP synthase subunit beta; mitochondrial*	*0.00082*	*1.66*	*0.0099*	*5.19*	*56*
370	*Q8CAQ8*	*Mitochondrial inner membrane protein (Mitofilin)*	*0.0013*	*1.42*	*0.015*	*6.1*	*84*

Protein abundance mapping of young group

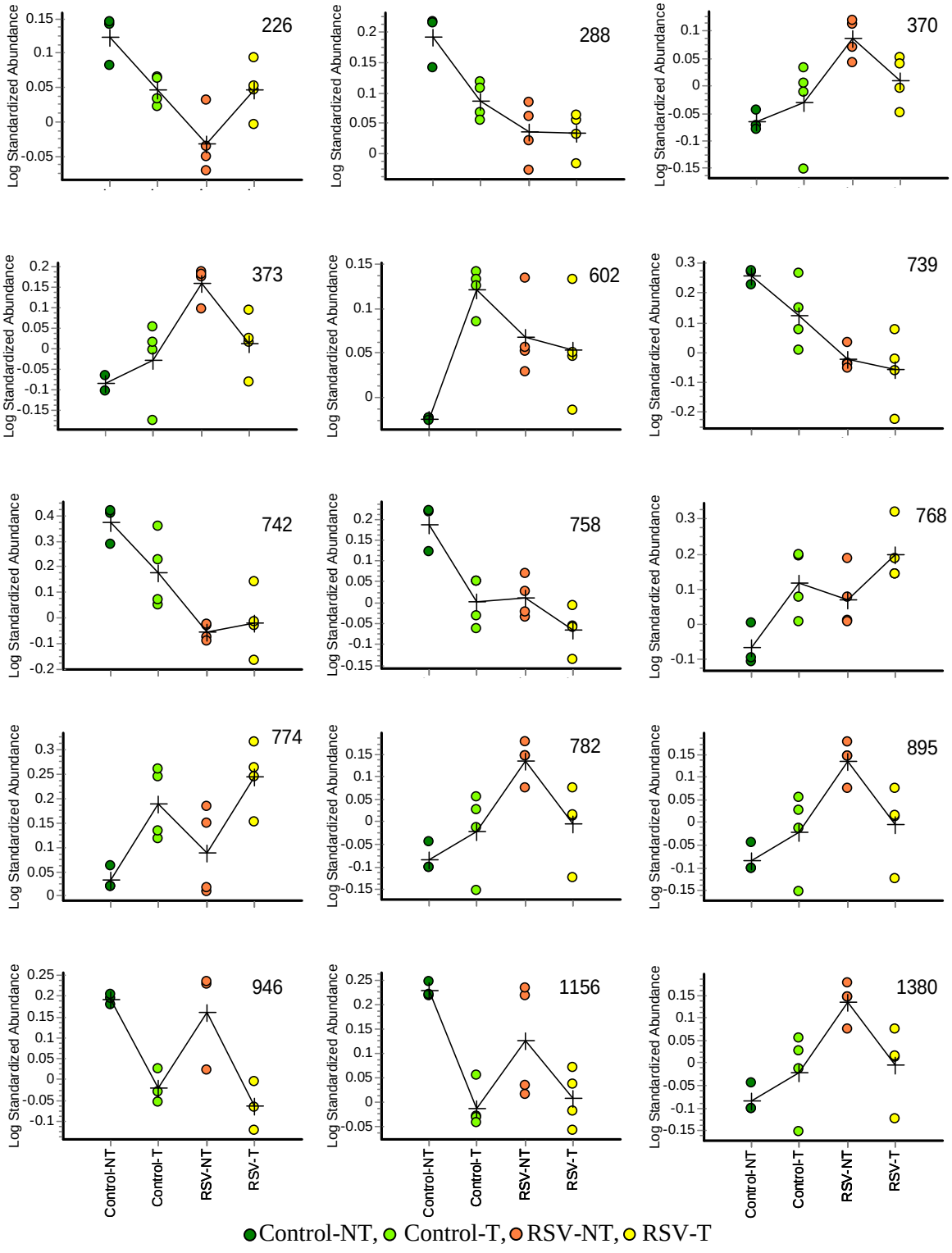


Table VI.3. *Lists protein identified that exhibited 1.3 or more change of expression in the mouse liver by effect of physical activity and/or RSV in mature group*

Master No.	***Protein AC***	***Name***	***T-test***	***Av. Ratio***	***1-ANOVA***	***pI***	***Mw (kDa)***
Down regulated proteins by exercise							
326	*Q8VC28*	*Aldo-keto reductase family 1 member C13*	*0.027*	*-1.58*	*0.005*	*6.67*	*37*
500	*Q8K2B3*	*Succinate dehydrogenase [ubiquinone] flavoprotein subunit; mitochondrial*	*0.0063*	*-1.52*	*0.0052*	*7.06*	*76*
Down regulated proteins by combination of exercise with RSV							
639	*Q91WU0*	*expressed sequence AU018778 (Protein Ces1f)*	*0.013*	*-1.30*	*0.012*	*6.4*	*62*
Down regulated proteins by RSV							
500	*Q8K2B3*	*Succinate dehydrogenase [ubiquinone] flavoprotein subunit; mitochondrial*	*0.039*	*-1.35*	*0.0052*	*7.06*	*76*
Up regulated proteins by RSV							
1257	*Q8VC28*	*Aldo-keto reductase family 1 member C13*	*0.008*	*1.33*	*0.0079*	*6.67*	*37*

Protein abundance mapping of mature group

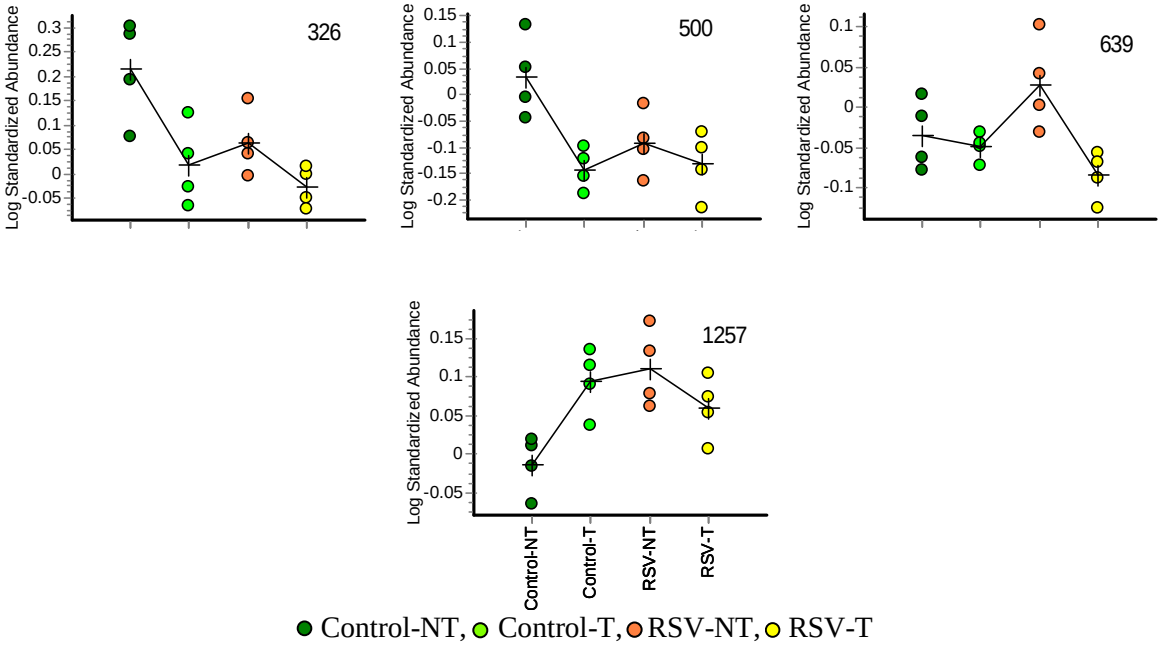
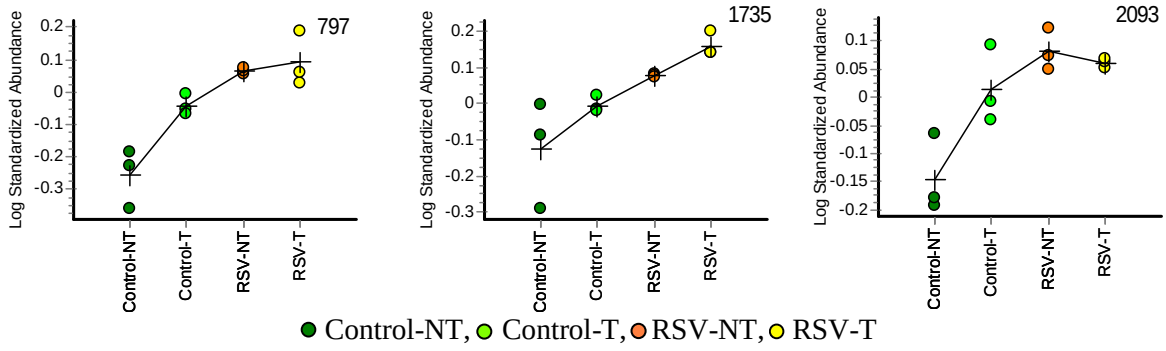


Table VI.4. *Lists protein identified that exhibited 1.3 or more change of expression in the mouse liver by effect of physical activity and/or RSV in old group.*

Master No.	***Protein AC***	***Name***	***T-test***	***Av. Ratio***	***1-ANOVA***	***pI***	***Mw (kDa)***
Up regulated proteins by exercise							
797	*P34914*	*epoxide hydrolase 2*	*0.018*	*1.62*	*0.00062*	*5.8*	*63*
Up regulated proteins bycombination of exercise with RSV							
1735	*P97328*	*ketohexokinase [Mus musculus]*	*0.016*	*1.21*	*0.01*	*5.8*	*33*
Up regulated proteins by RSV							
797	*P34914*	*epoxide hydrolase 2 [Mus musculus]*	*0.0037*	*2.07*	*0.00062*	*5.8*	*63*
2093	*Q99PT1*	*Rho GDP-dissociation inhibitor 1*	*0.0079*	*1.67*	*0.0031*	*5.1*	*23*
1735	*P97328*	*ketohexokinase [Mus musculus]*	*0.076*	*1.54*	*0.01*	*5.8*	*33*

Protein abundance mapping of old group



Discussion

Aging is a complicated process characterized by the changes in cellular functions and decline in the physiological conditions. Oxidative stress is increased with aging and leads to oxidative modifications of biomolecules during aging. Liver is the principal organ responsible for a number of metabolic processes. The liver mitochondria play a key role in metabolic process. It is not only the major resource of energy ATP but also the primary source of ROS. Accumulation of damaged proteins, lipids and DNA is key importance in liver dysfunction.

In this study we performed a liver 2D-DIGE proteomic approaches, investigate the proteins differentially expressed, identifying some of those proteins damaged during mouse liver aging. We also evaluate the impact of physical activity training and/or RSV on the proteins of liver during aging process. Here we will discuss below the possible implications of these differentially expressed protein with age-related changes observed in the liver and effect of RSV and exercise.

Redox regulation

Selenium-binding protein 2 was found decreased by aging and also by effect of RSV in young group. Selenium is an important oligo-element and has been shown to play a central role in redox processes in the cell (Haratake et al., 2005). The lack of selenium will suppress expression of various enzymes that will lead to cell abnormality and diseases. The protein response to oxidative radical intracellular was tightly regulated by its antioxidant responsive element (Lewis et al., 1988; Siedler et al., 1993). This protein plays important role in binding with xenobiotics and involved in hepatotoxicity. Decreased expression of this protein may as the result of increasing oxidative damage in aging process.

We found that aldehyde dehydrogenase (ALDH) was decreased significantly by aging, by RSV in young group, but increased by exercise in young group. ALDH is multifunctional enzyme. ALDH is involved in a variety of biological processes in organisms. ALDH is a key enzyme in cellular protection against oxidative damage. It has showed the capacity to decrease oxidative stress, particularly that caused by aldehydes (Chen et al., 2009; Vasiliou et al., 2004) as well as toxic lipid peroxides such as 4-hydroxynonenal and malondialdehyde. ALDH enzymes may also play important antioxidant roles by producing NAD(P)H and scavenging hydroxyl radicals via cysteine and methionine sulfhydryl groups (Estey et al., 2007; Lassen et al., 2006). Our data may contradict with previous report the ALDH activity increases with age in male mouse liver (Marshall et al., 2013). To our knowledge that oxidative damage during aging may lead to decrease the expression

protein of enzyme. In fact, recent study showed that the ALDH was decreased in rat liver (Bakala et al., 2013).

The enzyme peroxiredoxin 1 (PRDX1) was decreased in aging. PRDX1 is characterized as a typical 2-cysteine PRDX, meaning that it has two conserved and functionally important cysteines (Immenschuh and Baumgart-Vogt, 2005). PRDX1 has a role not only in limiting damage by oxidative stress, but has also been linked to the augmentation of natural killer cell cytotoxicity (Sauri et al., 1996) and regulation of cell proliferation, differentiation and apoptosis (Immenschuh and Baumgart-Vogt, 2005).

We have found the aldo-keto reductase (AKR) family 1 member C13 was decreased by exercise but increased by RSV it in mature group. The primary role of AKR may be to detoxify a range of toxic compounds produced during stress (Simpson et al., 2009). Its ability to accept multiple substrates is linked to a function in alleviating stress, and capable of detoxifying toxic carbonyls, including both endogenous stress-induced aldehydes, such as 4-hydroxy-2-nonenal (HNE), malondialdehyde (MDA), and methylglyoxal derived from lipid or sugar oxidation, and xenobiotic toxicants (Ellis, 2007; Jin and Penning, 2007).

Our data has showed that dihydrodiol dehydrogenase (DD) was down-regulated by aging. DD is cytoplasmic enzyme, which was first described to catalyze the oxidation of benzene dihydrodiol to catechol in the presence of $NADP^+$ in rabbit liver (Ayengar et al., 1959). DD has been implicated in the detoxication of polycyclic aromatic hydrocarbons (Penning, 1993).

3-Hydroxyanthranilate-3,4-dioxygenase (HAD) was increased by RSV in young group. This enzyme HAD belongs to the family of oxidoreductases, specifically those acting on single donors with O_2 as oxidant and incorporation of two atoms of oxygen into the substrate.

Interestingly, we found that enzyme peroxiredoxin 4 (PRDX4) was increased in aging. PRDX4 is an antioxidant protein which resides in the endoplasmic reticulum. This enzyme diminishes oxidative stress by reducing hydrogen peroxide to water in a thiol-dependent catalytic cycle (Wood et al., 2003) and has been linked to the regulation of the key pro-inflammatory transcription factor, nuclear factor kappa B (Yu et al., 2010). Increased expression of PRDX4 may counteract any possible decline in the activity of the enzyme due to oxidative damage and helpful to reduce the level of oxidative stress generated due to aging. In fact, other member of the antioxidant family of thioredoxin peroxidases, peroxiredoxin 3 was found overexpression in kidney in mice also during aging (Chakravarti et al., 2009).

Biosynthesis

We have showed the phosphomutases (PGM) was down-regulated significantly by aging and exercise in young group. PGM represents a key enzyme in carbohydrate metabolism, where it catalyzes the interconversion of glucose-1-phosphate and glucose-6-phosphate (Kotrba et al., 2001).

Ornithine aminotransferase (OAT) was down-regulated by aging. This enzyme OAT is a nuclear-encoded, 45 kDa mitochondrial matrix enzyme. OAT is a reversible enzyme expressed mainly in the liver, kidney and intestine. OAT controls the interconversion of ornithine into glutamate semi-aldehyde, and is therefore involved in the metabolism of arginine and glutamine which play a major role in N homeostasis (Dekaney et al., 2008).

Arginase 1 also was down-regulated by aging. Arginase is a cytosolic enzyme responsible for the cleavage of arginine. It is a rate-limiting enzyme in urea cycle, hydrolyzes l-arginine to urea and l-ornithine which is necessary for tissue repair processes. Arginase is the sixth and final enzyme of the hepatic urea cycle responsible for elimination of excessive nitrogen generated primarily by the metabolism of amino acids which are derived from the food intake or from endogenous protein catabolism (Crombez and Cederbaum, 2005).

Protein expressed sequence AU018778 (protein Ces1f) was decreased by combination of both RSV and exercise in mature group. The protein Ces1f has carboxylesterase activity. It catalyzes the hydrolysis of short-chain aliphatic and aromatic esters with broad substrate specificity and is inhibited by organophosphates. It plays an important part in the detoxification of organophosphorous compounds in mammalian systems and critical roles in drug metabolism and lipid mobilization (Xiao et al., 2012).

Epoxide hydrolases (EH) was found increased by exercise and RSV in old group. EH transforms epoxide-containing lipids to 1,2-diols by the addition of a molecule of water. EHs have important and diverse biological roles with profound effects on the physiological state of the host organism. It play a central role in regulating the levels of lipid epoxides (Morisseau, 2013).

Rho GDP-dissociation inhibitor α (RhoGDI- α) was up-regulated by RSV in old group. RhoGDI- α, one of group Rho GDP dissociation inhibitors in mammalian cells, belongs a major class of regulators of Rho GTPases by regulate the GDP/GTP exchange reaction of the Rho proteins. It plays essential roles in normal cell growth and malignant transformation (Zhang, 2006).

We found the 3-mercaptopyruvate sulfurtransferase (MST) was up-regulated by RSV in young group. MST is an enzyme believed to function in the endogenous cyanide detoxification system because it is capable of transferring sulfur from 3-mercaptopyruvate to cyanide, forming the less toxic thiocyanate (Jarabak, 1981).

Cysteine sulfinic acid decarboxylase (CSAD) was up-regulated by exercise and combination of both RSV and exercise in young group. Cysteine sulfinic acid is converted to hypotaurine by the action of CSAD and is subsequently oxidized to taurine (Davison, 1956).

Transport proteins

The serum albumin also was up-regulated by aging. Serum albumins (SAs) are multifunctional proteins which are highly conserved in both sequence and structure. The molecule, which has three domains, is very flexible and may change its conformation easily in order to bind many diverse ligands (Bujacz, 2012). Albumin is also found up-regulated in its expressions due to aging in kidney (Chakravarti et al., 2009).

Energy metabolism

Dimethylglycine dehydrogenase (MeGlyDH) was found down-regulated by aging. This enzyme is a mitochondrial matrix enzyme involved in choline degradation. MeGlyDH catalyzes the oxidative demethylation of dimethylglycine to yield sarcosine, 5,10-methylene-THF and FADH2 (Brizio et al., 2002).

Glycogen phosphorylase (GP) was found down-regulated by RSV in young group. GP catalyzes the reversible phosphorolytic cleavage of the α-1,4-glycosidic bonds at the non-reducing ends of the side chains of glycogen, resulting in the formation of glucose-1-phosphate and is one of the rate-limiting steps of glycogen breakdown (Klinov and Kurganov, 2001).

Succinate dehydrogenase (SDH) flavoprotein subunit was decreased by both exercise and RSV in mature group. SDH has a central role in mitochondrial metabolism as the only enzyme that is a component of both the TCA cycle and the electron transport chain. SDH catalyzes the oxidation of succinate to fumarate with the concomitant reduction of ubiquinone to ubiquinol (Hagerhall, 1997).

Enzyme ketohexokinase (KHK) was up-regulated by RSV and also by combination of RSV with exercise in old mice group. KHK catalyzes the phosphorylation of fructose on position C1 by

transferring a phosphate group from adenosine 5′-triphosphate to yield fructose-1-phosphate, which enters normal metabolic pathways (Raushel and Cleland, 1977).

The mitochondrial dysfunction associated with age-related alterations in several organ systems, particularly in the liver. We have found the NADH-ubiquinone oxidoreductase 75 kDa subunit was increased by RSV in young group. NADH:ubiquinone oxidoreductase or complex I is the first enzyme of the electron transport chain in organisms. It located in the inner mitochondrial membrane, is important for energy metabolism because it is the initial enzyme of the mitochondrial respiratory chain. It catalyzes the transport of electrons from NADH to ubiquinone for ATP synthesis (Smeitink et al., 1998).

The cytochrome b-c1 complex subunit 1, or complex III was found up-regulated by RSV in young group. The cytochrome bc1 complex subunit 1 is a component of the mitochondrial respiratory chain. It is a central component of the energy conversion machinery of respiratory and photosynthetic electron transfer chains. It catalyzes the reaction of electron transfer from ubiquinol to cytochrome c and couples this reaction to proton translocation across the membrane (Hunte et al., 2008).

We have found that ATP synthase subunit β, mitochondrial was found increased by RSV treatment in young group mice. Mitochondrial membrane ATP synthase produces ATP from ADP in the presence of a proton gradient across the membrane which is generated by electron transport complexes of the respiratory chain (Hubbard and McHugh, 1996; Weber and Senior, 2003). We recognize an increased level of ATP synthase subunit β, a rate-limiting enzyme that is closely related to a capacity for ATP production. Also increased of NADH-ubiquinone oxidoreductase, cytochrome b-c1 complex subunit, essential component in the electron transport chain which may strongly accelerated the electron transfer process by RSV. Then, the electron flux and ATP synthesis would be improved by RSV.

We found the 3-hydroxybutyrate dehydrogenase (βHBDH) was decreased in aging. βHBDH is involved in the synthesis of ketone bodies. βHBDH catalyzes the NAD(H)-dependent interconversion of acetoacetate to d-3-hydroxybutyrate (Watson and Lindsay, 1972).

Protein mitochondrial inner membrane protein, mitofilin, was up-regulated by RSV in young group. Mitofilin is an inner membrane protein that has been defined as a mitochondria-shaping protein in controlling and maintaining mitochondrial cristae remodeling (Gieffers et al., 1997).

To summarize, we found that most differentially expressed proteins were associated with the electron transport chain, urea cycle, enzymes oxidoreductases, regulatory factors in mitochondrial protein synthesis, aldehyde dehydrogenase family and antioxidant capacity in liver aging. Aging is accompanied by reduced enzymes activities. These functional changes contribute mainly to the alteration in the organism sensitivity to damaging action of stress factors during aging, to age-related modulation of the action of endogenous and finally, to the origin of diseases associated with aging. Our results provide insights about the effect of RSV treatment and/or physical activity at different ages and support the use of physical activity and/or RSV for the prevention of metabolic diseases related to aging.

GENERAL DISCUSSION

GENERAL DISCUSSION

Aging is an inevitable biological process that affects living organisms. Aging can be described as a time-dependent functional decline that leads to the cell's inability to withstand external and internal challenges. The causal factors underlying the time-dependent, deleterious processes of aging are not yet defined, and an adequate molecular explanation for aging is currently unavailable. The basic cellular and biochemical features of the aging process are complex and a large body of recent research have revealed main biological aging mechanisms and suggested potential aging interventions. These works provided new insights into the molecular processes and biological events that contribute to age-related deterioration.

Oxidative stress and chronic inflammation are two factors that have been linked to aging. According to Harman (1956, 1972) the aging process is the accumulation of oxidative damage in cells and tissues. The generation of reactive oxygen and nitrogen species during oxidative stress affects lipids, proteins, enzymes, carbohydrates, nucleic acids and other molecules within the cell, resulting in cellular damage. During the lifetime of an organism, the antioxidant network counteracts the deleterious effects of free radicals and reactive species on macromolecules. Then, aging is associated with the increased markers of oxidative stress, including increased levels of malondialdehyde and other lipid peroxidation products, oxidized DNA, and reduced enzymatic antioxidant activity (McLean and Le Couteur, 2004).

The polyphenolic compound RSV is synthesized by many plants, such as peanuts, blueberries, pine nuts, and grapes, and it protects the plants themselves against fungal infection and ultraviolet irradiation (Bavaresco, 2003). Because of its capacity to mimic CR and its antioxidant and anti-inflammatory properties, RSV has been considered a candidate for anti-aging studies. In fact, RSV was reported to significantly extend lifespan in yeast (Howitz et al., 2003), short-lived invertebrates, such as flies and nematodes (Bass et al., 2007) and metazoans (Howitz et al., 2003; Wood et al., 2004). RSV may effectively prevent senescence-related dysfunction, delay the onset of age-associated diseases, and treat chronic metabolic disorders.

Furthermore, the beneficial effect of exercise on lifespan has been demonstrated by several studies. Aerobic exercise is accompanied by an increase in the generation of active oxygen species in the skeletal muscle, which can result in tissue damage. However, regularly performed moderate exercise delays certain age-associated changes and protects against several metabolic disorders (Warburton et al., 2006). It has been also demonstrated that long-term moderate exercise retards

aging and extends the average lifespan of experimental animals (Holloszy, 1993). Furthermore, as a hormetic response, physical training reduces the effects of oxidative stress that are induced by intensive exercise and is accompanied by changes in antioxidant systems.

To date, the effect of long-term RSV treatment on age-associated oxidative damage has not yet clearly established. Moreover, the mechanisms for exercise's beneficial effects on aging are not well understood. Our study tested the hypothesis that long-term RSV supplementation alone or combination with exercise can counteract age-associated oxidative damage in mice. We administered RSV to mice for six months and followed exercise at different ages.

Aging is associated with increased free radical generation. The accumulation of oxidative damage is the cause of numerous diseases. Accumulation of unrepaired oxidative damage to biological macromolecules is one of the premises of the "free radical" theory of aging. Thus, high levels of oxidative damage in DNA, lipids, and proteins have been found in naked mole rats, the longest living rodent, compared to physiologically age-matched CB6F1 hybrid mice (Andziak et al., 2006). The extent to which oxidative stress contributes to aging may also vary between organisms, species, and tissues (Golden et al., 2002). Age-dependent increases in lipid peroxidation have been reported previously in some animal models (Barja, 2002). Examining the livers of mice, we found an age-associated accumulation of oxidative damage to lipids and proteins, measured by MDA and protein carbonyl groups, while the levels of total glutathione and sulfhydryl groups decreased. We have found that RSV and exercise reverse all these effects. It is therefore likely that RSV affects oxidative stresses through the activation of pathways that upregulate antioxidant defenses or radical-scavenging activity or that modulate free radical generation, e.g., by affecting mitochondrial function. We observed that RSV attenuated age-dependent increases in oxidative damage particularly in tissues in which the age-related increase in oxidative damage was most prominent, i.e., in the liver.

The age-related redox imbalance is likely caused by the net effect of weakened anti-oxidative defense systems, and the increasing production of reactive species, such as superoxide ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (H_2O_2), reactive nitric oxide, peroxynitrite ($ONOO^-$) and reactive lipid aldehydes. ROS and free radicals are important-mediators of several forms of tissue damage. There has been recent interest in the concept that oxygen and nitrogen free radicals play an important role in diseases. To protect against hostile oxidative environments, organisms have developed various antioxidant defenses including the antioxidant enzymes SOD, CAT, GPx, GR, $CytB_5$Rase, NQO1, TrxR and other endogenous free radical scavengers, including α-tocopherol,

ascorbic acid, vitamin E and GSH and CoQ. These antioxidants protect the cell against cytotoxic ROS such as superoxide anions, hydrogen peroxide and hydroxyl radicals. The overexpression of antioxidant enzymes or the activation of defensive mechanisms against oxidative stress has been effective in extending life span in some instances (Melov et al., 2000; Orr and Sohal, 1994; Schriner et al., 2005). As an endogenous antioxidant, GPx plays a critical role in intracellular antioxidant defense. GPx can scavenge ROS and prevent H_2O_2-induced hydroxyl radical formation. Therefore, the GPx level reflects one of the main antioxidant defense factors and maintains a high level of the cell antioxidant capacity. In the present study, RSV and training restored the GPx level in old animals; this phenomenon indicates that RSV contributes to increase the free radical scavenging capacity that participates in accelerating aging. We cannot forget that free radicals can also directly damage antioxidant enzymes such as SOD, and reduce their activities. In the present study, GPx and NQO1 levels and activities markedly increased in RSV and exercise training-treated groups.

Exercise increases the utilization of oxygen in the body and enhances the free radical formation in various tissues of animals. This higher free radical formation may lead to oxidative damage, such as lipid peroxidation. Numerous reports have documented increases in by-products of lipid peroxidation, especially MDA following exercise (Metin et al., 2003; Saiki et al., 2001). The amount of damage depends on exercise intensity, training state, and the tissue examined and could be reduced through dietary supplementation of antioxidants (Radak et al., 2004). As an hormetic response, the generation of ROS by exercise seem to increase the activity of antioxidant enzymes in muscle and in heart (Husain and Somani, 1997). Thus, exercise training increases the resistance against oxidative stress, providing enhanced protection (Alessio and Goldfarb, 1988). Animals frequently exposed to chronic exercise training have shown less oxidative damage after exhaustive exercise than untrained animals. This is largely due to the upregulation of endogenous antioxidant enzymes such as Mn-SOD, GPx, and γ-glutamylcysteine synthetase (GCS) (Radak et al., 2002). Regular exercise also increases antioxidant defenses in the skeletal muscle upregulating SOD and glutathione peroxidase gene expression, thereby adapting to stronger oxidative stresses (Leeuwenburgh and Heinecke, 2001). Antioxidant enzyme adaptation is considered to be one of the fundamental changes in skeletal muscle in response to exercise, much the same as mitochondrial oxidative enzyme adaptation (Jenkins, 1988). Elevated antioxidant enzyme activities with training have demonstrated clear benefits in preventing oxidative stress in a variety of experimental models and pathogenic conditions (Thirunavukkarasu et al., 2003). In this system, nuclear factor E2 p45-related factor 2 (Nrf2) is a transcription factor essential for the regulation of the expression of detoxifying and anti-oxidative genes in various tissues that plays an essential role in this response. It

has been suggested that a different response to oxidative stress in aging can be due to decay in the activation of the Nrf2 factor in tissues. RSV and exercise probably increase the activity of Nrf2 in tissues where it is dysregulated.

Inflammation may be also considered an essential process of aging because of its involvement in baseline aging and in the major degenerative diseases of later life, such as atherosclerosis, Alzheimer´s disease, and cancer. Along aging the incidence of diseases associated with inflammation increases (Gupta et al., 2006). It has been proposed that chronic molecular inflammation is a major biological mechanism that underpins the aging process and age-related diseases (Chung et al., 2006). Then, aging is associated with increased levels of CRP, as well as high inflammatory cell (neutrophils and monocytes) counts (Bruunsgaard et al., 2003). The key mediators of inflammatory reactions (i.e., IL-1β, IL-6, TNF-α, COX-2, and iNOS) have all been shown to be up-regulated during the aging process (Hager et al., 1994). COX activity and the production of TXA_2 and PGI_2 also increase during aging. Other pro-inflammatory proteins, such as AMs (VCAM-1, ICAM-1, P-, E-selectin), are also up-regulated during the aging process (Zou et al., 2003). It has been proposed that the continuous upregulation of pro-inflammatory mediators is induced during the aging process due by an age-related redox imbalance that activates the NF-κB signaling pathway (Chung et al., 2011). Then, the NF-κB transcription factor may be considered as the master regulator of the inflammatory process (Makarov, 2000). It is believed that NF-κB is constitutively activated at old age, which leads to the higher basal expression of pro-inflammatory cytokines, chemokines, adhesion molecules and ROS-generating enzymes.

RSV has been shown as an anti-inflammatory dietary phytochemical that antagonizes some catabolic effects of TNF-α and IL-1β via inhibition of NF-κB (Estrov et al., 2003). RSV is involved in the down-regulation of the inflammatory response through inhibition of synthesis and release of pro-inflammatory mediators, modification of eicosanoid synthesis, inhibition of activated immune cells, or inhibiting enzyme of inflammation such as iNOS and COX-2 via its inhibitory effects on NF-κB (Harikumar and Aggarwal, 2008; Pirola and Frojdo, 2008). Accordingly with these studies, in our study we have found that that RSV decreased the TNF-α, IL-1β, IL-6, and COX-2 in mouse liver.

The age-associated increase in the activity of the redox-sensitive transcription factor NF-κB can be also significantly attenuated by regular exercise (Radak et al., 2004). Regular exercise enhances the maintenance of the redox status and reduces NF-κB activity, thereby leading to suppression of pro-inflammatory mediators, such as cytokines, chemokines and AMs. Moderate regular physical

activity has been associated with reduced levels of TNF-α, IL-6, and CRP in healthy older adults. Furthermore, regular exercise of various duration and intensity decreases resting levels of TNF-α and CRP levels in young individuals. More recently, a number of studies reported that physical activity is associated with lower plasma concentrations of IL-6 and CRP in various age groups, from young adults to the elderly. Moreover, aerobic exercise was found to be associated with decrease serum levels of IL-6 and increased levels of IL-10, a potent anti-inflammatory cytokine, in healthy older men (Colbert et al., 2004; Jankord and Jemiolo, 2004; Taaffe et al., 2000). Interestingly, IL-10 has been shown to inhibit the synthesis of several pro-inflammatory cytokines, including TNF-α and IL-1β.

An increase in several "danger signals" associated with a chronic proinflammatory state is observed in aging. The NALP-3 inflammasome can be activated by different "danger-associated molecular patterns" (DAMPs) derived from damaged cells and organelles, and it induces inflammation by secretion of IL-1β and IL-18 (Martinon et al., 2002). NALP-3 inflammasome is an upstream target that controls age-related inflammation and offers an innovative therapeutic strategy that can be employed to lower NALP-3 activity to delay multiple age-related chronic diseases (Youm et al., 2013). The NALP-3 inflammasome is unique among innate immune sensors, as it can be activated in response to a diverse array of endogenous metabolic "danger signals" to induce sterile inflammation in absence of overt infection. In a recent study, Youm and colleagues have demonstrated that specific inhibition of NALP-3 inflammasome-mediated caspase-1 activation controls age-related functional decline, and IL-1β proinflammatory cascade is not the sole mediator of downstream effects of NALP-3 inflammasome activation during aging (Youm et al., 2013). Activation of the NALP-3 inflammasome in response to accumulation of various DAMPs induces systemic chronic inflammation in aging. Our results suggest that RSV or exercise aimed at dampening NALP-3 inflammasome activation may be efficient in disrupting age-related chronic diseases.

It has been proposed that the tissue content of CoQ decreases during aging (Lass et al., 1999), which may in part be responsible for the decline of energy metabolism. We have shown that, in response to aging, levels of CoQ decreased in brain but increased in liver and muscle tissues. Since aging is a complex biological process involving a progressive decline in the biochemical performance of specific tissues and organs, the potential for CoQ_{10} as an antioxidant, membrane-stabilizer and support for ATP synthesis that may slow down the physiological and biological decline that occurs with aging is considerable. This can indicate that dietary supplementation of

CoQ_{10} might be beneficial with aging. The supplementation of CoQ_{10}, which supports all living cells with ATP, may retard the bioenergy decline associated with senescence and illness. It has been demonstrated that CoQ_{10} is about as effective in preventing oxidative damage to lipids as α-tocopherol (Noack et al., 1994). CoQ_{10} has been demonstrated to spare α-tocopherol when the two antioxidants were present in the same liposomal membrane, as well as to recycle vitamin E (Crane, 2001). Pretreatment of rats with CoQ_{10} markedly suppressed the exercise-induced increase in the markers of lipid peroxidation in the heart, liver, and gastrocnemius muscle (Faff and Frankiewicz-Jozko, 1997). The results shown in this thesis have demonstrated that the expression of CoQ in brain, muscle and liver increases with RSV or exercise training in young, mature and old group mice. So, RSV and exercise training may improve the anti-aging abilities of old mice by enhancing the endogenous levels of CoQ.

Aging changes biological processes in many organs and tissues, leading to the development of age-associated diseases and deterioration of functions. The liver plays a pivotal role in the metabolism of nutrients, drugs, hormones, and metabolic waste products, thereby maintaining body homeostasis. The liver undergoes significant changes in structure and function in old age. Although the majority of liver functions seem to be maintained with age, the incidence of several liver diseases, including those associated with high triglyceride levels, non-alcoholic fatty liver diseases and hepatocellular carcinoma, increases with age. Age-related physiological changes, such as a reduction in liver mass, hepatic metabolising enzyme activity, liver blood flow and alterations in plasma drug binding may account for the decreased elimination of some metabolised drugs in the elderly. We have used the proteomic analysis to study the level protein expression changed by aging and by effects of exercise and/or RSV in mouse liver. Our results have revealed the positive changes in the most of the differentially expressed proteins are associated with the electron transport chain, urea cycle, enzymes oxidoreductases, regulatory factors in mitochondrial protein synthesis, aldehyde dehydrogenase family and antioxidant capacity by exercise and/or RSV. Interestingly, the response to RSV or exercise depends on the age of the animal at the beginning of the treatment with RSV or exercise. This different response may respond to epigenetic changes in the nuclei of cells during aging and may indicate changes in the response of organs to the same stimulus depending on the age of the individual.

To summarized of all we have discussed above, the possible action of RSV and exercise on aging was showed in figure VII.1

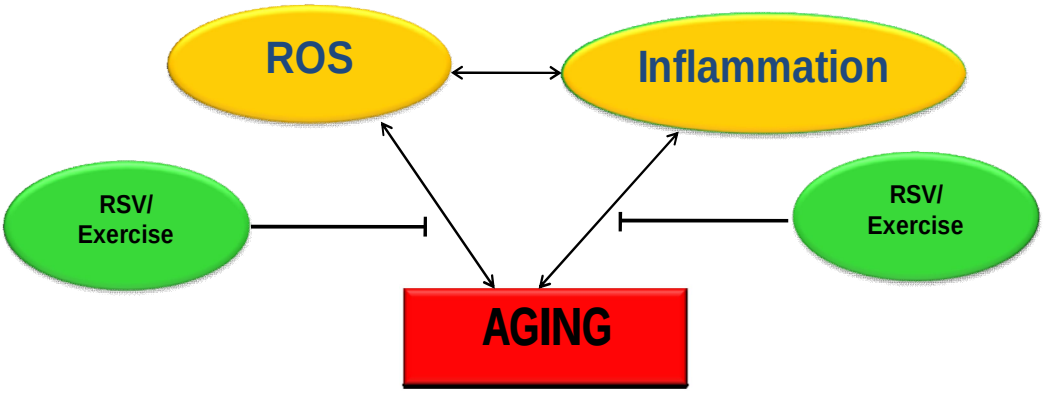


Figure VII.1. *The possible action of RSV and exercise on aging.*

CONCLUSION

CONCLUSION

1. RSV treatment in long-term and exercise training in short-term were not affecting the body weight of animals during experimental phase.

2. RSV treatment and/or exercise training reduces the oxidative damage caused by aging thanks to capacity to improve the protection by antioxidant system: SOD, CAT, GPx, GR, $CytB_5$Rase, NQO1, and TrxR.

3. The protein levels of COX-2, TNF-α, IL-1β, IL-6 increased in aging and can be decreased by RSV treatment and/or exercise training. With aging, the mRNA levels of components of ASC, CASP-1, NALP-1, NALP-3 inflammasome increases but are reduced by effect of RSV and exercise.

4. RSV treatment and exercise increases endogenous antioxidant enzyme systems in the major organs in old mice. Moreover, a decreased amount of malondialdehyde was observed in the liver, heart, and muscle of old mice by RSV and exercise.

5. With increasing age, the level of CoQ decreases in brain but increases in liver and muscle. RSV and exercise training can increase the level of CoQ in brain, liver and muscle in mouse.

6. The proteomic analysis in liver revealed that several differentially expressed protein associated with the electron transport chain, urea cycle, enzymes oxidoreductases, regulatory factors in mitochondrial protein synthesis, aldehyde dehydrogenase family and antioxidant capacity caused by aging and many of them were restored by RSV treatment and exercise.

CONCLUSIONES

1. El tratamiento de RSV, a largo plazo, y la realización de ejercicio, a corto plazo, no afectó al peso corporal de los animales según el procedimiento experimental seguido.

2. El tratamiento de RSV y ejercicio redujeron el daño oxidativo causado por el envejecimiento, gracias a su capacidad de incrementar la protección de los sistemas antioxidantes en el hígado: SOD, CAT, GPx, GR, CytB_5Rase, NQO1 y TrxR.

3. Los niveles de proteínasCOX-2, TNF-α, IL-1β, IL-6 aumentaron en los ratones envejecidos y disminuyeron tras el tratamiento de RSV y ejercicio. Con la edad, los niveles de ARN-mensajero de los componentes de ASC, CASP-1, NALP-1, NALP-3 del inflamasoma se incrementaron, pero este efecto fue reducido por el RSV y el ejercicio.

4. El tratamiento con el RSV y el ejercicio mejoraron los sistemas enzimáticos antioxidantes endógenos en los principales órganos de los ratones viejos. Por otra parte, se observó una disminución en la cantidad de malondialdehído en el hígado, corazón, y el músculo de los ratones viejos por el RSV y el ejercicio.

5. Al aumentar la edad, el nivel de CoQ disminuyó en el cerebro, pero aumentó en el hígado y músculo. RSV y ejercicio aumentaron el nivel de CoQ en el cerebro, hígado y músculo de ratón.

6. El análisis proteómico en el hígado reveló que las proteínas asociadas con la cadena de transporte de electrones, el ciclo de la urea, las enzimas oxidorreductasas, los factores de regulación de la síntesis de proteína mitocondrial, la enzima aldehído deshidrogenasa y otras relacionadas con la capacidad antioxidante se expresaban diferentes en el envejecimiento. Este efecto fue restaurado por el tratamiento con RSV y ejercicio.

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