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Review

Mitochondria and T2D: Role of Autophagy, ER Stress, and Inflammasome

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Type 2 diabetes (T2D) is one of the main current threats to human health. Both T2D and its numerous clinical complications are related to mitochondrial dysfunction and oxidative stress. Over the past decade, great progress has been made in extending our knowledge about the signaling events regulated by mitochondria. However, the links among mitochondrial impairment, oxidative stress, autophagy, endoplasmic reticulum (ER) stress, and activation of the inflammasome still need to be clarified. In light of this deficit, we aim to provide a review of the existing literature concerning the complicated crosstalk between mitochondrial impairment, autophagy, ER stress, and the inflammasome in the molecular pathogenesis of T2D.

Highlights

Oxidative stress, mitochondrial dysfunction, and type 2 diabetes are closely interconnected.

Mitochondrial impairment characteristic for type 2 diabetes is related to changes in the autophagic process, endoplasmic reticulum stress, and inflammation.

A new understanding of the pathophysiological mechanisms by which mitochondrial dysfunction and ROS, as essential signaling molecules, relate to other molecular pathways, could help to identify novel therapeutic targets for the treatment of type 2 diabetes.

The identification of key molecular targets for type 2 diabetes treatment can widen the pharmacological scope for clinical development.

Type 2 Diabetes (T2D) and the Involvement of Mitochondria in Its Pathophysiology

T2D is a serious and highly prevalent clinical condition that represents one of the main threats to human health in the 21st century [1,2]. It has been estimated that the number of T2D patients will increase to 700 million over the next 15 years. Furthermore, T2D is related to a high number of different chronic comorbidities that can undermine quality of life and life span. **Insulin resistance (IR)** (see Glossary), one of the main characteristics of T2D and cardiometabolic diseases, is related to various clinical complications, including obesity, polycystic ovary syndrome, metabolic syndrome, and atherosclerosis [3]. The main action of insulin is the maintenance of glucose homeostasis through stimulation of glucose uptake in peripheral tissues (primarily adipose tissue and skeletal muscle) and a reduction of liver gluconeogenesis (Figure 1). T2D is characterized by a metabolic imbalance in which ATP levels are compromised and mitochondria play a crucial role. Mitochondria are the main source of **reactive oxygen species (ROS)** and are critical to redox homeostasis, metabolism, and multiple cell functions, including apoptosis and maintenance of Ca^{2+} levels [4]. The relationship between mitochondria, metabolism, and inflammation, and the different signaling pathways involved in this complex interplay is of utmost importance. For example, alterations in mitochondrial metabolic routes [such as oxidative phosphorylation (OXPHOS) and tricarboxylic acid cycle (TCA)] can induce changes in gene expression that eventually lead to different outcomes in immune cells; for instance, M1 macrophages are impaired in the TCA cycle and enter a proinflammatory state, while M2 macrophages undergo β-oxidation and display anti-inflammatory responses [5]. Mitochondria play a fundamental role in the regulation of the immune response by modulating **autophagy**, **endoplasmic reticulum (ER) stress**, and **inflammasome** activation through different mechanisms, including ROS production and changes in mitochondrial DNA (mtDNA), which modulate and control the transcription of immune cells [5]. All these features highlight the importance of mitochondria in the modulation of the inflammatory response associated with T2D.

Mitochondrial Dysfunction and IR

Insulin plays a crucial role in controlling plasma glucose levels by different mechanisms, including glycogenolysis, glucose uptake, and gluconeogenesis. For example, skeletal muscle accounts for 60–70% of whole body insulin-stimulated glucose uptake and therefore helps to modulate

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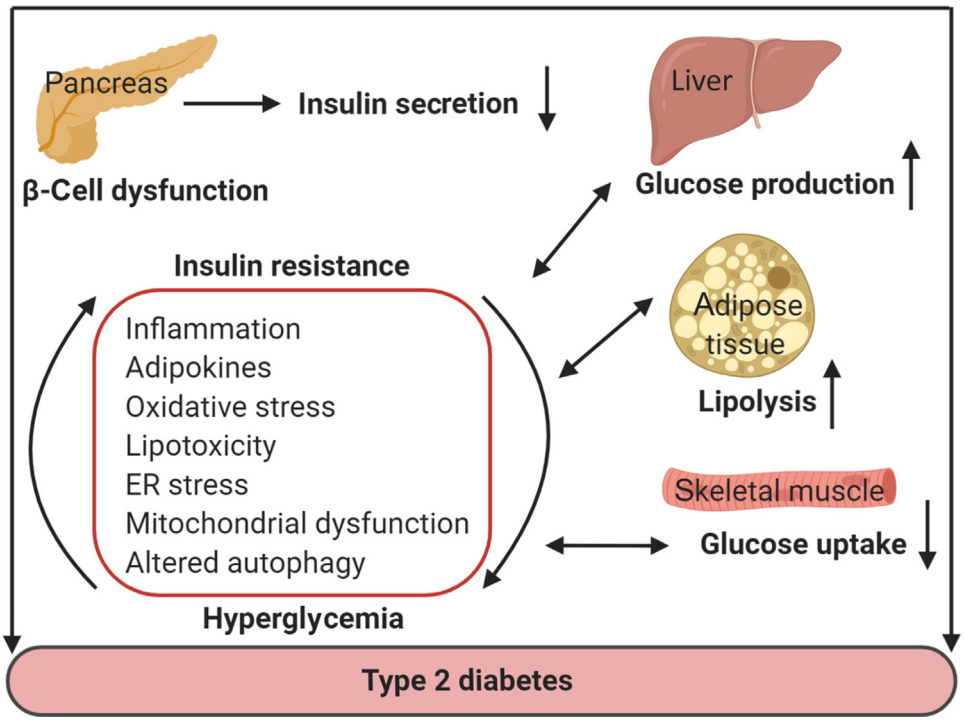

Figure 1. General Mechanisms of Type 2 Diabetes in the Pancreas and Target Organs (Liver, Adipose Tissue, and Skeletal Muscle). Diminished β-cell function is related to decreased insulin secretion by the pancreas and increased insulin resistance of peripheral tissues. Major biochemical alterations can be observed in skeletal muscle, liver, and adipose tissue, among other organs and tissues. The molecular mechanisms involved, which may be both cause and consequence of hypoinsulinemia and hyperglycemia, involve inflammation, lipotoxicity, mitochondrial dysfunction, and endoplasmic reticulum (ER) stress. This figure was created using BioRender (https://biorender.com/).

whole body energy homeostasis [6]. The specific regulation of insulin means that IR is involved in a large range of conditions, including hypertension, neuropathy, retinopathy, nephropathy, and cardiovascular diseases (CVD) [7]. In addition, T2D patients with hypertension often exhibit IR and are at greater risk of T2D than normotensive subjects [8]. It is well demonstrated that IR is a characteristic of T2D and results in a diminished capacity of cells to respond to fluctuating glucose levels. Different conditions can contribute to IR, such as excessive weight, metabolic syndrome, obesity, stress, and metabolic changes [9].

Dyslipidemia is typical of T2D and IR and both are related to **oxidative stress** (defined as an imbalance between ROS generation and antioxidant cellular defenses) and atherosclerosis. Novel approaches using antisense oligonucleotides and monoclonal antibodies may provide future therapies for diabetic dyslipidemia [10]. During dyslipidemia, a decrease in insulin-stimulated glucose disposal [11] and a drop in ATP levels have been described. In this context, abnormal lipid metabolism can undermine insulin signaling and therefore increase IR in different tissues [12]. With regard to this, Fayyaz *et al.* demonstrated that palmitate can be metabolized into sphingosine 1-phosphate (S1P) by hepatocytes and that S1P impairs insulin signaling by attenuating insulin-dependent protein kinase B (Akt) phosphorylation [13].

IR can be induced by multiple mechanisms, including an enhanced degradation of insulin receptor proteins [14], increased levels of serine phosphorylation of insulin receptor substrate (IRS) [15],

Glossary

Autophagy: highly evolutionarily conserved intracellular degradation system that delivers cytoplasmic constituents to the lysosome.
Cytokines: a group of small peptides that act as chemical messengers of the immune system. Can be produced by certain immune and non-immune cells.
Endoplasmic reticulum (ER) stress: a cellular phenomenon that occurs when the endoplasmic reticulum becomes saturated and its capacity to fold proteins diminishes. This effect may be caused by factors that impair protein glycosylation or disulfide bond formation, including mutations in the polypeptides entering the secretory pathway.
Inflammasome: a multiprotein complex that mediates the activation of the enzyme procaspase-1 involved in inflammation. Its activation is triggered by infections, tissue damage, or metabolic imbalances.
Insulin resistance (IR): a pathological condition in which cells fail to sense and respond normally to insulin, including the insulin-mediated uptake of glucose.
Mitochondrial dysfunction: a term used to describe the pathological functioning of mitochondria, including some or all of the following effects: decreased mitochondrial oxygen consumption, alterations of the mitochondrial membrane potential, diminished ATP production, and increased ROS generation.
Oxidative stress: imbalance between ROS generation and antioxidant cellular defenses.
Reactive oxygen species (ROS): highly reactive compounds that contain oxygen and are generated by diverse metabolic processes. At low concentrations ROS act as signaling molecules, but at higher concentrations they are deleterious.

elevated activity of phosphatases [16], or a decreased activation of insulin downstream signaling molecules such as Akt or PKC [17]. Phosphorylation of IRS at key target residues can reduce PI3K activation, thus playing an important role in the response to insulin levels [16], and a decrease in IRS tyrosine phosphorylation has been reported in different insulin-resistant animal models and humans [18]. In addition, proinflammatory **cytokines** and inflammation are related to IR. In human obesity, the expression of TNFα, interleukin (IL)-1, and IL-6 is increased in different tissues, including adipose tissue, and has been linked to systemic inflammation and accompanying IR [19].

ROS production is directly related to IR and **mitochondrial dysfunction** [2]. In fact, ROS production can modify signals that activate the serine kinases that phosphorylate IRS proteins [20]. There are different sources of ROS, including mitochondria and NADPH oxidase, that can contribute to ROS-induced phosphorylation of IRS-1 and impair insulin signaling [6]. In this sense, using uncouplers or inhibiting NADPH oxidase can enhance glucose metabolism and insulin signaling [21]. In fact, it has been shown that ROS, both total and mitochondrial, can promote damage in the electron transport chain (ETC), mainly at complex I [22], and hyperglycemia is known to induce metabolic changes in β-cells that markedly reduce mitochondrial metabolism and ATP synthesis [23].

Mitochondrial impairment, mitochondrial ROS (mtROS) production, and oxidative stress are related. However, the role of mtROS in the development of IR is unclear. Low levels of ROS can activate signaling pathways to initiate biological processes, while high levels of ROS can damage DNA, protein, and lipids and can aggravate the inflammatory process by enhancing IκKβ activation [24]. Furthermore, studies in humans and animal models have provided evidence for impaired OXPHOS in muscle mitochondria under conditions of IR. For example, Kelley *et al.*, [25] studied mitochondria isolated from human muscle biopsy specimens obtained from T2D, obese, and lean individuals, demonstrating a reduction in both NADH oxidoreductase and citrate synthase activity in the mitochondria of diabetic and obese subjects compared with lean subjects. Moreover, decreased mRNA expression of several genes associated with OXPHOS has been described in diabetic patients, including genes regulated by PGC-1α and nuclear respiratory factors [26,27]. This has been observed not only in subjects with T2D, but also in their first-degree relatives. Evidence at the protein level suggests that the muscle of subjects with T2D manifests impaired ATP production, suggested by reduced levels of ATP synthase and creatine kinase B [28].

In addition, mitochondria are known to be susceptible to a variety of genetic and environmental insults; the accumulation of mtDNA mutations and mtDNA copy number depletion can help to understand the prevalence of mitochondria-related diseases such as T2D. In this sense, a recent review has revealed the implication of novel mitochondrial biology effects in the progression of disease, such as mtDNA heteroplasmy, noncoding RNA (ncRNA), epigenetic modification of the mitochondrial genome, and epitranscriptomic regulation of the mtDNA-encoded mitochondrial transcriptome [29]. Indeed, there is growing interest in identifying genes and processes that could trigger IR beyond defects in the insulin signaling cascade itself. For example, Mercader *et al.*, [30] identified 286 genes that are associated simultaneously with insulin signaling and mitochondrial genes and which therefore may act as a molecular bridge between the two systems. Two strong candidates were identified, TRAF2 and NFKB1, and their connections to insulin genes and mitochondrial genes were verified based on literature accessed on PubMed. TRAF2 is reported to be connected to the insulin genes MAP3K1 (MEKK1) and CAV1 (caveolin 1) and to the mitochondrial genes MAP3K5 (ASK1) and CASP8 (caspase-8). A possible connection to MTOR (mTOR) has also been proposed.

The important role of ROS in IR has been reinforced by the use of different antioxidant treatments in human studies [31]. In addition, glucose homeostasis and insulin signaling can be improved by

activators of PKC such as diacylglycerol (DAG), which can activate and phosphorylate IRS. For example, PKCε contributes to lipid-induced IR through crosstalk with p70S6K and recently identified regulators of insulin signaling [32]. DAG can modulate PKCθ under IR conditions such as those in T2D and obesity [33]. In fact, PKCθ knockout mice do not suffer IR in hyperlipidemic conditions [34]. Together, this evidence highlights the importance of mitochondrial dysfunction in the induction of IR through activation of PKCs.

Other clinical studies have highlighted the relationship between IR and mitochondrial disturbance, including reduced number of mitochondria, altered mitochondrial morphology, and decreased functionality of ETC complexes [35]. In insulin-resistant patients, aerobic exercise can stimulate mitochondrial biogenesis and function, leading to increased insulin action [36]. Moreover, normal mitochondrial activity in skeletal muscle has been related negatively to hepatic lipid accumulation and positively to insulin sensitivity and glucose homeostasis [37]. The antioxidant alpha-lipoic acid can improve ER stress under IR conditions by enhancing mitochondrial function in hepatic cells [38], while the mitochondria-targeted antioxidant MitoQ can modulate mitochondrial function, ER stress, and insulin signaling in β-cells exposed to hyperglycemia [39].

Day *et al*. have demonstrated that alterations of DNA methylation in whole-blood are strongly associated with obesity and IR in humans [40]. They are currently developing a clinical trial to determine whether differences in human skeletal muscle DNA methylation patterns in the mitochondrial and nuclear genome explain the lower abundance of ETC mRNA and proteins observed in insulin-resistant skeletal muscle of obese and T2D patients (clinical trial NCT04126551; https://clinicaltrials.gov/ct2/show/NCT04126551). Another clinical trial has evaluated the function of mitochondria in T2D and revealed that reduced mtROS in peripheral blood mononuclear cells is associated with improved endothelial function and metabolic control in patients with T2D and periodontitis, pointing to mtROS as a therapeutic target to prevent CVD in T2D [41].

As already mentioned, hyperglycemia can induce mitochondrial dysfunction, β-cell impairment and T2D (Figure 2). Mitochondria control the production of insulin in β-cells in an ATP/ADP ratio-dependent manner; when the ATP/ADP ratio is high, ATP-dependent potassium channels are closed and insulin is secreted [42]. In fact, T2D can lead to a marked inhibition of the mitochondrial metabolism of β-cells and impede the secretion of insulin [23]. Under hyperglycemia conditions, the levels of intracellular Ca^{2+} increase in β-cells, enhancing ADP consumption and increasing mitochondrial membrane potential ($\Delta\Psi_m$), which in turn increases ROS production [42]. In addition, in T2D, mitochondria become damaged and their dysfunction can alter Ca^{2+} transport, thus enhancing mitochondrial permeability transition (MPT) and leading, eventually, to apoptosis [42]. Furthermore, dysregulation of intracellular Ca^{2+} homeostasis due to mitochondrial dysfunction or defects in the function of mitochondria-associated ER membranes (MAMs) has been implicated in the pathogenesis of insulin insensitivity and T2D. These results underline the importance of Ca^{2+} levels for β-cell function and insulin action and release in T2D [43].

Therefore, there is considerable accumulated evidence that mitochondria play a key role in IR and are thus key targets for its treatment. Lipodystrophy can also induce T2D and IR in humans [44], by interfering with the molecular pathways involved in T2D, such as autophagy, ER stress, and inflammasome activation.

Autophagy and the Pathophysiology of T2D

Eukaryotic cells possess two major machineries for degradation: the ubiquitin-proteasome system and autophagy-lysosomal pathways (ALPs). Autophagy, a phenomenon preserved during evolution, from yeast to mammals, is a homeostatic process that controls cytoplasm quality by

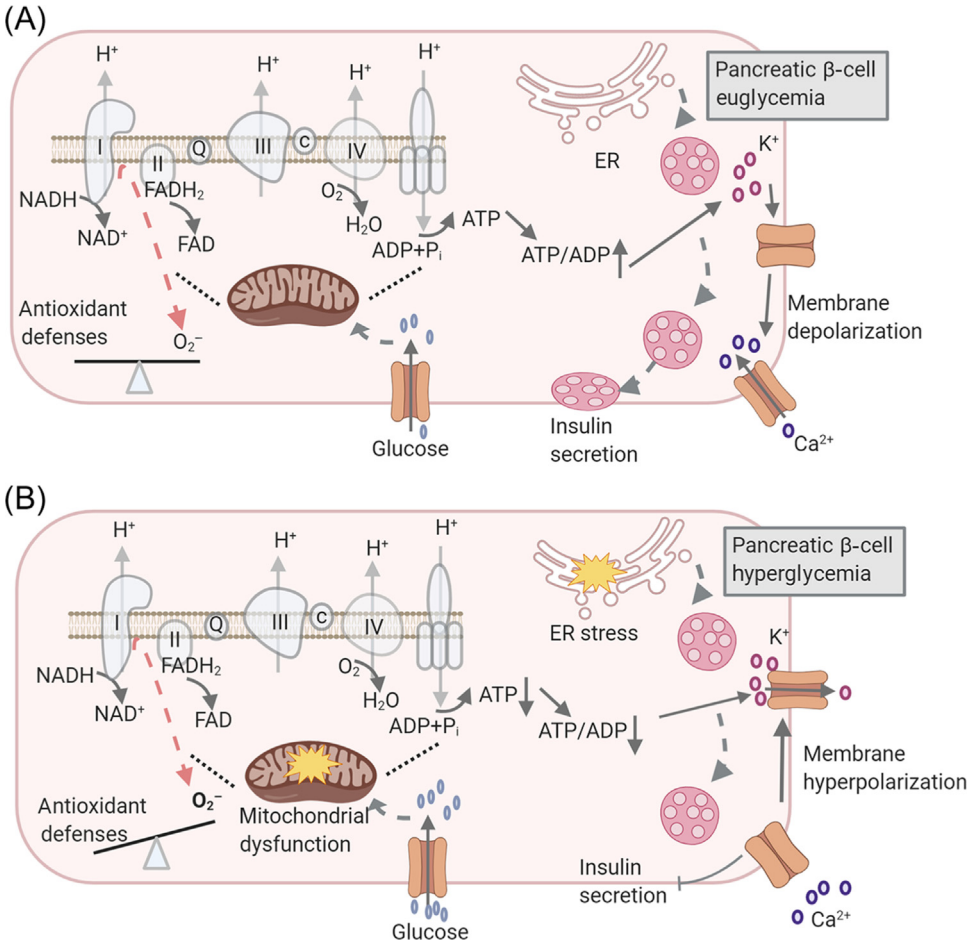

Figure 2. β-Cell Function in Normal Conditions and in Type 2 Diabetes (T2D). (A) Under normal conditions, glucose is metabolized by glycolysis and mitochondria generating ATP. Therefore, the ATP/ADP ratio is high and the ATP-sensitive K^+ channels are closed; the plasma membrane is depolarized and favors Ca^{2+} influx in the cell, which eventually promotes the release of endoplasmic reticulum (ER)-synthesized insulin. (B) In T2D conditions, hyperglycemia promotes mitochondrial dysfunction in β-cells, as well as ER stress. ATP synthesis is undermined and ATP-sensitive K^+ channels can be opened; the plasma membrane is hyperpolarized and Ca^{2+} channels are closed, blocking Ca^{2+} influx and, as a consequence, impeding insulin secretion. This figure was created using BioRender (https://biorender.com/).

eliminating misfolded proteins/protein aggregates, damaged or unnecessary organelles, or even pathogens by their degradation in the lysosome [45]. The term 'autophagy' encompasses several mechanisms classified as macroautophagy, chaperone-mediated autophagy, and microautophagy.

Macroautophagy is the major component of the ALPs and is therefore often simply termed 'autophagy' (a nomenclature that is also used in this review). The molecular regulation and machinery of general autophagy has been extensively described in several other reviews [46] and will not be discussed in further depth here. In this process, a double-membrane vesicle called the autophagosome (which carries the cytoplasmic cargo to be degraded) fuses with the lysosome, giving rise to a structure denominated the autophagolysosome, in which the cargo is proteolytically digested. The degraded products are then transported back to the cytosol, where they can be reused for biosynthesis or energy production. Apart from bulk autophagy, which is

considered a nonselective mechanism (usually triggered by nutrient deprivation), more than 15 other types of selective autophagy have been described, including the elimination of specific targets such as organelles (ER, ERphagy; mitochondria, mitophagy; peroxisomes, pexophagy), subcellular structures (lipid droplets, lipophagy; proteasome, proteaphagy; glycogen, glycophagy), and extracellular complexes (pathogens, xenophagy).

Autophagy and β-Cell Function

Over the past decade, specific functions of autophagy have been discovered in many tissues and organs and it has become evident that abnormal upregulation or downregulation of autophagy is an attribute of a variety of pathologic conditions [47].

Numerous *in vitro* and *in vivo* studies have shown that autophagy is critical for the maintenance of β-cell function and survival under normal conditions and acts as an adaptive mechanism in stress conditions by preventing β-cell failure. Impaired autophagy results in reduced β-cell mass and pancreatic insulin content, due to increased apoptosis and decreased proliferation of β-cells [48]. Autophagy plays an active role in the regulation of insulin homeostasis, in addition to its role in β-cell survival [49], as inhibition of autophagy by small interfering RNA-mediated knockdown of *Atg5/7* leads to increased secretion of proinsulin and insulin. Likewise, pharmacological stimulation of autophagy also reduces insulin secretion from mouse and human islets [50].

Multiple stress stimuli are known to upregulate and be alleviated by autophagy in β-cells, including ER stress, oxidative stress, glucolipotoxicity, and mitochondrial dysfunction (Figure 3). A functional ER is critical for the normal functioning of β-cells, since these cells face a constantly high protein-folding burden by which they synthetize proinsulin, converting it into its mature form and secreting it. Numerous insults (oxidative stress and dysregulation of Ca^{2+} homeostasis, among others) can produce ER stress in β-cells, which is manifested as protein translation interruption, unfolded/misfolded protein overload, and molecular chaperone synthesis. β-cell ER stress has been described as a stimulus of autophagy, which mediates degradation of misfolded proteins or dysfunctional regions of the ER, thus promoting the homeostasis of β-cells [51]. The opposite has also been reported in these cells, as insufficient autophagy compromises the unfolded protein response (UPR), thereby promoting diabetes [52]. β-cells are also highly susceptible to oxidative stress, particularly of the hyperglycemia-induced type, which leads to impaired insulin secretion and cell death [53]. Induction of the antioxidant nuclear factor erythroid 2-related factor 2 (Nrf2) in these cells prevents cellular damage and apoptosis through the transcription of autophagy genes, while its knockout results in lower β-cell mass [54]. Interestingly, autophagy can also promote Nrf2 activation through p62/SQSTM1-mediated disruption of its interaction with the Kelch-like ECH-associated protein 1 (KEAP1) [55], a repressor that binds to Nrf2 and promotes its degradation via the ubiquitin-proteasome pathway.

Autophagy also combats oxidative stress through the degradation of dysfunctional mitochondria via mitophagy. The best understood mitophagic pathway is PINK1-Parkin-mediated mitophagy, which is stimulated by a reduction in $\Delta\Psi_m$, often linked to moderate elevations in ROS [56]. The kinase PINK1 accumulates on the outer mitochondrial membrane (OMM) of depolarized mitochondria, resulting in the recruitment of its target, the E3 ubiquitin ligase Parkin. Parkin then ubiquitinates multiple proteins on the OMM, leading to the recruitment of both proteasomes and autophagosomes.

β-cells are highly susceptible to changes in nutrient availability, and autophagy has been described as a fundamental metabolic regulator of these cells. Interestingly, short-term nutrient deprivation stimulates a secretory insulin granule-specific autophagic process called β-cell

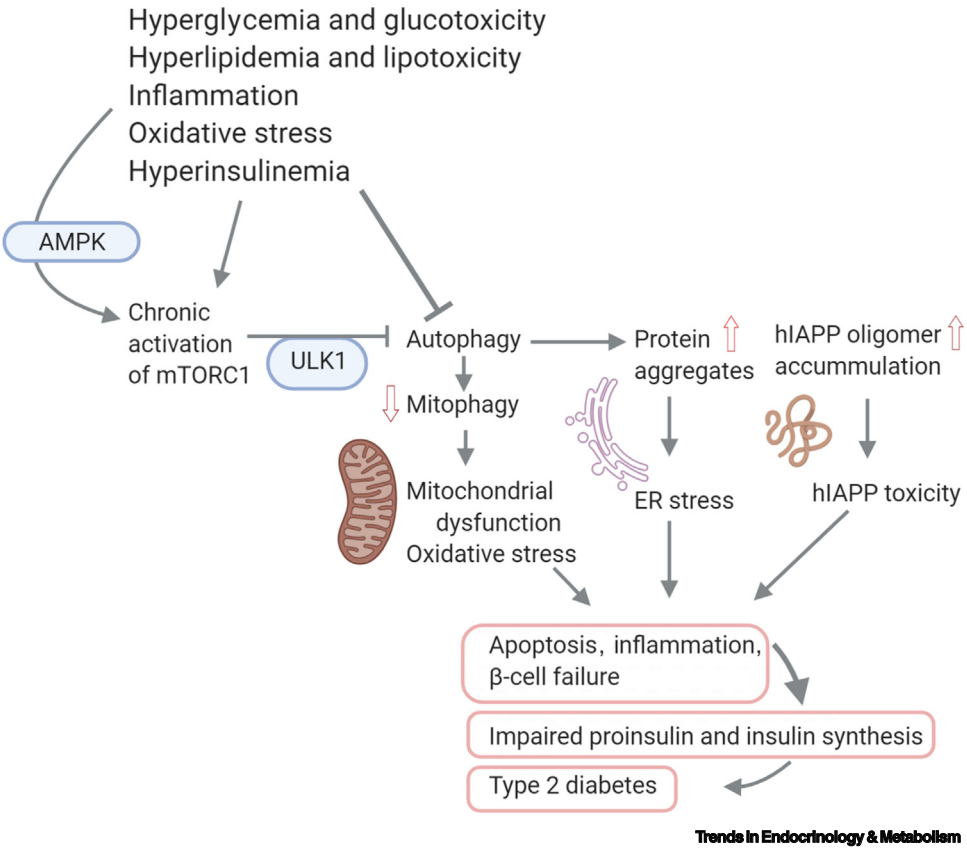

Figure 3. Autophagy and the Pathophysiology of Type 2 Diabetes (T2D). Dysregulated/impaired autophagy is viewed as an important contributing factor to the pathogenesis of T2D. In β-cells, it occurs as a result of the action of multiple stressful stimuli, including glucotoxicity, lipotoxicity, inflammation, presence of oxidative stress, and hyperinsulinemia, which can activate AMPK (AMP-activated protein kinase), a major bioenergetic sensor and autophagic regulator in the cell. Furthermore, mTORC1 inhibits autophagy via phosphorylation of ULK1 (Unc51-like kinase, hATG1). Poor autophagy results in accumulation of protein aggregates, including human islet amyloid polypeptide (hIAPP), and of damaged and dysfunctional mitochondria, due to impaired selective removal of mitochondria through mitophagy. These effects leads to β-cell failure and cell death, both hallmarks of diabetes. This figure was created using BioRender (https://biorender.com/). Abbreviations: ER, Endoplasmic reticulum.

crinophagy, while longer-lasting starvation induces classic autophagy [57]. Moreover, β-cell autophagy is stimulated by free fatty acids including palmitate [58], cholesterol [59], omega-3 fatty acids [60], and vitamin D [61], thereby playing a protective role and preventing the activation of apoptosis. Finally, glucagon-like peptide-1 (GLP-1) receptor agonists (exendin-4 and liraglutide), as well as a dipeptidyl peptidase-4 (DPP-4) inhibitor (MK-626), are known to stimulate β-cell autophagy and improve β-cell function *in vitro* and *in vivo*, protecting them against glucolipotoxicity and tacrolimus-induced β-cell death [61,62].

Altered Autophagy as a Contributing Factor of T2D

Dysregulated/impaired autophagy has been described as an important contributing factor to the pathogenesis of T2D [63]. An increased number of autophagosomes and accumulation of p62/SQSTM1 have been detected in rodent β-cells from insulin-resistant and diabetic models and in β-cells of T2D patients [57]. The causal mechanism for the blockage of β-cell autophagic flux may involve lysosomal defects in response to chronic glucolipotoxicity [64]. The importance of autophagy in this context, particularly in regard to metabolic adaptation of β-cells, has

been highlighted. Atg7$^{f/f}$: RIP-Cre mice display reduced serum insulin levels and impaired glucose tolerance [65]; however, Atg7$^{+/-}$ heterozygote mice show no anomalies in their systemic metabolic profile in a basal metabolic state, though they do develop severe diabetes when crossed with leptin-deficient (*ob/ob)* mice or fed a high-fat diet (HFD) [66]. Numerous reports have provided evidence that HFD and metabolic stress upregulate β-cell autophagy as a protective mechanism; thus, impairment of β-cell autophagy leads to deleterious metabolic effects [67]. In line with this, the overexpression of Atg5, which enhances autophagic activity, improves the metabolic profile of aged mice, including insulin sensitivity [68].

The progression to T2D is characterized by an increasing alteration of mitochondrial morphology and function. One of the proposed underlying mechanisms involves long-term exposure to glucose and fatty acids, which leads to glucolipotoxicity and high levels of ROS and metabolic stress. This overwhelms the mitochondrial homeostatic mechanisms, impairing mitophagy and causing an accumulation of dysfunctional and damaged mitochondria (Figure 3). Impaired mitophagy has been related to reduced glucose-responsive insulin secretion in β-cells [69]. Glucolipotoxicity also causes accumulation of cytoplasmic p53, which blocks the mitochondrial translocation of Parkin and inhibits mitophagy [70]. Human subjects with T2D exhibit a decreased expression of components of the mitophagy pathway [71], while mutations in mitophagy-related genes, including *PINK1*, *PARKIN*, *CLEC16A*, and *PDX1*, have been associated with both T1D and T2D in humans [72]. Extensive bibliography points to the enhancement of mitophagy as a potential mechanism to preserve β-cell function and delay the progression of T2D.

Another phenomenon related to diminished autophagic clearance in β-cells is human islet amyloid polypeptide (hIAPP) aggregation (Figure 3), which is thought to be one of the main contributors to pancreatic demise during T2D in humans, but not in rodents [73].

In summary, it is evident that autophagy is vital for the normal functioning of β-cells and that T2D is related to diminished pancreatic autophagy. In fact, the protective effects of the antidiabetic drug metformin against the demise of β-cells has also been linked to autophagy enhancement, related to mTORC1 inhibition, with or without implication of AMPK activation, and other mechanisms [74].

Besides the pancreas, altered autophagy has been described in several other organs implicated in the pathogenesis of T2D; indeed, an important role in the development of typical diabetic complications, including diabetic nephropathy, retinopathy, cardiomyopathy, and neuropathy, has been suggested. The process of adipogenesis, in which adipocytes differentiate from primary fibroblasts, requires profound cytoplasmic reorganization, achieved through massive autophagy. T2D and obese patients have been shown to display increased autophagic flux in the adipose tissue in comparison with lean individuals without diabetes [75]. The authors of the study in question suggested that increased autophagy in visceral adipose tissue of obese patients represents a protective effect against adipose tissue apoptosis, which converts to increased adipose tissue apoptosis under conditions of obesity-related T2D.

Skeletal muscle is the major site of insulin-stimulated glucose uptake and is thus an important site of IR in obesity and T2D. IR in human skeletal muscle is characterized by decreased insulin-stimulated glucose disposal and metabolism, triacylglycerol accumulation, and reduced content and functional capacity of mitochondria [76]. Studies in transgenic mice have demonstrated that insufficient autophagy is associated with an impaired function of insulin-sensitive tissues, including skeletal muscle [77], and autophagy-deficient skeletal muscle displays many of the same characteristics as insulin-resistant muscle, including mitochondrial dysfunction. At present, it seems that autophagy signaling is undermined by IR, but it is unclear if this is due to an intrinsic

defect in autophagy or a response to hyperinsulinemia. Moreover, the emerging autophagy-glycemia-insulinemia regulatory network is extremely complicated. Insulin has been shown to inhibit autophagy in human skeletal muscle; however, autophagy continues to be responsive to the suppressive effects of insulin in insulin resistant and obese mice [78]. In addition, infusion with physiological insulin concentrations has been shown to decrease the skeletal muscle protein content of LC3B-II, a key marker of autophagosome formation, in both lean and obese individuals. This response was absent in diabetic patients in a state of euglycemia and became normalized when they were under conditions of fasting hyperglycemia [79]. A potential mechanism by which IR suppresses autophagy is the activation of mTORC1 by phosphorylation at Ser^{2448} [80]. Attempts to assess whether basal autophagy is dysregulated in skeletal muscle of T2D patients have provided conflicting findings, with both downregulation [81] and no difference being reported with respect to healthy controls [79].

ER Stress and Mitochondrial Dysfunction in T2D

ER stress occurs when protein biosynthesis, the secretory pathway, folding capacity, and the cell death pathway are drastically compromised [82]. Over-nutrition [83], obesity [84], diabetes [85], and their related metabolic disorders can lead to the accumulation of misfolded proteins in the ER, consequently leading to ER stress. The core component of the process is a triad of stress-sensing proteins, inositol-requiring enzyme 1 (IRE1), protein kinase R-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6 (ATF6), which participate in the transcriptional regulation of UPR, in protein synthesis and in promoting the interaction between inflammation and apoptosis [86]. Interestingly, oxidative stress disturbs the ER redox state, thereby disrupting disulfide bond formation and protein folding, causing ER stress and mitochondrial dysfunction [87].

The induction of cell death is a key component of unresolved ER stress. The most significant ER stress-induced apoptotic pathway is mediated through CHOP/GADD153, which is a bZiP transcription factor induced through the ATF6 and PERK pathways [88] (Figure 4). CHOP activates the transcription of *Gadd34*, *Ero1*, *Dr5* (death receptor 5), *Trb3* (Tribbles homolog 3), and carbonic anhydrase VI and represses the transcription of the antiapoptotic Bcl2 proteins. Bcl2 family proteins are also fundamental to ER stress-induced apoptosis (Figure 4). The activation of Jun kinase (JNK) though IRE1/TRAF2/apoptosis-signal-regulating kinase 1 (ASK1)/TNF-R signaling [89] also induces phosphorylation of Bim (Bcl2-interacting mediator of cell death), inhibiting its binding to dynein and myosin V motor complexes [89]. It then translocates to the ER membrane and activates caspase-12 in response to ER stress, whereas an antiapoptotic factor, BCL-xL (Bcl2-like 1), binds to BIM and inhibits its translocation [90]. The pathway is activated when ROS disrupts the redox-sensitive ASK1-thioredoxin complex, leading to activation of JNK, p38 MAP kinase, and cell death [91]. In addition, BAX and BAK oligomerization and the increase in Ca^{2+} release from the ER, induce depolarization of the inner mitochondrial membrane (IMM), cytochrome *c* release, activation of apoptosis protease-activating factor 1 (APAF-1), and procaspase-9-dependent apoptosis [92]. The induction of oxidative stress also promotes the leaking of Ca^{2+} from the ER lumen [93]. In addition, the increase of Ca^{2+} release stimulates calpain-dependent ER-associated caspase-12 activation [94].

Increases in cytosolic Ca^{2+} can stimulate mtROS production through multiple mechanisms. As previously described, ROS are generated during the normal physiological activity of the mitochondrial ETC, which can be increased during mitochondrial Ca^{2+} loading [95]. Ca^{2+} opens the MPT pore to release cytochrome *c* from the IMM, blocking the electron flow through complex III that leads to the accumulation of ubisemiquinone radical intermediate ($QH^{\bullet}$) and promotes site-specific superoxide anion generation. The increase of Ca^{2+} also stimulates the TCA cycle, which increases the electron mitochondrial flow; this, in turn, enhances the risk of oxidative stress,

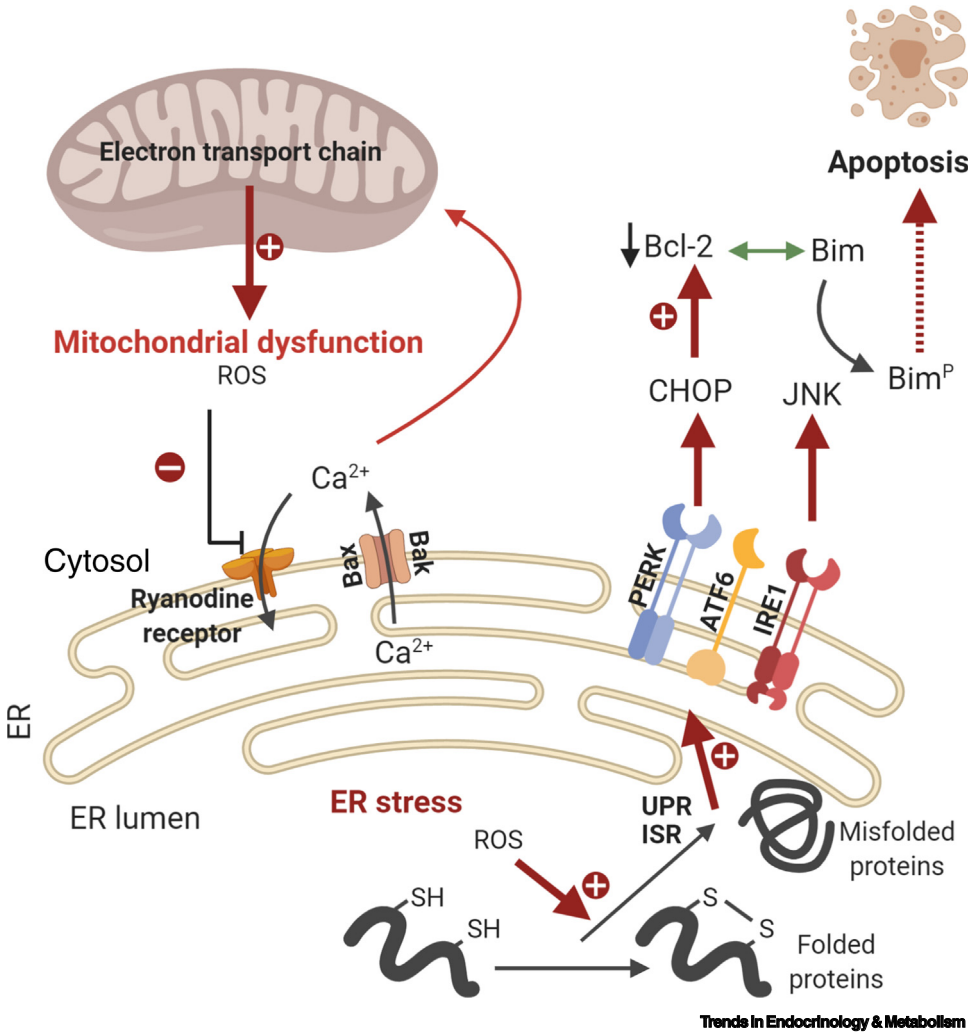

Figure 4. Crosstalk between Endoplasmic Reticulum (ER) and Mitochondria. The induction of ER stress by accumulation of misfolded proteins activates the three branches of the unfolded protein response (UPR), PERK, ATF6, and IRE1, and can lead to cell death. Mitochondria-dependent oxidative stress promotes ER transitory calcium release through activation of proapoptotic Bcl-2 family member, Bax and Bak, which further promotes mitochondrial dysfunction and sustained ER stress. This figure was created using BioRender (https://biorender.com/). Abbreviations: ATF6, Activating transcription factor 6; IRE1, inositol-requiring enzyme 1; ISR, integrated stress response; JNK, Jun kinase; PERK, protein kinase R-like endoplasmic reticulum kinase; ROS, reactive oxygen species.

stimulates nitric oxide release (thus inhibiting complex IV of the ETC), and induces glutathione (GSH) leaking upon opening of the MPT pore. The exacerbation of Ca^{2+} release during oxidative stress is also promoted through oxidation of critical thiol, which leads to inactivation of the ryanodine receptor, thereby enhancing Ca^{2+} release from the ER [96].

The Inflammasome and Mitochondrial Dysfunction and Their Role in T2D

T2D patients exhibit a low-grade chronic inflammatory state, with enhanced levels of cytokines and adipokines and activation of proinflammatory pathways [97]. Several studies have demonstrated that increased expression of proinflammatory cytokines plays an important role in T2D complications such as retinopathy, neuropathy, nephropathy, and CVD [98]. Two fundamental cytokines involved in these complications are IL-18 and IL-1β, both of which promote IR, impair

β-cell function, and induce apoptosis [99]. These mediators are generated by a set of intracellular sensors of immune response, after recognition of cytoplasmic pathogen patterns (PAMPs) and damage-associated molecular patterns (DAMPs) as exogenous and endogenous factors, respectively [100]. This initiates an innate immune response (IIR) from immune cells [101] and the inappropriate unleashing of IIR can exacerbate T2D complications [102]. The aforementioned intracellular sensors are known as the inflammasome protein complex [103]. Inflammasome assembly and activation requires a pattern recognition receptor (PRR) as a sensor, usually the ASC adaptor (apoptosis-associated spec-like protein containing a CARD) and caspase-1. PRRs include membrane-bound Toll-like receptors (TLRs) and C-type lectins (CTLs), which scan the extracellular milieu for PAMPs and DAMPs [100]. These intracellular receptors are known as NLRPs [nucleotide oligomerization domain (NOD), leucine-rich repeat (LRR), and pyrin domain (PYD)] and also as NALP [103].

A NOD-like receptor has been identified, and to date there are at least 20 known human NLR genes [103]. In addition, it has been described that the biological function of inflammasomes is to activate caspase-1, which leads to the maturation of IL-1β and IL-18 and the induction of pyroptosis [104].

NLRP3 is the most studied inflammasome complex and plays an important role in the proinflammatory response. To become completely activated, NLRP3 requires a priming stage, which is mediated by PAMPs and DAMPs or by inflammatory cytokines, and enhances the expression of inflammasome components [100]. The activation stage leads to NLRP3 inflammasome and caspase-1 maturation [105].

The NLRP3 inflammasome, an innate immune signaling complex, is the key mediator of IL-1 family cytokine production in atherosclerosis. NLRP3 is activated by various endogenous danger signals that abound in atherosclerotic lesions, such as oxidized low-density lipoprotein and cholesterol. Consequently, NLRP3 inflammasome activation contributes to the vascular inflammatory response that drives atherosclerosis development and progression [106]. Furthermore, inflammation is an important promoter of atherosclerosis, the underlying pathology of CVD; therefore, the therapeutic targeting of inflammatory pathways may improve cardiovascular outcome in patients with CVD. This idea has been pushed by the Canakinumab Anti-Inflammatory Thrombosis Outcomes Study (CANTOS) trial group, which demonstrated the therapeutic potential of the monoclonal IL-1β-neutralizing antibody canakinumab [107].

Under noninfectious conditions, a broad array of external stimuli, metabolism-related molecules, and cellular events have been implicated in the activation of the NLRP3 complex, including environmental or industrial particles and nanoparticles, extracellular ATP, K^+ efflux, Ca^{2+} signaling, ROS, monosodium urate crystals, amyloid-beta fibers, mitochondrial dysfunction, and lysosomal rupture structures [108].

In this sense, disturbance of intracellular metabolites can lead to mitochondrial dysfunction and apoptosis [109]. For example, these complexes can produce pro- and anti-inflammatory signals by modifying the levels of molecules generated in the TCA cycle [110]. Metabolic events in mitochondria also seem to have significant effects on immunity [111]. Consequently, mitochondria are perfectly positioned to regulate and control NLRP3 activation, functioning as a signaling hub for the activation of IIR [112]. Although the exact molecular mechanism that triggers inflammasome activation by mitochondrial metabolites is unclear, mitochondria are known to function as an effective scaffold for NLRP3 inflammasome assembly [113]. Recent research has established a link between the NLRP3 inflammasome and mitochondria, suggesting that the activation of

NLRP3 induces the transfer of NLRP3 from the ER to MAMs, where it forms a functional inflammasome complex linked with ASC and caspase-1 [113].

Several studies have demonstrated the critical role of mitochondrial damage in triggering the NLRP3 inflammasome complex in various diseases [114]. The accumulation of damaged mitochondria and higher concentrations of mtROS in response to autophagy/mitophagy inhibition have been associated with the release of IL-1β by macrophages. The silencing of autophagic machinery in mice promotes the accumulation of damaged mitochondria, augmenting mtROS and promoting NLRP3 inflammasome activation [115]. Stimulating mitophagy in macrophages is also thought to lead to the suppression of NLRP3 inflammasome activation and enhancement of β-cell dysfunction [116] (Figure 5). Therefore, mitophagy could be a preventive tool against

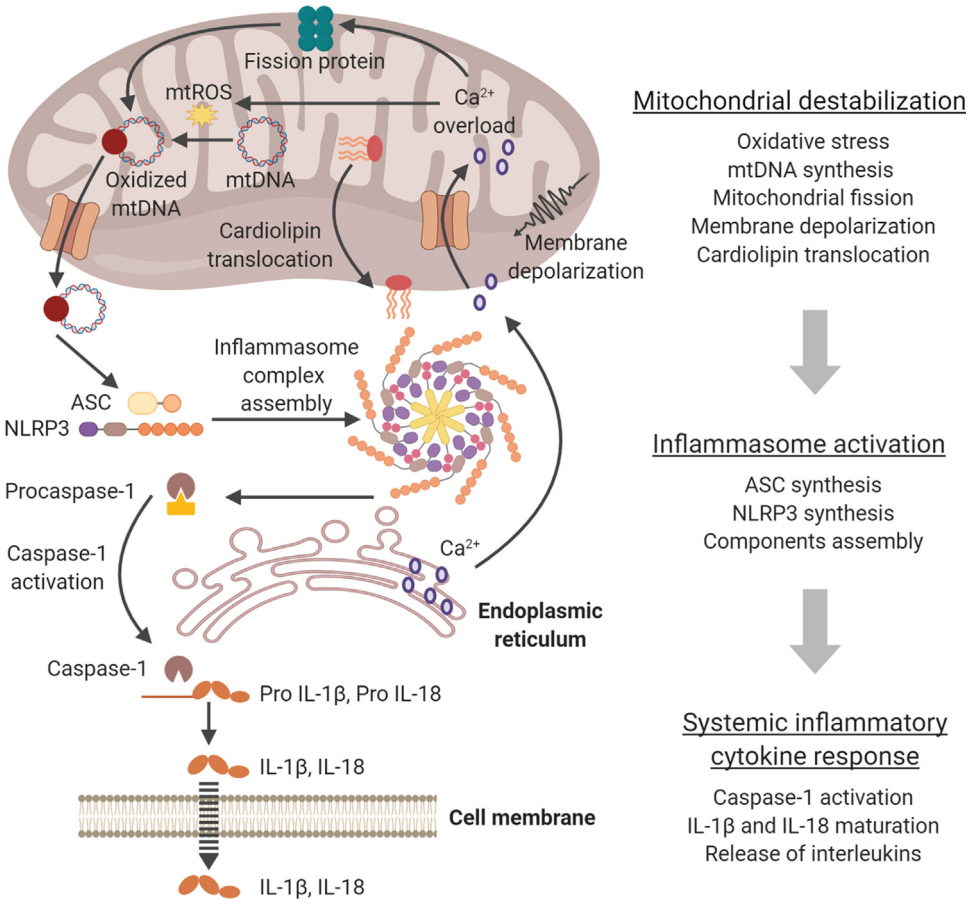

Figure 5. Mitochondria as a Signaling Hub for NLRP3 Inflammasome Activation and Cytokine Production in Type 2 Diabetes (T2D). T2D is characterized by a cluster of metabolic factors that induce mitochondrial destabilization through increased reactive species generation, a higher mitochondrial fission, increased mitochondrial DNA (mtDNA) synthesis, membrane depolarization, and cardiolipin translocation. This metabolic situation activates the synthesis and assembly of the different inflammasome complexes (ASC and NLRP3). Subsequently, the inflammasome complex triggers procaspase-1 activation, which is followed by interleukin (IL)-1β and IL-18 maturation. Finally, interleukins are released into the systemic circulation, thus promoting a state of chronic inflammation in T2D. This figure was created using BioRender (https://biorender.com/). Abbreviations: ASC, Apoptosis-associated spec-like protein containing a CARD; mtROS, mitochondrial reactive oxygen species; NLRP, nucleotide oligomerization domain (NOD), leucine-rich repeat (LRR), and pyrin domain (PYD).

NLRP3 inflammasome activation. In the same way, mitochondrial dynamics, specifically fusion and fission processes, are altered in the leukocytes of T2D patients [117]. In fact, recent investigations have revealed that poor glycemic control during T2D can alter mitochondrial dynamics and NLRP3 and enhance CVD [118]. The authors in question described an increased expression of several proteins involved in mitochondrial fission and a decrease in the mitochondrial fusion accentuated in patients with poor glycemic control, suggesting that glycemic control plays a key role in the immune response of diabetic subjects through NLRP3 alteration [118].

NLRP3 may also be activated directly by mitochondria-derived effector molecules such as mtDNA and the phospholipid cardiolipin (CL). It has been reported that inflammasome activation depends on the binding of mtDNA to the NLRP3 inflammasome complex [119]. mtDNA is released into the cytosol during NLRP3 inflammasome activation by ATP, probably through the MPT pore [119]. In line with this evidence, it has been shown that NLRP3 can co-immunoprecipitate with mtDNA. Other studies have demonstrated that blocking ANT1, a central protein mediating MPT, prevents a collapse of $\Delta\Psi_m$ and the subsequent release of mtDNA and mtROS, thus avoiding hyperactivation of the NLRP3 inflammasome [120]. Recent research shows that the new synthesis of mtDNA and its further oxidation by ROS plays a critical role by triggering IIR and activating the NRLP3 inflammasome [121,122]. The authors highlighted the fact that mtDNA acts as a DAMP [122], describing how mitochondrial sensing of innate-immunity triggers can lead to mtDNA synthesis and that activation of TLR4 triggers a pathway that drives expression of the enzyme CMPK2, which is required to produce the nucleotide cytidine triphosphate (CTP). The authors hypothesized that newly synthesized mtDNA is particularly susceptible to DNA damage by ROS due to a lack of protection from the nucleoid proteins that usually protect mature mtDNA [121]. In this sense, novel investigations have shown that serum cell-free mtDNA in T2D subjects contributes to NLRP3 inflammasome-mediated chronic inflammation [123] and could be a promising serum marker of the inflammasome activation.

CL is a bioactive lipid located in the IMM and has a unique structure among phospholipids, as it is formed by two diacylated phosphatidyl groups linked by a glycerol bridge [124]. Some studies show that CL co-immunoprecipitates with NLRP3 from mitochondrial extracts after cell treatment with various ROS-dependent or -independent triggers, suggesting an interplay between CL and NLRP3. The authors in question also proved that preventing CL synthesis reduces NLRP3 activation [125]. Another study described that mitochondrial damage is enough to promote CL externalization to the OMM [126]. Iyer *et al*. postulated that, because CL is found only in mitochondria and bacteria, it functions as an endogenous PAMP that is revealed upon mitochondrial dysfunction and is detected by NLRP3 [125]. Furthermore, several studies in humans, animal, and cell models have demonstrated that T2D and its comorbidities are characterized by an alteration in cellular CL levels [127]. Specifically, in the human heart of T2D patients, a higher rate of incorporation of docosahexaenoic acid into CL has been related to a decrease in mitochondrial mass, an alteration in mitochondrial morphology, and an increase in mtROS production. Toledo *et al*. reported that physical activity and weight loss restore CL levels in muscle cells, showing that intensive short-term lifestyle modifications can modify mitochondrial content and functional capacity [128].

However, Horng has put forward an interesting theory which holds that a broad range of stimuli represent an intermediate step in the process of destabilization of mitochondria by the Ca^{2+} signaling pathway, by generating mitochondrion-associated ligands that activate the NLRP3 inflammasome complex [129]. It is important to point out that ER Ca^{2+} release is closely coordinated with mitochondrial Ca^{2+} uptake to control mitochondrial function. In fact, a high Ca^{2+} influx can lead to mitochondrial damage, increasing mtROS levels and MPT. Suppression of Ca^{2+} translocation reduces mtROS production and mitochondrial membrane depolarization, as well

as inhibiting inflammasome activation. Research has also shown that the triggering of NLRP3 causes a drop in $\Delta\Psi_m$, further implicating mitochondria in the activation of the NLRP3 inflammasome. Bañuls *et al*. demonstrated that $\Delta\Psi_m$ depolarization of leukocytes correlates with the presence of comorbidities in metabolically unhealthy obese versus healthy obese subjects [130].

When the evidence is considered as a whole, inhibition of the NLRP3 inflammasome signal by different molecules appears to be an effective mechanism for attenuating some neurologic diseases, metabolic disorders (i.e., obesity and T2D), and chronic inflammatory diseases [131]. In addition, metformin, the most widely employed oral antidiabetic drug, has been described as a critical regulator of the NLRP3 inflammasome [132]. Although the exact molecular mechanism is not fully understood, Iannantuoni *et al*. demonstrated an important inhibition of NLRP3 in T2D patients treated with metformin, as well as a decrease in proinflammatory cytokine production [118]. This emerging role for the NLRP3 inflammasome as a sensor of metabolic stress with important functions in the development of T2D and associated comorbidities has attracted much attention. Deciphering the molecular events underlying NRLP3 inflammasome activation and its relation with systemic inflammation could be the key to a promising pharmacological strategy to treat T2D complications.

In conclusion, the T2D phenotype is related to mitochondrial damage and mitochondrial function impairment, and increased mitochondrial turnover and biogenesis due to an accumulation of oxidized molecules and enhanced levels of systemic inflammation. The increased rate of mitochondrial disturbance/replacement can effect NLRP3 inflammasome regulation in different tissues and cell types, thus playing a critical role in T2D complications. These findings reaffirm the link between mitochondria and inflammatory signaling in the IIR, which may echo the organelle's early evolutionary origins as a bacterial cell.

Concluding Remarks and Future Perspectives

T2D is a leading cause of multiple comorbidities and its high prevalence demands new treatments and targets. Mitochondrial impairment and damage are closely related to T2D; therefore, it is vital to develop new pharmacological and nonpharmacological strategies in order to modulate biogenesis, recycling, and mitochondrial metabolism, as well as ROS production.

Autophagy is an important regulatory signaling pathway for T2D and its complications, as it controls glucose and lipid metabolism and insulin secretion, and affects many other tissues besides β-cells. There is abundant evidence that autophagy promotes β-cell survival by enabling adaptive responses to prevent or mitigate the detrimental effects of ER stress, mitochondrial dysfunction, and oxidative stress. Thus, identifying mechanisms of β-cell autophagy regulation under these conditions, in order to better understand β-cell survival and develop therapies that target β-cells directly, should be a major research goal.

Finally, higher rates of mitochondrial disturbance/replacement can effect NLRP3 inflammasome regulation in different tissues and cell types, thus playing a critical role in T2D complications. The intriguing link between mitochondria and inflammatory signaling, including effects on the innate immune system, underlies this relationship, which may echo the organelle's early evolutionary origin as a bacterial cell. Although our knowledge about the molecular mechanisms of diabetes has been largely improved, many fundamental issues related to the topic of this review remain unsolved (see Outstanding Questions) and are currently in the focus of intense research.

Outstanding Questions

Which specific molecular triggers govern the activation of the NRLP3 inflammasome in β-cells of T2D patients?

Which is the causal relation between T2D and ER stress?

Is there a specific autophagy-related molecular target in β -cells with potential therapeutic relevance in T2D?

Is mitochondrial dysfunction and damage the truly critical step, or the point-of-no-return, in β-cell demise in T2D?

What clues can help solve the insulin resistance-oxidative stress as the metabolic chicken and egg paradox?

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