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Review

Effects of estrogens in mitochondria: An approach to type 2 diabetes

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Abstract: Type 2 diabetes (T2D) is characterized by a state of hyperglycemia in the blood due to insulin resistance developed by organs such as muscle, liver, and adipose tissue. A common factor in individuals with T2D is mitochondrial dysfunction. Mitochondria are dynamic organelles responsible for energy and antioxidant metabolism in the cells. Estrogens, such as 17β-estradiol (E2), are steroid hormones that have shown a great capacity to regulate mitochondrial function and dynamics through estrogen receptors (ERs), modulating the expression of mitochondrial biogenesis-related genes and cell signaling mechanisms. The accumulation of reactive oxygen species, the low capacity for ATP synthesis, and morphological alterations are some of the mitochondrial processes impaired in T2D. Insulin signaling and secretion by pancreatic β-cells, ATP-dependent processes, are also altered in T2D. In this review, mitochondria were exposed as the central axis for the action of estrogens in individuals with T2D. Estrogens increased glucose uptake, insulin signaling, and mitochondrial bioenergetics, and decreased ectopic lipid accumulation in non-adipose tissues and oxidative stress, among other processes, in various preclinical and clinical models of diabetes. The development of strategies to target compounds to mitochondria could represent a novel therapeutic alternative to potentiate the effects of estrogens on this organelle in patients with insulin resistance and T2D.

Keywords: type 2 diabetes; mitochondria; estrogen; estrogen receptor; insulin resistance; mitochondrial dysfunction

1. Introduction

Type 2 diabetes (T2D) is a chronic disease that affects the way the body uses glucose. In people with T2D, there is insulin resistance, a condition in which the cells of the body have a reduced response

to the action of insulin. As a result, hyperglycemia develops. In the early stages of the disease, the pancreatic β cells secrete enough insulin for uptake in muscle and liver; however, an inability of the body to reduce blood glucose levels is noted. The intermediate state, before T2D, is called metabolic syndrome and is characterized by obesity, hypertension, high blood lipids, and slightly elevated fasting blood glucose [1]. If untreated, this can cause complications such as neuropathy, nephropathy, cardiovascular diseases, and eye diseases, such as retinopathy and eye loss [1]. The causes of T2D are not completely understood yet, but there is strong evidence that environmental factors, such as obesity and physical inactivity, contribute to the causes and disturbances of this disease. It is well known that a balanced diet, as well as a moderate intake of alcohol and a low intake of sugar-sweetened beverages, are related to a reduced risk of the development of T2D [2]. The International Diabetes Federation estimated in 2021, that 537 million adults aged 20-79 years old worldwide had diabetes, showing that the prevalence of diabetes in women is slightly lower than in men (10.8% vs 10.2%). In 2045, the number of adults affected is expected to rise to 783 million [3]. This makes T2D one of the diseases with the highest impact worldwide nowadays.

T2D is associated with changes in energy substrate levels in different tissues. This is because there is a correlation between T2D and mitochondrial dysfunction [4]. Mitochondria are the site for many critical cellular processes such as energy metabolism, regulation of reactive oxygen species (ROS) levels, and programmed cell death events. Mitochondrial dysfunction may contribute to the development of insulin resistance. Reduced efficiency in ATP generation and increased ROS production disturbs the ability of cells to respond adequately to insulin signaling. In addition, mitochondrial dysfunction may affect lipid metabolism and contribute to lipid accumulation in non-adipose tissues, such as the liver and muscle, which is associated with T2D. The alteration in all these mitochondrial processes, results in an impaired metabolism in vital organs such as the liver, muscle, and brain, as well as alterations in insulin secretion by pancreatic β-cells [4]. Estrogens, such as 17β-estradiol (E2), have shown great capacity to regulate events associated with mitochondrial dysfunction. In recent years, evidence has accumulated that points to estrogens as an attractive alternative to mitigate the pathophysiological aspects of T2D. This review integrates the evidence that shows how estrogens may represent an important pharmacological tool to mitigate the adverse effects of mitochondrial dysfunction with an approach to potential treatments for individuals with T2D.

2. Mitochondrial function and dynamics

Mitochondria are complex organelles made up of two membranes: the inner mitochondrial membrane (IMM), which forms the cristae and encloses the mitochondrial matrix; and the outer mitochondrial membrane (OMM), which separates the entire organelle from the cytoplasm. Between both membranes is a small subcompartment known as the intermembrane space (IMS). The mitochondrial matrix is the space where most of the cellular metabolism processes converge [5]. This organization plays an important role in the regulation of mitochondrial function and cellular response to energy and environmental demands. As discussed below, the major bioenergetic and antioxidant processes occur in this organelle.

2.1. Mitochondrial bioenergetics

Cells metabolize nutrients such as carbohydrates, fatty acids, and amino acids to form intermediates for the generation of energy in the form of ATP in the mitochondria. The oxidation of glucose and some glucogenic amino acids generates pyruvate. This metabolite must be transported to the mitochondrial matrix where it is transformed into acetyl-CoA in a reaction catalyzed by the pyruvate dehydrogenase complex (PDH) [6]. Then, acetyl-CoA is condensed with oxaloacetate to generate citrate. This reaction is catalyzed by citrate synthase (CS), which is an important marker to assess the metabolic state of cells and their mitochondrial content [7,8]. The main function of the TCA cycle is to generate the reducing molecules NADH and $FADH_2$ that deposit electrons in the electron transport chain (ETC) present in the IMM and provide the energy needed for proton pumping and subsequent ATP generation.

The ETC is composed of four large protein complexes (I-IV) and the electron carriers ubiquinone (coenzyme Q) and cytochrome c. This system uses the flow of electrons to pump protons towards the IMS. Complex I, also called NADH dehydrogenase (ND), is an enzymatic complex that catalyzes the transfer of a hydride ion from NADH and a proton to ubiquinone, and it pumps four protons from the matrix to the IMS [9]. On the other hand, complex II, also known as succinate dehydrogenase, catalyzes the oxidation of succinate (TCA cycle reaction) and transfers electrons to ubiquinone, through FAD and various Fe-S centers within the complex [10]. Subsequently, electrons pass from the reduced ubiquinone to cyt c through complex III (also called cytochrome bc_1 complex). The last reaction in the respiratory chain is catalyzed by complex IV, also called cytochrome c oxidase (COX). This complex transports electrons from cyt c to molecular oxygen, reducing it to H_2O. The transfer of electrons from NADH to O_2, through the ETC, allows protons to be pumped towards the IMS by complexes I, III, and IV. This creates a difference in proton concentration and electrical charge inside and outside the matrix. The energy stored in this electrochemical gradient is called the proton-motive force. This force is harnessed by the F_1F_O-ATP synthase complex (also referred to as complex V) to generate ATP in a process called oxidative phosphorylation (OXPHOS). The measure of the electrochemical gradient through the IMM is called the mitochondrial membrane potential ($\Delta\Psi_m$) and is generated due to the difference in ion distribution on both sides of the IMM. This gradient is essential for ATP generation [11]. The integral action of ETC and OXPHOS allows the generation of the greatest amount of ATP in the cell, and many diseases have been related to its dysfunction, including T2D [12,13].

Mitochondria are also the site of many important biosynthetic pathways, including that of fatty acids and cholesterol, which is the main precursor for steroid hormone biosynthesis [14]. Reactions for the synthesis of nucleotides, amino acids, heme, calcium homeostasis, mitophagy, regulation of cell death events, and inflammation also take place in this organelle [15]. The maintenance of mitochondrial function is vital for tissues and organs that are highly energy-dependent such as the liver, heart, and brain, as well as high energy-demanding tissue such as skeletal muscle. In skeletal muscle, the rate of oxidative respiration, and therefore mitochondrial content, can vary depending on the type of muscle fiber. Type I fibers called “slow-twitch fibers” can use the ATP produced by oxidative respiration and harness the energy for long-duration activities. On the contrary, type II fibers, or “fast-twitch fibers” use the ATP produced in glycolysis. Although glycolysis produces less ATP compared to OXPHOS, its production is very rapid and can be used immediately [16]. This energy-dependence of tissues and organs is why mitochondrial dysfunction is strongly linked to metabolic diseases such

as obesity and metabolic syndrome; as well as chronic diseases such as cancer, cardiomyopathies, neurodegenerative diseases, and primary mitochondrial rare diseases [17].

2.2. Mitochondrial homeostasis: The balance between biogenesis and mitophagy

Depending on the energy requirements of the tissue and environment, cells must coordinate a balance between the generation of new mitochondria by mitochondrial biogenesis, and the replacement of defective or damaged mitochondria by mitophagy. The generation of new mitochondria begins with the activation of peroxisome proliferator-activated receptor γ coactivator (PGC)-1α, considered the master regulator of mitochondrial biogenesis. PGC-1α stimulates the action of nuclear transcription factors such as nuclear respiratory factor 1 (NRF1), NRF2, and oestrogen-related receptor α (ERRα); as well as mitochondrial transcription factors (TFAMs) [18]. TFAMs are transported to the mitochondrial matrix to act as mediators of the transcription of genes encoded in mitochondrial DNA (mtDNA). There are 13 polypeptides encoded in mtDNA and they are part of the ETC and OXPHOS complexes [19]. Translation is coordinated by mitochondrial ribosomes, initiation factors (mtIF2, mtIF3), elongation factors (mtEF-TU, mtEF-TS, mtEF-G1), and termination factors (mtRRF1, mtRRF2), and it takes place in the mitochondrial matrix [20]. Proteins synthesized in the cytosol that must be imported to the mitochondria are associated with the TIM23 translocase [21]. All of these processes imply that there must be a very well-coordinated communication between the nucleus and the mitochondria. As will be described later, impaired mitochondrial biogenesis is one of the major processes associated with metabolic diseases, such as obesity and T2D.

On the other hand, mitophagy is a catabolic process where autophagosomes selectively degrade mitochondria in response to stress conditions. A deficit in the uptake of nutrients, a state of oxidative stress due to an accumulation of ROS, or a high rate of mutations in mtDNA can drive mitophagy. During mitophagy, autophagosomes engulf the damaged mitochondria forming mitophagosomes. These structures fuse with lysosomes, where the mitochondria are degraded by lysosomal enzymes [22]. Mitophagy is initiated when a stress situation or mitochondrial damage causes an accumulation of the mitochondrial serine/threonine kinase PINK1 activating the PINK1/Parkin pathway. Subsequently, a series of phosphorylation reactions are activated, including those acting on PINK1, allowing the recruitment of mitophagic proteins such as LC3 to the labeled mitochondria. LC3 participates in the formation of the autophagosome membrane that forms around the mitochondria. Once the autophagosome is formed it fuses with lysosomes for degradation and subsequent recycling of cellular components. Due to its importance, this pathway is strictly regulated [22,23]. Integrating the above, the maintenance of mitochondrial homeostasis, to preserve the energetic, metabolic, and antioxidant balance, depends on the coordination of mitochondrial biogenesis and mitophagy. These processes ensure the efficient and controlled generation and elimination of mitochondria contributing to the maintenance of cellular homeostasis.

2.3. Mitochondrial antioxidant system

The oxidative processes required for ATP synthesis in mitochondria generate about 50% of all ROS produced in the cell. ROS levels in the cell depend on the specific mitochondrial activity of each tissue and the cell type. The resulting physiological ROS concentrations are highly regulated and in homeostatic balance [24]. ROS are generated by the reduction of oxygen (O_2), mostly by complexes I

and III in the ETC. The major ROS in the cell context is superoxide radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$). The mitochondrial isoform of superoxide dismutase (MnSOD) catalyzes the conversion of superoxide to H_2O_2 [25]. Mitochondria have an antioxidant system consisting of peroxiredoxins (Prx3 and Prx5), thioredoxins (Trx2), and thioredoxin reductase (TRXR2) that sense and regulate cellular H_2O_2. This antioxidant system does not eliminate ROS but rather maintains them at adequate levels since ROS have a role in cell signaling to mediate proliferative and differentiation processes [26]. It has also been shown that low levels of oxidative stress in mitochondria are needed by cells.

Exposure to some types of mitochondrial stress, such as mild oxidative stress, can lead to adaptive responses that enhance mitochondrial function. This phenomenon is known as mitochondrial hormesis or mitohormesis [27]. This could help counteract processes associated with mitochondrial dysfunction in T2D. Moreover, exposure to mitochondrial stress can activate cellular signaling pathways, such as the AMP-activated protein kinase (AMPK) pathway and the sirtuin pathway, which are associated with the regulation of metabolism and insulin resistance [27,28]. These examples emphasize the importance of using mitohormesis as a strategy to explore cellular pathways that may reduce insulin resistance and the progression to T2D.

2.4. Mitochondrial dynamics

Mitochondria are not static or rigid organelles but are constantly changing in number, morphology, and distribution in the cell. This occurs mainly due to two key processes: Mitochondrial fusion and fission. Fusion implies the union of two mitochondria and is regulated by the mitofusin proteins (MFNs) 1 and 2, present in the OMM. MFN2 proteins can interact with each other or oligomerize with MFN1 to promote mitochondrial fusion [29]. MFN2 is also involved in the physical interaction between mitochondria and the endoplasmic reticulum, which requires Ca^{2+} signaling events [30]. These interactions occur through the mitochondria-associated endoplasmic reticulum membranes (MAMs), which have shown an important role in insulin signaling [31]. In turn, the protein optic atrophy 1 (OPA-1) participates in the fusion of the IMM of both mitochondria. The proteolytic cleavage of OPA1 results in the formation of two polypeptide chains: Long-OPA1 (L-OPA1) and short-OPA1 (S-OPA1) [32]. This cleavage is carried out by two metalloproteases: OMA-1 and YME1L, with OMA1 controlling the availability of the two OPA1 isoforms [33]. Additionally, prohibitin (Phb) helps to maintain mitochondrial OPA1 levels. Defects in both OMA1 and Phb alter L-OPA1 levels, which can result in impaired IMM function, and consequently fragmentation of mitochondria [34]. Also, OPA-1 participates in the maintenance of the cristae structure [35]. This shows how fusion is not a random event, quite the contrary, it is a highly regulated series of processes that sense environmental conditions to respond to the energetic and metabolic requirements of the cell.

On the other hand, fission involves the division of a mitochondrion in two, without altering the integrity of the membranes. This occurs thanks to the dynamin-related protein 1 (DRP1), which forms a ring-shaped structure that contracts both mitochondrial membranes in a GTP-dependent process. Mitochondrial fission 1 (FIS1) is anchored to the OMM and seems to participate in the recruitment of DRP-1, along with other factors such as mitochondrial fission factor (MFF), and the mitochondrial dynamic proteins MiD49 and MiD51 [29,36]. Fusion and fission are processes that participate in the regulation of mitochondrial respiratory activity, mtDNA distribution, apoptosis, and Ca^{2+}-mediated signaling. They also occur in response to an energy imbalance. For example, an excess of glucose or

fatty acid oxidation promotes fusion and decreases fission. The balance between fusion and fission is controlled by a system of dynamin-related GTPases that act as energy sensors [37]. As will be discussed later, metabolic diseases such as T2D are associated with a disturbance in this balance, which leads to higher levels of oxidative stress, mitochondrial dysfunction, and insulin resistance.

2.5. Mitochondrial calcium homeostasis

Calcium is an important regulator of several mitochondrial processes, contributing to energy production. As mentioned above, the PDH complex is a crucial step in connecting glycolysis with the Krebs cycle. The PDH complex is regulated, among other mechanisms, by mitochondrial calcium through two enzymes that control its activity by phosphorylation: pyruvate dehydrogenase kinase (PDK), and its counterpart, pyruvate dehydrogenase phosphatase (PDP). When Ca^{2+} concentration in the mitochondrial matrix increases, PDP activity increases, leading to dephosphorylation and activation of PDH, contributing to energy metabolism. In contrast, under conditions of low Ca^{2+}, PDK increases its activity, phosphorylates PDH, and inactivates it [38]. This calcium-mediated regulation allows cells to coordinate the activity of the PDH complex with their metabolic needs. In addition, calcium also regulates the activity of Krebs cycle enzymes, including isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and malate dehydrogenase [38,39].

Ca^{2+} also participates in the ETC function by regulating the activity of complexes I and II by participating in the activation of their substrates, NADH and $FADH_2$, respectively [38]. Additionally, the abundance of ETC complexes also participates in the maintenance of calcium homeostasis. A decrease in the levels of complexes I and II reduces mitochondrial Ca^{2+} levels. This was observed in MCF-7 cells [40]. In addition, dysregulation of complex I causes an increase in mitochondrial calcium uniporter (MCU), increasing calcium levels as a compensatory effect to mitochondrial dysfunction [41]. Therefore, it is important to point out that an adequate mitochondrial calcium balance is essential for energy production. As will be mentioned later, the alteration of this balance may contribute to the pathophysiology of T2D by altering processes such as the correct secretion of insulin by the plasma cells.

Since many diseases are correlated with mitochondrial dysfunction, much biomedical research is currently focused on the discovery of bioactive molecules that can restore mitochondrial function. Among the molecules that have been shown to have these regulatory effects are estrogens.

3. How do estrogens regulate mitochondrial function

Estrogens are female steroid hormones that play important roles in reproductive and non-reproductive processes. 17β-estradiol (E2) is the main product of the biosynthetic pathway of estrogens from cholesterol and is the most important estrogen during the premenopausal period in women. Its lipophilic nature allows it to diffuse freely through cell membranes. E2 is synthesized in various tissues, mostly in the ovaries, the corpus luteum, and the placenta [42]; however, it also has effects in non-reproductive tissues such as the liver, pancreas, smooth muscle, adipose tissue, and brain [43–45]. Nervous tissue actively synthesizes steroid hormones, including estrogens. This process is known as neurosteroidogenesis [46]. On the other hand, there are synthetic analogs of estrogens. 17α-ethinyl estradiol (EE), for example, is a synthetic estrogen derived from E2 in which the OH on C-17 is replaced by an ethynyl group, giving it greater resistance to degradation in the liver [47]. EE is

frequently given in the clinic in combination with progestins for the preparation of oral contraceptives and in hormone replacement therapy [48]. However, the use of this hormone is contradictory since there is evidence that demonstrates how the pharmacodynamics of other drugs are altered when co-administrated with EE [47]. Both E2 and EE have been used in various models and have been shown to be bioactive compounds for regulating mitochondrial function, as will be described below.

3.1. Estrogen receptors

The effects of estrogens and their analogs in the cell are mainly due to their interaction with estrogen receptors (ERs). In humans, three types of receptors have been characterized: ERα, ERβ, and the G protein-coupled estrogen receptor (GPER) [49,50]. In the genomic, or "classic" ER pathway, ERs act as transcription factors that stimulate or repress the expression of target genes. Activation of ERs requires their binding with E2. This causes the dimerization of the ERs and their translocation from the cytoplasm to the nucleus, allowing their interaction with the transcriptional machinery and their binding to specific DNA sequences known as estrogen response elements (EREs) [51]. However, it is reported that there are genes that do not contain EREs in their promoter regions (close to 35% of the estrogen response genes) and are also regulated by E2 but without direct binding of ERs to DNA. In this indirect genomic signaling, ERs may act through protein-protein interactions with other transcription factors and response elements [52]. In addition, estrogen signaling can also start from the plasma membrane. E2 can interact with GPERs by activating signaling pathways such as the MAP kinase and phosphoinositide 3-kinase (PI3K) pathways, as well as pathways dependent on second messengers such as cAMP and Ca^{2+} [51,53]. These pathways participate in the regulation of cell proliferation, migration, and cell death by apoptosis. Also, ERs have been shown to participate in regulating the elevated bioenergetic demands required in several important physiological processes such as lactation [54], regulation of cardiovascular function, and neuroprotective events [55]. On the other hand, the synthetic derivative EE can bind to both isoforms of the ERs, as well as to GPER. It has been reported that EE has a higher affinity to ERα than E2 [56,57], and therefore a greater capacity to activate estrogenic signaling pathways.

ERs are also present in the mitochondria of a wide variety of cells and tissues. Monje and Boland were the first to suggest the presence of ERs in this organelle [58]. After that, Chen and coworkers delved into the study of the mechanism of action of these receptors in mitochondria. Specifically, they studied rat liver mitochondria and isolated mitochondria from the hepatocarcinoma cell line HepG2. In the same study, it was observed that mitochondrial superoxide production increases after treatment with the synthetic analog EE [59]. Subsequently, the presence of ERβ in the mitochondrial fractions of the HepG2 cell line was confirmed previously [60]. Over the years, the presence of mitochondrial ERs (mtERs) has been confirmed in other in vitro models, such as the lens cell line HLE-BE [61], osteosarcoma cell line SaOS-2 [60] and in breast adenocarcinoma cell lines MCF-7 [62] and MDA-MB-231 [63]. In MCF-7 cells, the presence of both mtERs (ERα and ERβ) is reported and it was determined that they are located in the mitochondrial matrix, playing a possible role in the regulation of the expression of mitochondrial respiratory chain genes encoded in the mtDNA [62]. Also, mtERβ can bind to sequences similar to EREs in the mtDNA, suggesting a direct action of mtERs in mitochondrial transcription [63,64]. In addition, the association between mtERβ and the ATP synthase complex has been confirmed, suggesting a possible regulatory mechanism of the complex by estrogens [65]. In a systemic context, ERs have been identified in mitochondria of ovaries, uterus,

neurons, cardiomyocytes, hippocampus, skeletal muscle, liver, and heart [66]. The mtERs are being identified in more and more tissues as research associated with estrogen signaling advances.

Specifically, in the context of metabolic homeostasis, ERα plays a central role in metabolic regulation after food uptake and energy expenditure through estrogens. ERα regulates visceral adipose tissue levels, insulin levels, and glucose tolerance. The deletion of ERα in a mouse model results in an obese phenotype manifested as hyperplasia and hypertrophy of adipose tissue, which is linked to decreased energy production and insulin resistance [67]. In hypothalamic neurons, ERα allows the maintenance of normal body weight and is essential in the control of energy balance [68]. Energy generation and metabolic activation after food uptake are stimulated by E2 through effects similar to those of leptin in the hypothalamus [69]. On the other hand, there is evidence that ERβ negatively regulates glucose metabolism and insulin signaling in a mechanism that involves PPARγ in adipose tissue from mice subjected to HFD, suggesting pro-diabetogenic effects [70]. It is interesting how ERα and ERβ show opposite effects in regulating glucose metabolism and insulin response. Knowledge of these mechanisms of metabolic switches mediated by ERs could reinforce the use of specific antagonists or agonists as a potential auxiliary in the treatment of diabetes.

3.2. Estrogenic effects on mitochondria

Regardless of its action through the ERs, E2 improves mitochondrial respiratory function at the level of ETC and ATP production, regulates redox homeostasis in cells (GSH/GSSG), and restores the microviscosity of mitochondrial membranes (that is, the flow of substrates on both sides of the membrane) in skeletal muscle [71]. Interestingly, E2 has been localized to mitochondrial membranes, and E2 treatment in vitro and in vivo (in ovariectomized rats) has shown its translocation and accumulation from the plasma membrane to mitochondrial membranes [71]. Ovariectomy (OVX) is the surgical removal of the ovaries and is performed in animal models to study the effects of the absence of estrogen (produced mainly by the ovaries). E2 appears to have different effects on the tissue and mitochondrial ETC complex. For example, in the liver and skeletal muscle of OVX mice, E2 treatment restores the activity of complex I (NADH oxidation, ubiquinone reduction, and H_2O_2 production) and complex III, while complex II activity decreases in the liver, and increases in skeletal muscle [71,72]. On the other hand, it has been reported that E2 can inhibit and destabilize ATP synthase in hepatic mitochondria [73]. This shows integrated mechanisms of the action of E2 in the regulation of bioenergetic homeostasis in mitochondria.

It is well known that females live longer than males in many species, including humans. This could be in part because of the hormone profile and the mitochondrial theory of aging. In females, estrogens can impact the antioxidant capacity of the cell. Vina and coworkers explained how estrogens improve the expression of MnSOD and GPx enzymes [74,75]. This was associated with the activation of MAPK and NF-κB pathways, as described in the MCF-7 cell line exposed to oxidative stress [76]. These antioxidant effects are also observed in isolated mitochondria of the brain and liver in a rat model [77]. In this study, treatment with E2 at 0.02 nM (intracellular concentration) prevented the formation of ROS, protected mitochondrial integrity, and increased the $\Delta\psi_m$. In parallel, this treatment prevented the release of cyt c and mitochondrial-related apoptotic events. Also, treatment with E2 prevents the opening of the mitochondrial permeability transition pore (MPTP) and reduces the translocation of the pro-apoptotic protein Bax to mitochondria [78]. Other mitochondrial antioxidant markers, such as Prx3 and Trx2 also increase with E2 treatment in rat WAT and in the adipocyte cell

line 3T3-L1 [79]. All this evidence strongly suggests that E2, in a variety of biological models stimulates the expression and activity of mitochondrial antioxidant enzymes and regulates the apoptotic events in mitochondria.

Mitochondrial fusion and fission processes are also regulated by estrogens and this can impact physiological processes such as those associated with the nervous, cardiovascular, and endocrine systems; as well as in pathologies such as cancer, Alzheimer's disease, and diabetes [80,81]. In MCF-7 breast adenocarcinoma epithelial cells, E2 causes an increase in the expression of genes associated with mitochondrial fusion (mfn1, mfn2, and opa1) in an ER-dependent mechanism [82]. The same genes are upregulated in response to E2 in U87-MG glioblastoma cells [83]. At the protein level, E2 can modify MFN2 S-nitrosylation, causing changes in bioenergetic regulation, redox, and iron homeostasis, as well as mitochondrial morphological changes. These modifications improve the processes of the cardiovascular system [84]. The ERα also participates in regular events of mitochondrial dynamics. An ERα knockdown model of pancreatic β cells showed elongated mitochondria, suggesting increased fusion. In turn, ERα acts as a repressor of the expression of OMA1 and CHOP [85]. This is achieved because ERα interacts with ERE present in the promoters of both genes, resulting in an inhibition of their expression. However, it is not yet clear if E2 is involved in this process. This mechanism maintains adequate levels of fusion/fission events, which is associated with a decrease in oxidative stress and endoplasmic reticulum stress, contributing to β cell survival and insulin secretion [85]. The impact of estrogens in regulating mitochondrial dynamics involves the role of ERs in regulating the expression of key genes in fusion/fission processes. Research into understanding these mechanisms may be relevant to improving treatments for diseases associated with mitochondrial dysfunction.

The last effect of estrogens on mitochondria that we discuss is their ability to regulate mitochondrial calcium homeostasis. Calcium homeostasis is due, in large part, to the mitochondrial calcium uniporter (MCU) located in the IMM. Activation of ERs, mainly ERα, increases the rate of mitochondrial Ca^{2+} uptake in HeLa cells and MCF-7 cells, suggesting a possible mechanism of estrogen and ER-mediated regulation of mitochondrial Ca^{2+} levels [86]. Estrogens can also interact with cellular signaling pathways involved in the regulation of calcium levels in the cell and in the mitochondria. Some protein-coding genes involved in the PI3K/Akt signaling pathway are regulated at the transcriptional level by estrogens through activation of the nuclear ER pathway. A direct interaction between ERs and PI3K can also occur, activating the enzyme by changes in its conformation [87]. Activation of this pathway regulates the activity of numerous proteins, including MCU. Akt can phosphorylate the N-terminal region of MCU containing a regulatory subunit of the transporter, modulating mitochondrial Ca^{2+} content [88]. This represents an indirect example of how estrogens influence mitochondrial Ca^{2+} levels.

4. Mitochondrial dysfunction in T2D

T2D is a multifactorial disease involving genetic and environmental factors. This disease is characterized by pancreatic β-cell dysfunction and insulin resistance, primarily in muscle and liver. Insulin resistance is the inability of insulin to increase glucose uptake and utilization in an individual. One of the major factors contributing to the inhibition of insulin secretion is the resistance of β-cells to incretin hormones, such as glucagon-like peptide 1 (GLP1) and gastric inhibitory polypeptide (GIP). Altered insulin and glucagon secretion, as well as increased hepatic glucose production, are

characteristic factors in patients with T2D. An alteration in glucose uptake in skeletal muscle is also observed, with an increase in lipolysis, which raises the levels of free fatty acids (FFA) present in the plasma [1]. FFAs are associated with an increase in ROS levels and the consequent development of oxidative stress. This has been observed as a contributing factor to metabolic diseases such as non-alcoholic fatty liver disease (NAFLD) and obesity-related metabolic disorders [89,90].

Insulin resistance has direct effects on glucose homeostasis, mostly in skeletal muscle, liver, and white adipose tissue (WAT) [91]. In normal conditions, the binding of the insulin receptor (INSR) to its ligand causes the activation of tyrosine kinases which phosphorylate proteins of the insulin receptor substrate (IRS) family. IRS1 and IRS2 phosphorylation allow their binding to different targets including PI3K which promotes the translocation of the glucose transporter GLUT4 to the plasma membrane in muscle cells [92,93]. This signaling cascade allows glucose uptake. In patients with T2D, insulin resistance is associated with increased phosphorylation of IRS proteins, impairing downstream signaling, mainly PI3K. This causes low glucose uptake in the muscle [94].

There is strong evidence of the relationship between mitochondrial dysfunction and insulin resistance (Figure 1). A couple of decades ago it was discovered that elderly patients with T2D had diminished mitochondrial activity compared to healthy young people, by measuring the flow of the TCA cycle and the ATP synthesis by OXPHOS [95], and an impaired lipid metabolism with a decreased mitochondrial content in skeletal muscle of individuals with obesity-related insulin resistance [96–98]. These studies gave one of the first indications of the role of mitochondrial dysfunction in diabetes. From here, many human studies began to yield interesting data. T2D is a disease that affects the metabolism of multiple organs and tissues; however, in this section, we will focus on skeletal muscle, liver, adipose tissue, and pancreas, as there is a strong correlation between these organs and mitochondrial dysfunction in the context of T2D [4,99,100].

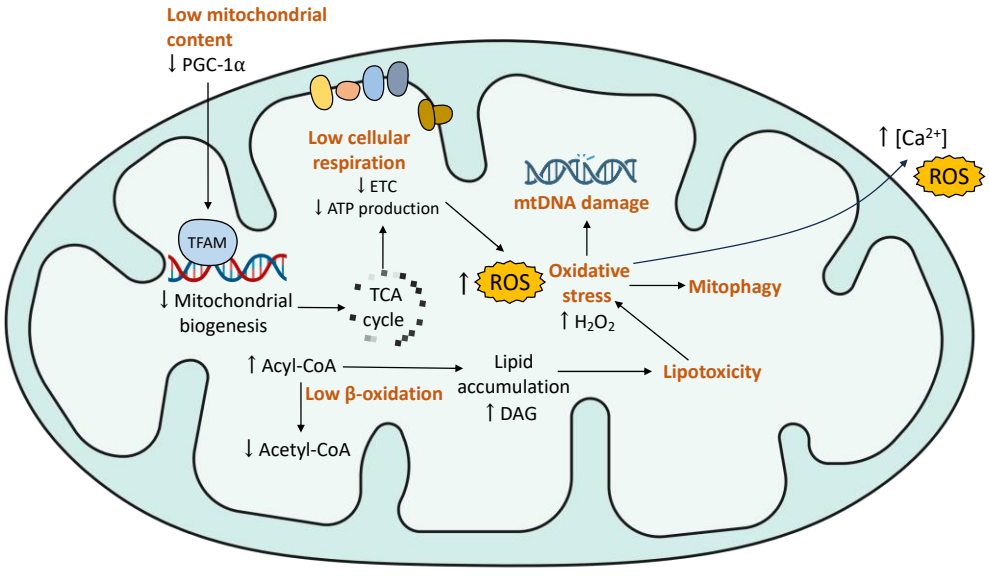

Figure 1. Altered mitochondrial processes in insulin resistance.

Note: Insulin resistance has been associated with low mitochondrial content and altered mitochondrial biogenesis due to low levels of biogenesis-stimulating factors such as PGC-1α. Likewise, there is a low rate of

cellular respiration, low levels of subunits of the ETC complexes, and low ATP production. An alteration in cellular respiration also causes a high production of ROS, generating oxidative stress. Furthermore, the decreased rate of fat oxidation causes lipid, such as DAG, accumulation, and lipotoxicity. Oxidative stress is associated with mitophagy, apoptosis, the activation of pro-inflammatory pathways, alteration in calcium homeostasis, and mtDNA damage. PGC1α, Peroxisome proliferator-activated receptor-gamma coactivator - 1alpha; TFAM, transcription factor A, mitochondrial; ETC, electron transport chain; TCA cycle, tricarboxylic acid cycle; ROS, reactive oxygen species; DAG, diacylglycerol. Created with BioRender.com.

In the skeletal muscle of patients with T2D, a lower mitochondrial density has been found compared to healthy people. Furthermore, this correlates with a decrease in both the phosphorylation of IRS-1 and the activation of Akt, showing a clear alteration in insulin signaling [101,102]. Individuals with T2D and metabolic syndrome have decreased mitochondrial content in both the intramyofibrillar and subsarcolemmal regions of skeletal muscle [102,103]. Lower mitochondrial content correlates with metabolic inflexibility; that is, an inability of the cell to respond to changes that alter its metabolism (e.g., fuel-energy switching). An example of this may be the inability of mitochondria to oxidize fat, which leads to lipid accumulation in the skeletal muscle. A reduced oxidative capacity in the mitochondria can lead to an accumulation of intramyocellular fatty acids. Increased levels of diacylglycerols, sphingolipids, and ceramides, as well as alterations in their subcellular localization, are also correlated with insulin resistance [104,105]. These fatty acids can be precursors for the synthesis of toxic lipids such as ceramide and diacylglycerol. The accumulation of lipotoxicity caused by these molecules is also associated with insulin resistance (Figure 1) [106,107]. It has also been discovered how ETC activity, cellular respiration, and the expression of genes related to mitochondrial bioenergetic activity are decreased in skeletal muscle samples from patients with T2D [108–110]. In particular, Asmann and coworkers demonstrated that the levels of PGC-1α were decreased in these samples, indicating an impaired mitochondrial biogenesis [108]. This evidence indicates a clear association between energy metabolism dysfunction in mitochondria and insulin resistance in skeletal muscle, which may help explain the pathophysiological aspects of T2D in this tissue.

Alterations in hepatic mitochondrial processes have also been reported in diabetic animal models and patients with T2D. An increase in hepatic fat storage in the form of non-alcoholic steatosis (NASH) in diabetic patients is notorious [111,112]. The increase in fatty acid oxidation is associated with a deficiency in the enzymatic activity of the long-chain acyl-CoA dehydrogenase (LCAD), a mitochondrial enzyme. This leads to an accumulation of diacylglycerol accompanied by insulin sensitivity in liver cells [113]. Interestingly, mice deficient in very long-chain acyl-CoA dehydrogenase (VLCAD) show greater resistance to obesity induced by a high-fat diet and a decrease in insulin resistance in muscle and liver [114]. On the other hand, obese individuals with NASH show alterations in mitochondrial function, associated with a high resistance to hepatic insulin. These alterations include a decrease in ATP levels, decreased maximal respiration, and increased hepatic oxidative stress [115,116]. In obese people, there is an increase in hepatic respiratory capacity compared to lean people, due to the high oxidative demand. However, in patients with NASH and with T2D this mitochondrial adaptation is lost. In obese individuals with T2D, there is a decrease in the oxidative capacity associated with complex II, compared to obese individuals without T2D, accompanied by increased H_2O_2 production [115,117]. The mechanisms by which this mitochondrial adaptation is altered in individuals with T2D remain unknown and their understanding could open up new intervention strategies for the treatment of T2D at different stages of the disease.

In adipocytes, mitochondrial oxidative metabolism is highly active, as ATP generation is required to drive processes such as triacylglycerol synthesis, fatty acid esterification, and gluconeogenesis [118,119]. In obese subjects, the activity of the ETC complexes, the mitochondrial membrane potential, and the levels of ATP in subcutaneous adipose tissue decrease [120]. Also, there is a decrease in the expression of important genes in catabolic pathways such as β-oxidation, ketolysis, the degradation of branched-chain amino acids, and the TCA cycle [121]. Interestingly, in non-obese subjects with T2D, these changes in mitochondrial parameters are not detected, suggesting that obesity is a condition that modulates T2D-associated mitochondrial dysfunction. From another perspective, it has been shown that there is greater mitochondrial oxidative capacity in adipocytes of metabolically unhealthy obese patients (characterized by insulin resistance, hepatic steatosis, and subclinical inflammation) as a possible compensatory effect to deal with insulin resistance, an effect that is lower in healthy obese people [122]. Physical activity has an inverse effect on adipose tissue since it causes mitochondrial remodeling, which prevents the decrease in mitochondrial content, its fragmentation, and the accumulation of ROS [123].

In mouse models of T2D, this connection also exists between mitochondrial dysfunction and obesity. Obese diabetic mice show low levels of mitochondrial protein and impaired respiration and fatty acid oxidation in adipocytes [124]. Likewise, the transcription of genes associated with ATP production, mitochondrial ribosomal proteins, mitochondrial heat-shock proteins, and uncoupling proteins is decreased [125]. By treating these mice with rosiglitazone, a compound that enhances insulin sensitivity, all of the effects above were ameliorated [124,125].

Insulin secretion by pancreatic β cells depends on the ability of mitochondria to synthesize ATP through OXPHOS. The generated ATP causes the closure of ATP-sensitive K^+ channels, which in turn causes the opening of voltage-depended Ca^{2+} channels. In the cytosol, intracellular Ca^{2+} stimulates the secretion of insulin granules in response to glucose [126]. Therefore, an impaired function and morphology of mitochondria in β cells may be key factors in the pathophysiology of T2D, and this has been verified in patients with manifestations of the disease. In patients with T2D, an increase in the expression of ETC complexes I and V is reported in pancreatic β cells [127]. This increase in the ETC could be the cause of excess ROS production, generating a state of oxidative stress. Also, an increase in the expression of Uncoupling protein 2 was found. Elevated Ca^{2+} concentration is also correlated with excessive production of mitochondrial ROS such as superoxide and other free radicals in the cell. There are also genetic variations in mtDNA associated with T2D and other metabolic diseases. Some mtDNA single nucleotide polymorphisms (SNPs) have been reported in various genes coding for tRNA, rRNA, proteins, and even in non-coding regions in subjects with diabetes [128]; however, this information requires further physiological evaluation to assess the role of these SNPs in the association with T2D. On the other hand, two genome-wide associated studies (GWAS) have identified variants in the gene encoding mitochondrial transcription factor B1 (TFB1M) in individuals with T2D, correlated with mitochondrial dysfunction and impaired insulin secretion [129,130]. All of the above indicate that the secretion of insulin by β cells requires the integral functioning of mitochondria and that an alteration in mitochondrial homeostasis may be related to the development and progression of T2D.

Metabolic stress and the accumulation of ROS, products of T2D-associated mitochondrial dysfunction, are related to other adverse effects on the cell, such as deregulation of apoptotic pathways, mitophagy, and alterations in mitochondrial dynamics. In pancreatic β cells, mitochondrial damage is associated with inflammatory signals, which initiate pro-apoptotic pathways. Mitochondrial damage

associated with oxidative stress induces mitophagy in the presence of proinflammatory cytokines. This mechanism protects β cells and prevents diabetes by counteracting inflammatory lesions [131].

T2D is also associated with dysfunction of mitochondrial dynamics, which impairs the maintenance of cellular metabolic homeostasis [132]. In diabetic patients there is an increased activity of DRP1 and FIS1 proteins causing an increase in fission events and contributing to mitochondrial fragmentation, further compromising the metabolic state [133]. The localization of DRP1 is also modified. Under normal conditions, DRP1 is located in the cytosol and translocates to the mitochondria, but in a state of lipid overload, characteristic of obesity or metabolic syndrome, DRP1 acetylation increases, stimulating its translocation above normal levels and promoting fission events [134]. On the other hand, mitochondrial fusion is a process that contributes to the maintenance of glucose homeostasis. Deletion of Mfn1 and Mfn2 in β cells decrease respiratory function and glucose tolerance. Interestingly, these proteins also participate in the maintenance of mtDNA by regulating TFAM expression [135], suggesting to be possible targets of interest to treat impaired glucose levels in diabetic patients.

On the other hand, Ca^{2+} homeostasis is another of the altered processes in T2D (Figure 1). The instability of MAMs causes the altered connection between mitochondria and the endoplasmic reticulum, which has been correlated with impaired glucose homeostasis and insulin signaling in myotubes from T2D patients and mice [31,136,137]. The endoplasmic reticulum and mitochondria have many sites close to each other in β cells. At these sites ryanodine receptor Ca^{2+} release channels (RyRs) are abundant and evidence suggests that these channels are regulated by mitochondrial oxidative metabolism and that Ca^{2+} flux is critical for metabolic control. In a diabetic state, intracellular Ca^{2+} influx is compromised, which deregulates the activity of RyRs and impairs communication between mitochondria and the endoplasmic reticulum [138]. In T2D, Ca^{2+} levels in the endoplasmic reticulum are lower, limiting Ca^{2+} availability in mitochondria and increasing Ca^{2+} leaked by the mitochondrial transition pore (mPTP). This was observed in vascular smooth muscle cells [139]. Calcium is an important factor in intracellular communication; thus, it is necessary to understand the mechanisms by which Ca^{2+} dysregulation develops in diabetes. This may generate possible therapeutic approaches based on restoring calcium flux to improve mitochondrial function and altered metabolic processes in diabetes and related diseases.

5. Mitochondria as a target for estrogens in diabetes

Estrogens have protective effects in preventing the development of T2D, and maintaining insulin sensitivity in various organs and tissues, mostly in skeletal muscle, liver, and adipose tissue (Figure 2). Historically, it has been known that men are more susceptible to developing T2D than premenopausal women, and this protection in women is significantly reduced when their estrogen levels decline or when they enter menopause [140–142]. Premenopausal women have an enhanced insulin sensitivity and reduced incidence of T2D compared with age-related men. Menopause is characterized by a progressive decline in E2 levels, decreased insulin sensitivity, and disrupted glucose homeostasis as well as a higher risk for T2D [140]. Clinical studies have shown that postmenopausal women are more susceptible to weight gain, dyslipidemia, altered fat transport and distribution, and impaired glucose tolerance compared to premenopausal women [142]. This has been correlated with the estrogen profile and its circulating levels. Hormone replacement therapies (HRT), which consist of replacing the estrogens that the body no longer produces after menopause with synthetic hormones, have shown to

be efficient in reducing metabolic syndrome; however, they have also shown clinical variations in the homeostasis of the glucose and insulin sensitivity, so its benefits are controversial [143,144]. The results obtained by an HRT show that it includes a lot of the stage of menopause (early or late), the mode of administration, and the hormonal profile administered [145].

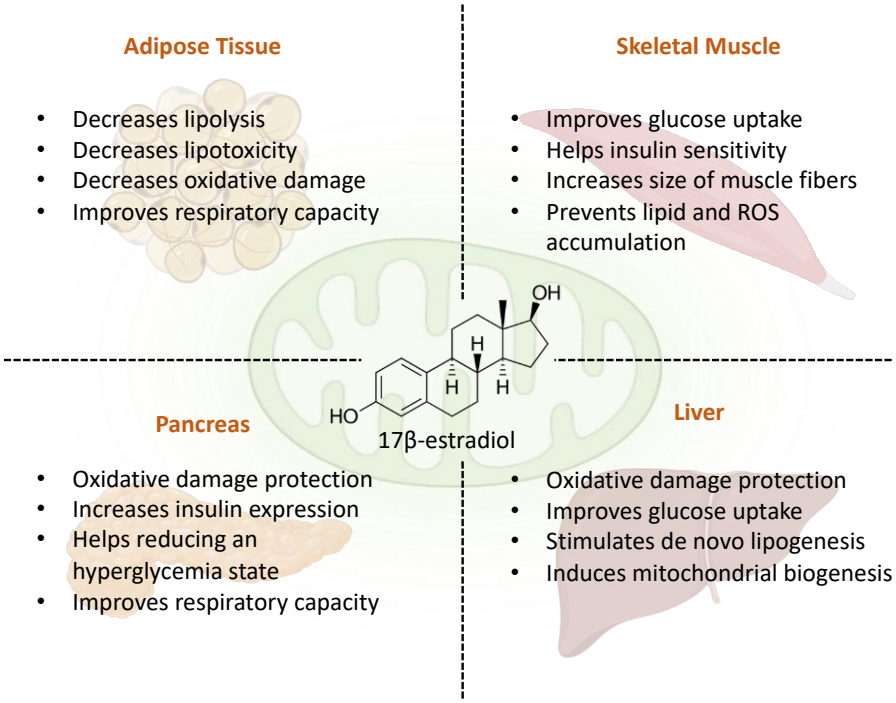

Figure 2. Mitochondria-related effects of 17β-estradiol in tissues affected in T2D. Mitochondrial-associated processes that have been shown to improve after treatment with E2 in various contexts are shown in adipose tissue, skeletal muscle, pancreas, and liver. (Created with BioRender)

As mentioned in the previous section, skeletal muscle presents alterations in energy metabolism, insulin signaling, mitochondrial morphology, and dynamics in T2D. Using specific ER agonists, it was shown that activation of ERα increases the expression of the glucose transporter GLUT4, while activation of ERβ increases the size of muscle fibers [146]. Proper glucose uptake in muscle helps stimulate mitochondrial bioenergetic demand and prevent the development of T2D. In addition, Rivas and coworkers demonstrated that a reduction in ERα in muscle decreases oxidative metabolism, increases lipid and ROS accumulation, and alters mitochondrial morphology and dynamics [147]. This indicates that ERα helps maintain mitochondrial function and insulin sensitivity. Although it is not clear, it could be intuited that E2 could cause these processes by activating ERα. E2 has already demonstrated the ability to restore and protect the OVX-induced reduction of mitochondrial CI activity in skeletal muscle [72]. Therefore, it is clear that the protective mechanisms exerted by E2 in skeletal muscle mitochondria require more attention to exploit its benefits in metabolic alterations such as insulin resistance and T2D.

As in muscle, the liver is one of the organs that increases its glucose uptake in the presence of E2 [146]. In the liver, E2 exerts protection against oxidative damage. Using in vivo models, Díaz and coworkers demonstrated that females produce lower levels of H_2O_2 and have lower xanthine oxidase

activity, an enzyme of purine catabolism associated with oxidative stress, compared to males and OVX females. In addition, lipid oxidation and glutathione levels are lower in males, indicating a higher oxidative stress profile [148]. Oxidative damage is one of the major characteristics of the pathophysiology of T2D, therefore, the hepatic antioxidant effect of E2 could function as an auxiliary alternative in the treatment of this disease and requires further preclinical and clinical studies.

Estrogens have positive effects in regulating pancreatic β-cell mitochondrial function in T2D. E2 protects β-cells from oxidative damage through an ERα-dependent mechanism [149]. This signaling also increases the expression and content of insulin in the same model [150]. Both studies laid the foundation for suggesting that ERα is an important regulatory node in T2D in the context of pancreatic β-cells. The relevance of ERα in reducing oxidative damage and a diabetic state was confirmed by inducing hyperglycemia in cultured islets and in an in vivo rat model [151]. Using KO mice for ERs, it was shown that the action of E2 is due to extranuclear mechanisms that depend on ERα and GPER. In addition, in β-cells from MIN6 mice, a model for diabetes, a predominance of ERα was observed in both the plasma membrane and mitochondria [152]. Since mitochondria are the main site of antioxidant defense in the cell, these studies suggest a possible role of mtERs in the regulation of processes associated with oxidative damage in T2D; however, more studies are required to corroborate this association.

As discussed above, one of the events associated with insulin resistance is increased lipolysis in adipose tissue. In murine models, it is known that females have greater protection against lipid-induced insulin resistance than males [153]. Camporez and coworkers used this background to demonstrate that E2 decreases lipolysis in WAT, showing an impaired reduction in insulin resistance associated with obesity in mice fed with a high-fat diet (HFD). In addition, they demonstrated that E2 treatment reduces ectopic lipid levels in the liver and muscle [154]. Complementing the above, another study found that the selective activation of ERβ by a specific agonist induces lipolysis in the liver and increases *de novo* lipogenesis in the liver and WAT, decreasing the levels of hepatic steatosis in a murine model. Interestingly, this agonist reduces PGC-1α levels, suggesting stimulation of mitochondrial biogenesis [155]. It is known that in female rats there are higher levels of mitochondrial biogenesis markers (TFAM, PGC-1α, and β) and that HFD decreases the levels of these markers in males. In addition, E2-induced mitochondrial biogenesis is known to increase $\Delta\Psi_m$ and decrease oxidative stress levels associated with lipotoxicity [156]. To combat oxidative stress, E2 is known to increase the expression of components of the mitochondrial antioxidant system (Prx3 and Trx2) in an *in vitro* model of WAT [79]. All this evidence points to E2 as a possible modulator that decreases lipolysis induced in adipose tissue in a T2D context, which allows for a decrease in the accumulation of lipids in muscle and liver, reducing the adverse effects of lipotoxicity.

6. Targeted strategies using estrogens

Some strategies have been developed to focus the action of estrogens on specific tissues or structures. In this last section, we will focus on two of them. The first one consists of fusing the E2 structure with a peptide derived from the incretin GLP1. An incretin is a hormone that stimulates the secretion of insulin after the ingestion of a meal. GLP1, together with GIP, is responsible for lowering blood glucose levels. These two molecules have been the basis for the creation of new agents that mimic the action of incretins (such as GLP1 receptor agonists) [157,158]. This has made GLP1 a therapeutic agent with the potential for treating diabetes. GLP1 enhances glucose-stimulated insulin

secretion and stimulates insulin expression in β cells. Furthermore, GLP1 acts as a growth factor to promote β cell proliferation and survival [159]. The selective action of GLP-1 on β cells has been exploited to target E2 and concentrate its protective and antidiabetic effects. The covalent union of GLP1 with E2 (GLP1-E2) resulted in insulinotropic effects, since it increases insulin sensitivity and glucose homeostasis concerning its parent monoagonists separately [160], notably indicating a synergistic effect when combining both hormones. In addition, it was found that the action triggered by GLP1-E2 depends on the activation of ERα and the GLP1 receptor (GLP1R) in a pathway that depends on its internalization by clathrin vesicles and lysosomal acidification [161]. This strategy combines the effects of E2 in improving energy production and lipid and glucose metabolism, and when directed to pancreatic cells, it could reduce the effects associated with a systemic hormonal imbalance in men and women, which are the product of hormone replacement therapies. So far GLP1-E2 has shown positive results in T2D and metabolic syndrome models [161,162]. This strategy represents yet another example of how the effects of estrogens can be harnessed in the clinic to stimulate aspects of energy metabolism.

The development of new molecules that accumulate in mitochondria and promote their function has emerged as a promising alternative for the treatment of diseases associated with mitochondrial dysfunction. The use of mitochondria-targeted antioxidants (MTAs) has shown beneficial effects by reducing ROS levels in diseases associated with oxidative damage, including Parkinson's disease, cardiovascular diseases, and cancer progression [163]. The study of the effects of MTAs is scarce in patients with insulin resistance and T2D. The most widely used strategy for this purpose consists of covalently conjugating the bioactive compound of interest to the lipophilic triphenylphosphonium cation (TPP). This positively charged carrier takes advantage of the predominantly negative charge of the mitochondrial matrix and migrates against its concentration gradient, taking advantage of the mitochondrial membrane potential [164]. In our group, we are working on the development and characterization of novel mitochondria-targeted estrogens based on the covalent coupling of the TPP group to estrogens such as E2 and EE. Currently, some antioxidant molecules have used this strategy. Among the most studied are MitoQ® (derived from coenzyme Q or ubiquinone), Mito-vitamin E (derived from vitamin E), and SkQ1 (derived from plastoquinone) [164–166]. We believe that the use of mitochondria-targeted bioactive compounds such as estrogens or antioxidants represents a strategy that could generate new therapeutic alternatives to mitigate the adverse effects of mitochondrial dysfunction in diseases such as T2D, metabolic syndrome and other conditions associated with impaired metabolism and aging.

7. Conclusions

Estrogens, mainly E2, are compounds with the capacity to regulate mitochondrial function. Some of these estrogen-regulated mitochondrial processes are mediated by ERs. E2 has shown protective effects against oxidative damage, lipotoxicity, calcium homeostasis alteration, impaired bioenergetics, and mitochondrial dynamics; processes associated with the pathophysiology of T2D in specific tissue and organs. Furthermore, insulin secretion and signaling are processes dependent on mitochondrial homeostasis. Therefore, the mitochondrial protective effects of estrogens could be exploited to generate important therapeutic targets that serve as auxiliary treatments in patients with T2D.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare that they do not have conflicts of interest in the creation of this article.

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