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Mitochondrial bioenergetics and redox dysfunctions in hypercholesterolemia and atherosclerosis

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ABSTRACT

In the first part of this review, we summarize basic mitochondrial bioenergetics concepts showing that mitochondria are critical regulators of cell life and death. Until a few decades ago, mitochondria were considered to play essential roles only in respiration, ATP formation, non-shivering thermogenesis and a variety of metabolic pathways. However, the concept presented by Peter Mitchell regarding coupling between electron flow and ATP synthesis through the intermediary of a H^+ electrochemical potential leads to the recognition that the protonmotive force also regulates a series of relevant cell signalling processes, such as superoxide generation, redox balance and Ca^{2+} handling. Alterations in these processes lead to cell death and disease states. In the second part of this review, we discuss the role of mitochondrial dysfunctions in the specific context of hypercholesterolemia-induced atherosclerosis. We provide a literature analysis that indicates a decisive role of mitochondrial redox dysfunction in the development of atherosclerosis and discuss the underlying molecular mechanisms. Finally, we highlight the potential mitochondrial-targeted therapeutic strategies that are relevant for atherosclerosis.

1. Background

Aerobic eukaryotic cells oxidize their organic fuels completely to CO_2 and H_2O in a process called cell respiration. All the enzymatic steps in the oxidative degradation of these organic fuels converge into a final stage in which energy-rich electrons removed from dietary carbohydrates (glycolysis and the tricarboxylic acid (TCA) cycle) or fats (β-oxidation and the TCA cycle) flow to molecular oxygen, yielding free energy that is used to generate ATP from ADP and inorganic phosphate. In the TCA cycle, four pairs of hydrogen atoms are transferred by specific dehydrogenases from isocitrate, alpha-ketoglutarate, succinate and malate, in each turn of the TCA cycle, to NAD^+ and FAD. These coenzymes are the major carriers of hydrogen atoms in the process of oxidation of fuel molecules, such as glucose, fatty acids and, to a much lower extent, some amino acids that undergo loss of their amino groups and enter the TCA cycle. From these coenzymes, the hydrogens are separated into H^+ and "energy-rich" electrons, which are transferred through a sequence of electron carriers called the electron transport chain (ETC) or respiratory chain to the final electron acceptor, molecular oxygen. The free energy resulting from this process is conserved with high efficiency in the form of ATP in a process called oxidative phosphorylation. Three ATP molecules are formed for each NADH that is oxidized by molecular oxygen via the respiratory chain; thus, originating the expression P/O ratio equals 3. A P/O ratio of approximately 2 is obtained from the respiratory substrate succinate when electrons flow from $FADH_2$ to O_2. These ratios express the efficiency of oxidative phosphorylation. The P/O ratio of 3 was first proposed by the Nobel laureate Professor Severo Ochoa (1943).

After Eugene P. Kennedy and Albert L. Lehninger (Kennedy and Lehninger, 1949) demonstrated that isolated liver mitochondria contain the entire set of β-oxidation, TCA cycle and oxidative phosphorylation enzymes and coenzymes, the organelle was called the "power plant" of the cell. The pioneering electron microscopy observations of George Palade (1952) and Fritjof Sjostrand (Sjöstrand, 1956) revealed that a typical mitochondrion is approximately 1.0 μm in length and 0.5 μm in diameter despite great variations in shape, size, and arrangement of substructures that were frequently observed. It is now known that mitochondrial fusion and fission are highly regulated events and that mitochondrial dynamics are relevant to several physiological and pathophysiological processes (Sebastián et al., 2017; Moore et al., 2019; Kowaltowski et al., 2019). The organelle matrix has significant electron density and fine granularity and is surrounded by two membranes, an

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outer membrane and an inner membrane. The outer membrane is smooth and contains proteins (porins) that confer non-specific permeability to solutes with molecular weights lower than 10 kD (Zalman et al., 1980). The inner membrane is highly folded, forming internal ridges called cristae and is largely impermeable to ions and polar molecules. This membrane is very rich in proteins such as the components of the ETC and carriers and channels that are responsible for the flux of ions and metabolites that move in and out of the mitochondrial matrix.

Until a few decades ago, mitochondria were considered to play essential roles only in respiration, ATP formation, non-shivering thermogenesis (thought to occur exclusively in brown adipose tissue) and a variety of metabolic pathways, such as the TCA cycle, fatty acid β-oxidation, amino acid metabolism, ketogenesis, gluconeogenesis, ureagenesis and other metabolic activities. By the 1960s and 1970s, one of the main challenges in the field of bioenergetics was understanding of the mechanisms by which the oxidation of substrates by mitochondria were used to drive ADP phosphorylation. It was already known (Racher, 1976) that the catalysis of oxidative phosphorylation occurs through two distinct protein assemblies. The first is a multi-enzymatic system, the ETC, which is embedded in the inner mitochondrial membrane where pairs of electrons flow thermodynamically "downhill" from $NADH^+H^+$ (−0.32 V) to complex I (NADH dehydrogenase) or from $FADH_2$ to complex II (succinate dehydrogenase) and sequentially to ubiquinone (CoQ10) to generate ubiquinol ($CoQH_2$). Ubiquinol transfers two electrons to complex III (ubiquinol-cytochrome c oxidoreductase), from complex III via cytochrome *c* to complex IV (cytochrome *c* oxidase) and finally to molecular oxygen (+0.82 V) to generate water. It is now known that each ETC complex contains multiple electron carriers that differ in each species and that complexes I, II and III contain iron-sulfur (Fe–S) centres. The second protein assembly, ATP-synthase (complex V), is also characterized as the coupling device and catalyses the "uphill" ATP formation from ADP and Pi using the free energy released from electrons flowing through the ETC. There are three segments of the ETC called energy-conserving sites that provide enough free energy to generate ATP located at different segments along the ETC. The first site is located between NADH dehydrogenase and CoQ10, the second is between cytochromes *b* and *c*1, and the third is between cytochrome *c* and oxygen. Respiration and phosphorylation are tightly coupled in intact mitochondria; therefore, the concentrations of intra-mitochondrial ADP and Pi (in the physiological range) determine the rate of mitochondrial respiration. In the absence of ADP, the rate of respiration is slow (resting respiration) and regulated by proton leakage. Other mechanisms that regulate the rates of respiration are outlined below.

Electron transfer through the respiratory complexes does not require physical linkage; however, early biochemical studies and more recent in vivo structural studies have demonstrated that in the native membranes, the ETC components associate to form so-called supercomplexes (Brzezinsk, 2019). The higher-level organization seems to be dynamic, presenting a distribution of free complexes and supramolecular assemblies of different compositions. Understanding the functional significance of the higher-level organization is presently a challenge and suggests a possible relevant structural crosstalk between the complexes (Milenkovic et al., 2017; Letts and Sazanov, 2017; Davies et al., 2018).

During a period of at least three decades (1950s–1970s), there was an intense debate among researchers involved in the investigations of the mechanisms by which redox free energy was conserved in the form of ATP. Three different hypotheses received the most attention: (a) the chemical, (b) the conformational and (c) the chemiosmotic hypotheses (Racher, 1976). Since conformational changes are essential features of enzyme catalysis, we will consider that the conformational hypothesis proposed by Boyer (1975) satisfies both the chemical and chemiosmotic hypotheses outlined below.

The chemical hypothesis was originally proposed by Slater (1953) and states that oxidative phosphorylation occurs analogously to the glycolytic substrate-level phosphorylation catalysed by glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase. This hypothesis predicts the formation of three "high-energy" intermediates (Ã X) as derivatives of the respiratory chain at the levels of the three "energy conservation sites". These energy-rich intermediates would be further converted to (X ~ Y), which would be converted by phosphorolysis to X ~ Pi, the putative donor of the phosphate group to ADP to generate ATP. Since the Ã X intermediates were considered derivatives of the respiratory chain, they were likely to be detected by spectroscopic techniques. Although the search for such intermediates was not successful regarding the elucidation of the molecular mechanisms of oxidative phosphorylation, the spectral analyses obtained were fruitful in recognizing the ETC composition and the order of interactions among its components (Racher, 1976). It should be emphasized that, according to the chemical hypothesis, oxidative phosphorylation is catalysed by a simple flat fragment of the inner membrane or even by solubilized enzymes (Racher, 1976). In contrast, Peter Mitchell (Mitchell, 1961, 1976) introduced the concept of coupling between respiration and ADP phosphorylation through the formation of a proton-motive force generated by the downhill flow of electrons. The pumping of protons out of the inner membrane through complexes I, III and IV when electrons flow through the respiratory chain generates a negative inside electrical membrane potential ($\Delta P = \Delta pH + \Delta\Psi$) that is defined by chemical and electrical components of the proton-motive force. According to Peter Mitchell, this proton-motive force provides the energy for ATP synthesis, suggesting that ATP synthase functions as a coupling device operating in reverse; that is, the H^+ backflow through the proton channel (Fo) of ATP synthase provides the energy to catalyse ATP synthesis from ADP and Pi bound in the F1 subunit of the enzyme and release ATP in the matrix.

In contrast to the chemical model, Peter Mitchell's hypothesis required both (a) compartments that permit the formation of the proton-motive force and (b) an inner membrane that is asymmetrically organized and highly impermeable to H^+ and other ions (Mitchell, 1961, 1976). This hypothesis had previous support from other systems, such as the reversible Ca^{2+} pump of the sarcoplasmic reticulum (Makinose and Hasselbach, 1971) and the Na^+-K^+ pump of the plasma membrane (Garrahan and Glynn, 1967), which use ion gradients to generate ATP. Whether the proton-motive force satisfies quantitative thermodynamic considerations, such as a P/O ratio of 3, was a matter of intense controversy mainly due to difficulties in experimentally determining the H^+/ATP, ATP/O and H^+/O stoichiometries (Reynafarje et al., 1976; Vercesi et al., 1978). Acceptance of the chemiosmotic hypothesis culminated with Peter Mitchell being awarded a Nobel Prize in Chemistry in 1978.

2. Mitochondria after the chemiosmotic theory

The main reason we briefly revisited these hypotheses on energy coupling between respiration and ADP phosphorylation was to draw the attention of students and non-specialists in the field to the reasons that the Mitchell Theory (the concept of coupling between electron flow and ATP synthesis through the intermediary of a H^+ electrochemical gradient) has generated excitement and caused exponential growth in research on mitochondria around the world. In this respect, it is worth noting that according to the Mitchell Theory, mitochondrial bioenergetics require various properties of the inner membrane that were neglected by the chemical hypothesis (Slater, 1953; Mitchell, 1961, 1976). Moreover, it is now amply recognized that the proton-motive force, in addition to being the driving force for ADP phosphorylation, is also the driving force for several energy-requiring processes, such as (***a***) Ca^{2+} and other ion transport across the membrane (Vercesi et al., 2018), (***b***) non-shivering thermogenesis (Nicholls and Locke, 1984; Klingenberg, 2017), (***c***) reduction of NADP + by NADH that is catalysed by nicotinamide nucleotide transhydrogenase, the main source of reducing power in mitochondrial antioxidants enzyme systems

(Rydström, 2006), (*d*) import of cytosolic proteins and substrates for matrix metabolic pathways (MacKenzie and Payne, 2007), (*e*) K^+ influx through the ATP sensitive channel (mitoK$_{ATP}$) (Garlid and Paucek, 2003), and (*f*) ADP/ATP exchange via the adenine nucleotide translocator (Klingenberg, 2008). As a corollary, inner membrane proteins such as the uncoupling proteins (UCPs) (Nicholls and Locke, 1984), the mitoK$_{ATP}$ channel (Garlid and Paucek, 2003) and possibly the H^+ transport activity of the ANT translocator (Bertholet et al., 2019) that directly or indirectly catalyse the slow return of protons to the matrix (mild uncoupling) are able to promote a fine tuning of the proton-motive force. As proposed by Professor Skulachev (1996), this regulated mild uncoupling protects against excess mitochondrial oxidant generation and its deleterious effects on mitochondria and cell functions (Skulachev, 1998; Facundo et al., 2006; Cunha et al., 2011; Figueira et al., 2013; Ježek et al., 2018).

The endogenously regulated uncoupling mechanisms best studied are the UCPs and mitoK$_{ATP}$. UCPs are integral membrane proteins with apparent molecular masses ranging from 30 to 33 kDa that dissipate the electrochemical proton gradient generated by respiration as heat. The mammalian UCP, now named UCP1, was believed to exist only in the brown adipose tissue (BAT) of mammals as a late evolutionary acquisition. For decades, the only physiological role attributed to UCP was its involvement in transient thermogenesis in newborn, cold-acclimated, and hibernating mammals (Nicholls and Locke, 1984; Klingenberg, 2017). In the presence of fatty acids (FAs), UCPs facilitate the re-entry of protons extruded by the respiratory chain into the matrix, bypassing ATP synthase (Klingenberg, 2017; Skulachev, 1996). The discovery of the plant counterpart of UCP in 1995 (Vercesi et al., 1995, 2006) initiated a search for UCP homologs. Between 1997 and 2000, several homologs of UCP1 were identified in all mammalian tissues (Ježek et al., 2018). It is now accepted that, except for UCP1, these new UCPs are not thermogenic but are widespread in eukaryotes, and they may have various physiological roles, including the regulation of cellular redox signalling (Brandalise et al., 2003; Woyda-Ploszczyca and Jarmuszkiewicz, 2017; Ježek et al., 2018).

Potassium uptake into the mitochondrial matrix through the mitoK$_{ATP}$ channel is accompanied by phosphate and water and results in mitochondrial swelling. This activates a K^+/H^+ antiporter that generates a futile cycling of K^+ across the inner mitochondrial membrane (Garlid and Paucek, 2003). For each cycle of K^+ moving in and out of the matrix, there is a net influx of H^+ that causes a small drop in ΔP. K^+ transport by mitoK$_{ATP}$ is quite slow and permits only mild uncoupling. Similar to the case of UCPs, this decreases the reduction state of respiratory complexes I, II and III and, as a consequence, decreases the generation of superoxide by the ETC. The mitoK$_{ATP}$ channel is sensitive to ATP, glybenclamide, and 5-hydroxydecanoate and is stimulated by diazoxide (Liu et al., 2001). Increased mitoK$_{ATP}$ activity is protective against ischaemia-reperfusion injury and hyperlipidaemic metabolic and redox stress (Facundo et al., 2006; Alberici et al., 2006, 2009).

Other potassium channels have been found in the inner mitochondrial membrane of various cells. The activity of a large-conductance calcium-activated potassium channel (mitoBKCa) has been studied in brain cells and cardiomyocytes (Balderas et al., 2015) and in human dermal fibroblast mitochondria (Kicinska et al., 2016). Although the activity of the mitoBKCa channel protects the heart from ischaemia and glioma cells from cell death, the impact of the mitoBKCa channel in mitochondrial biology and disease remains to be determined (Balderas et al., 2015).

Considering the importance of the integrity of the inner membrane in sustaining membrane electrochemical potential, it should be emphasized that any agent that is able to bind to this membrane and specifically or non-specifically alter the proton gradient may partially or totally disrupt membrane electrochemical potential, thus compromising ATP synthesis and other energy-dependent processes. These agents include traditional uncouplers such as dinitrophenol and FCCP but also a large number of natural, commercial, pharmaceutical and an increasing number of environmental chemicals that transiently or irreversibly affect mitochondrial functions (Wallace and Starkov, 2000; Meyer et al., 2018). Since changes in the proton-motive force also regulate relevant cell signalling processes such as superoxide generation (Hamanaka and Chandel, 2010), redox balance and mitochondrial Ca^{2+} handling (Glancy and Balaban, 2012; Vercesi et al., 2018), the new concepts proposed by the chemiosmotic theory included mitochondria as the centre of a multitude of essential cellular functions. Therefore, alterations in ATP synthesis, Ca^{2+} transport and oxidant generation lead to cell death and disease states (Vaseva et al., 2012; Figueira et al., 2013; Wallace, 2015).

Moreover, mitochondria contain their own genome, a 16.5 kb circular DNA molecule that encodes 13 peptides that are components of four of the oxidative phosphorylation complexes. Inherited defects in the mitochondrial genome cause diseases for which diagnosis is difficult, and treatments are largely palliative (Wallace, 2015). The location of mtDNA molecules in the proximity of the sites of oxidant production exposes them to very high mutation rates, thus generating a mixed intracellular population of mtDNA, a state known as heteroplasmy (Wallace, 2015). These DNA mutations accumulate during normal ageing and result in complex diseases of great relevance to public health, including cancer, diabetes, neurodegeneration and many others (Wallace, 2015).

Overall, these new concepts developed in light of the Mitchell Theory increased the general interest in mitochondrial research, and newly discovered processes were key in understanding the mechanisms of ageing and programmed or accidental cell death under pathologic conditions (Balaban et al., 2005; Rottenberg and Hoek, 2017; Vercesi et al., 2018). The mitochondrial cell death-regulatory machinery includes highly regulated processes such as oxidant production (Vercesi et al., 1997; Kowaltowski et al., 2009), the Ca2+ transport system (Vercesi et al., 2018) and membrane permeability transition pore (mPTP) formation (Zoratti and Szabò, 1995; Javadov et al., 2017; Vercesi et al., 2018).

3. Ca^{2+} transport and membrane permeability transition (MPT)

A calcium uniporter (MCU) present in the inner mitochondrial membrane mediates the uptake of Ca^{2+} down its electrochemical gradient, while Ca^{2+} efflux occurs via two separate and independent pathways (Nicholls and Akerman, 1982). The molecular nature of the channel was only recently identified (Baughman et al., 2011; De Stefani et al., 2011). The efflux pathways promote Ca^{2+} release even when $\Delta\Psi$ is sufficiently high to preclude Ca^{2+} efflux from the matrix by reversal of the MCU (Nicholls and Akerman, 1982). At high mitochondrial Ca^{2+} loads, the cations stimulate oxidant generation (Castilho et al., 1995a,b) that synergises with Ca^{2+} to promote the opening of a proteinaceous mega-channel, the membrane permeability transition pore (mPTP) (Zoratti and Szabò, 1995).

Despite the efforts of many research groups, the molecular structure of the mPTP remains controversial (Vercesi et al., 2018; Carraro et al., 2019). Various mitochondrial matrix and membrane proteins have been suggested to participate in the mega-channel structure, such as adenine nucleotide transporter (ANT), cyclophilin D (CypD), voltage-dependent anion channel (VDAC), aspartate-glutamate, hexokinase, phosphate carriers (PiC) and the spastic paraplegia 7 protein (SPG 7). In addition, other data indicated that the lateral stalk or the c-ring of the F1Fo ATP synthase forms the mPTP (Carraro et al., 2019). Although data from John Walker's laboratory largely disagree with the participation of these ATP synthase subunits in the Ca^{2+}-induced mega-channel (He et al., 2017a, 2017b), more recent data from Bernardi's group support the conclusion that the mPTP originates from a Ca^{2+}-dependent and reversible conformational change in F-ATP synthase (Carraro et al., 2019; Urbani et al., 2019).

At a high conductance state, the mPTP permits the flux of solutes up

to 1500 Da, thus eliminating all mitochondrial energy-linked functions (Zoratti and Szabò, 1995; Vercesi et al., 2018). It has also been proposed that the mPTP also occurs at a low conducting state in which it may display some physiological functions that include regulation of mitochondrial Ca^{2+} release and mitochondrial volume (Vercesi, 1984, 1985; Bernardes et al., 1986; Ichas and Mazat, 1998). The transition of the pore from a low to high conductance state seems to be dependent on mitochondrial redox balance (Zago et al., 2000). Indeed, induction of the mPTP by Ca^{2+} is stimulated by depletion of mitochondrial NADPH (Vercesi, 1987; Zago et al., 2000), thiol oxidants (Fagian et al., 1990; Valle et al., 1993; Bernardes et al., 1994; Halestrap et al., 1997) and exogenous (Hermes-Lima et al., 1991; Castilho et al., 1995a; Kowaltowski et al., 1996) and endogenous oxidant-generating systems (Carbonera et al., 1988., Castilho et al., 1995b) and is protected by antioxidants (Kowaltowski et al 1995, 1998). The evidence that cyclosporin A (CsA), a pore-opening inhibitor (Crompton et al., 1988; Broekemeier et al., 1989), prevents cell death under different pathological conditions (Griffiths and Halestrap, 1993; Bernardi et al., 2006) supports the participation of this pore in the pathogenesis of ischaemia/reperfusion, heart and neurodegenerative diseases, traumatic brain injury, muscular dystrophy, inflammation, dyslipidaemias, drug toxicity and ageing (Griffiths and Halestrap, 1993; Bernardi et al., 2006; Halestrap and Pasdois, 2009; Vaseva et al., 2012).

The redox hypothesis for mPTP regulation is further supported by the protection against it opening by several antioxidants (Vercesi et al., 2018) or the absence of molecular oxygen (Castilho et al., 1995a). In addition, evidence has shown that exogenous catalase (Valle et al., 1993; Castilho et al., 1995a; Kowaltowski et al., 1996), peroxiredoxin (Kowaltowski et al., 1998) or o-phenanthroline (Castilho et al., 1995a) prevents opening of the mPTP. This strongly supports the notion that H_2O_2 participates in this process due to its ability to promote protein dithiol formation (Fagian et al., 1990; Kowaltowski et al., 2001). This proposition supports data indicating that redox signals mediated through cysteine oxidation via sulfenylation, S-glutathionylation and S-nitrosylation regulate opening of the mPTP (Mailloux et al., 2014), thus suggesting that the mPTP is not a molecularly defined channel but rather a permeability transition formed by protein-thiol crosslinking (Vercesi et al., 2018). Mitochondrial dysfunctions that lead to the mPTP and consequently to cell death have been implicated in the pathogenesis of several metabolic and ageing diseases, including atherosclerosis, as discussed in the next sections.

4. Atherosclerosis and mitochondrial dysfunction

In the following sections, we will discuss mitochondrial dysfunctions in the context of atherosclerosis, with a particular emphasis on hyperlipidaemia-induced atherosclerosis. Atherosclerosis develops because of predisposing risk factors such as primary or secondary dyslipidaemias, diabetes, obesity, hypertension, smoking and infections. Except for genetic hyperlipidaemias, all other conditions are complex and involve several simultaneous metabolic, hormonal and immunological disturbances. Therefore, genetic dyslipidaemia models are useful for investigating whether excess lipid in the circulation and therefore inside the cells might affect mitochondrial function and the development of atherosclerosis.

4.1. Atherosclerosis, oxidative stress and mitochondria

Multiple lines of incontrovertible evidence have indicated a causal role for excess low-density lipoprotein (LDL) cholesterol in atherosclerosis. However, as elegantly demonstrated by Brown and Goldstein in the early 1980s, the disease culprit is not native LDL, since most cell types have effective defence mechanisms against an overflow of LDL cholesterol. When a sufficient amount of LDL cholesterol is internalized by cells, LDL receptors and de novo cholesterol synthesis are shutdown, thus preventing cholesterol overload (Goldstein and Brown, 1990) and subsequent cell death. Brown and Goldstein also demonstrated that chemically modified LDL particles are recognized by a family of macrophage receptors (scavenger receptors), leading to the formation of cholesterol-laden macrophages (foam cells) that accumulate in the arterial intima (Brown and Goldstein, 1983), which is a hallmark of atherosclerosis. Steinberg's and Chisolm's groups showed that the most relevant chemical modification of LDL that occurs in vivo is oxidation and proposed the “LDL oxidative modification hypothesis for atherosclerosis” (Chisolm and Steinberg, 2000). In early lesions, oxidized LDL (oxLDL) acts as inflammatory stimulus within the vessel wall, activating endothelial cells and recruiting circulating monocytes, which efficiently phagocytose these oxLDL particles and become macrophage foam cells that undergo cell death. Dead foam cells that are ineffectively cleared result in the perpetuation of inflammatory stimuli within the intima, propagating atherosclerosis (Kavurma et al., 2017; Kasikara et al., 2018; Geovanini and Libby, 2018). Thus, oxidative stress, cell death and inflammation are key processes driving the initiation and progression of atherosclerosis.

There is strong support for oxidation of LDL taking place in vivo because oxLDL is present in human and mouse atherosclerotic lesions (Yurdagul et al., 2016). In addition, oxLDL and electronegative LDL are elevated in the plasma of patients with hypercholesterolemia and coronary artery disease (Hulthe and Fagerberg, 2002; Oliveira et al., 2006; Vasconcelos et al., 2009; Yang et al., 2017). However, the mechanisms that drive in vivo systemic and vascular wall oxidative stress during atherogenesis are less well understood. Since oxidative stress is considered the heartwood of the disease and represents a unifying mechanism of a wide range of risk factors (metabolic, haemodynamic and immunological), mitochondrial redox dysfunction has become an attractive hypothesis to explain an early event in atherogenesis (Oliveira et al., 2005; Puddu et al., 2005). In fact, in the last two decades, a growing number of experimental studies (Fig. 1) have associated mitochondrial dysfunctions with atherosclerosis (Madamanchi and Runge, 2007; Peng et al., 2019). One pioneering study by Ballinger et al. (2002) showed that oxidative mitochondrial DNA (mtDNA) damage was positively correlated with the extent of atherosclerotic lesions in arteries from humans and from hypercholesterolemic apoE knockout mice and that this damage preceded the phenotypic establishment of the disease in these mice. mtDNA undergoes cumulative oxidative damage from oxidants generated by the nearby respiratory chain (Shokolenko et al., 2009; Bratic and Larsson, 2013). Once mtDNA defects are present, they lead to decreased respiratory subunit formation, impaired mitochondrial respiration and increased oxidant production, establishing a vicious cycle between mtDNA damage and mitochondrial dysfunction. A more recent proof of concept was shown in hyperlipidaemic apoE knockout mice with deficient mitochondrial polymerase-γ proofreading activity. These mice exhibited extensive mtDNA damage, defects in oxidative phosphorylation and increased atherosclerosis (Yu

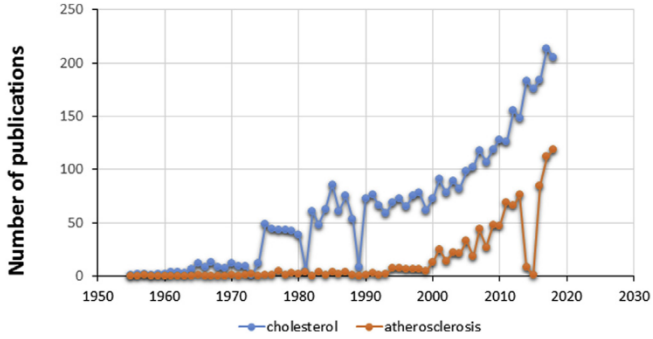

Fig. 1. Scientific publications through the years with the key words mitochondria* and cholesterol (blue line) and mitochondria* and atherosclerosis (orange line). PubMed searches on May 9, 2019, limiting the results by the presence of key words in the Title and Abstract.

et al., 2013).

We previously investigated the possible contribution of mitochondria to cellular oxidative stress in a familial hypercholesterolemia model, atherosclerosis-prone LDL receptor knockout mice (LDLr−/−). Mitochondria from several tissues in these mice generate more oxidants than controls and are more susceptible to Ca^{2+}-induced mitochondrial permeability transition (MPT) (Oliveira et al., 2005). These findings reveal that mitochondrial redox imbalance may be involved in two key events in atherosclerosis: i) as a source of oxidants that oxidize LDL and ii) as the mitochondrial pathway for cell death (Vercesi et al., 2007). We also confirmed in naïve hypercholesterolemic subjects that oxidants derived from peripheral blood monocytes, preferentially from mitochondria, were increased along with oxLDL plasma levels (Vasconcelos et al., 2009). Mitochondrial oxidative stress and enhanced MPT response were later shown in the myocardium of a hypercholesterolemic pig model (McCommis et al., 2011). We demonstrated that mitochondrial oxidative stress in LDLr−/− mice is associated with the depletion of mitochondrial NADP-linked substrates, which leads to insufficient amounts of reducing equivalents (NADPH) to reconstitute the H_2O_2-scavenging function of the glutathione and thioredoxin reductase/peroxidase system (Paim et al., 2008). Mitochondrial NADPH deficiency, oxidant accumulation and MPT are partially reversed by treatments with isocitrate and catalase (Paim et al., 2008) and with the natural antioxidants mangiferin and vimang (Pardo-Andreu et al., 2008; Dorighello et al., 2018a). The NADPH deficit in LDL receptor-defective cells is partially explained by the augmented cholesterol synthesis in these cells (Oliveira et al., 2005), which is a pathway that consumes large amounts of NADPH (24:1 M ratio). Using several in vivo treatments (citrate, pravastatin, citrate + pravastatin) in an attempt to spare mitochondrial NADPH content in LDLr−/− mice, we modulated the mitochondrial oxidant production rates, which correlated with the severity of atherosclerosis (Dorighello et al., 2016). The positive correlation between mitochondrial oxidant production rates and the size of aortic atherosclerotic lesions in this model was also verified in the context of ageing (Dorighello et al., 2018b). In agreement, the increased MPT response to Ca^{2+} in hypercholesterolemic pigs was associated with decreased levels of reduced glutathione (GSH) and antioxidant enzyme activities (MnSOD, thioredoxin and peroxiredoxin) (McCommis et al., 2011).

Accelerated atherosclerosis and elevated mitochondrial oxidant production were observed in experiments involving the deletion of components of the mitochondrial antioxidant system in atherosclerosis models, reinforcing the connection between mitochondrial oxidants and atherogenesis. Attenuated superoxide dismutase 2 activity enhanced atherogenesis in apoE knockout mice that were exposed to environmental tobacco smoke or filtered air (Harrison et al., 2011) and plaque instability in aged apoE knockout mice (Vendrov et al., 2017). On the other hand, strategies directed at preserving mitochondrial antioxidant mechanisms such as the mitochondrial ectopic expression of catalase (mCAT) neutralized mitochondrial oxidants and reduced lesion areas and inflammatory markers in LDLr−/− mice that were transplanted with mCAT transgenic mouse bone marrow (Wang et al., 2014).

4.2. Cell cholesterol content, mitochondria and cell death

Excess intracellular lipids cause mitochondrial redox dysfunction, permeability transition and cell death in metabolic disturbances that predispose individuals to atherosclerosis (Vercesi et al., 2018). On the other hand, increasing cell oxidants induces lipid peroxidation and glycoxidation reactions and protein and mtDNA oxidative damage, which, if not detoxified or cleared by ubiquitin-proteasome and autophagy pathways, lead to death in many cell types, including those of the arterial wall.

Regarding cell cholesterol content, loading macrophages with free cholesterol is associated with widespread mitochondrial dysfunction and activation of the mitochondrial apoptosis pathway (Yao and Tabas, 2001). In addition, oxysterols, particularly 7-ketocholesterol, present in oxLDL and generated by autoxidation (Zarrouk et al., 2014), are also cytotoxic to vascular wall cells by inducing calcium-dependent activation of several pro-apoptotic pathways (Berthier et al., 2005). Although mitochondria possess a limited amount of cholesterol in their membrane bilayers, the regulated transport of cholesterol into mitochondria plays physiological roles in steroidogenic and non-steroidogenic tissues through the cytochrome P450 enzymes (García-Ruiz et al., 2017). Mitochondrial sterol 27-hydroxylase (CYP27A1) is widely distributed in numerous tissues. The 27-hydroxylation of cholesterol by CYP27A1 is part of bile acid synthesis in the liver and regulates cholesterol homeostasis in non-steroidogenic tissues (Adams et al., 2004). In macrophages, 25- and 27-hydroxycholesterol downregulate cholesterol synthesis through the SREBP pathway and enhance the cell efflux of cholesterol via LXR (Fu et al., 2001; Graham, 2015), thus alleviating cholesterol overload. However, mitochondrial cytochrome P450 enzymes consume NADPH to metabolize cholesterol and thus decrease the reducing power of mitochondria. In addition, mitochondrial membranes that are enriched with cholesterol have increased membrane order parameters, which negatively affect specific membrane carriers, such as the GSH transport system, without affecting others, such as the adenine nucleotide translocator (García-Ruiz et al., 2017). This results in GSH depletion in the mitochondrial matrix, enhancing mitochondrial oxidants induced by different stimuli. Replenishment of GSH using a GSH precursor that freely diffuses through membranes, such as GSH ethyl ester (GSH-EE), protects against oxidative stress in steatohepatitis (von Montfort et al., 2012).

Apart from the mitochondrial permeability transition pathway of apoptosis, other death pathways are triggered by intracellular excess cholesterol, such as impaired autophagy and inflammasome activation. Cholesterol-loaded macrophages found in advanced atherosclerotic lesions have impaired autophagy due to the accumulation of lipoprotein components inside lysosomes, which include cholesteryl esters and free cholesterol, and alkalization of organelle contents (Cox et al., 2007). Autophagy impairment prevents the turnover of organelles, such as dysfunctional mitochondria, and compromises overall cell function (Mizushima and Komatsu, 2011). This leads to foam cell death and contributes to the development of a more complex atherosclerotic lesion. A recent study supports the hypothesis that autophagy is useful in vascular disease prevention by stimulating vascular cell cholesterol efflux, which leads to inhibition of necrotic core formation and lipid accumulation (Michiels et al., 2016).

Oxidative stress conditions, including mitochondrial redox dysfunction and mtDNA damage, provoke and potentiate the inflammatory response, a key event in atherosclerosis. Previous studies indicate that increased oxidant production induces the assembly of multiprotein inflammatory complexes called inflammasomes, which are implicated in the induction of regulated cell death modes (De Vasconcelos et al., 2016). The nod-like receptor protein 3 (NLRP3) subset of the inflammasome is the major immune sensor for cellular stress signals. NLRP3 activation triggers caspase-1-mediated maturation of the precursors of the cytokines IL-1β and IL-18 (Salminen et al., 2012). Experimental approaches have demonstrated that autophagic uptake capacity regulates mitochondrial integrity, oxidant production, and subsequent NLRP3 activation (Nakahira et al., 2011; Zhou et al., 2011). Zhou et al. (2011) demonstrated that inhibition of autophagy triggers the accumulation of damaged, oxidant-generating mitochondria, which augments the activation of NLRP3 inflammasomes in human macrophages. Cholesterol crystals, observed at very early stages of diet-induced atherosclerotic lesions, directly activate NLRP3 inflammasomes in human macrophages by causing lysosomal damage and cathepsin B release (Rajamäki et al., 2010; Duewell et al., 2010). Cholesterol crystals induce inflammation mostly in macrophages and possibly also in other endocytotic cells such as endothelial cells. Activation of macrophage inflammasomes promotes atherosclerosis and its subsequent complications in both mice and humans (Tall and Westerterp, 2019).

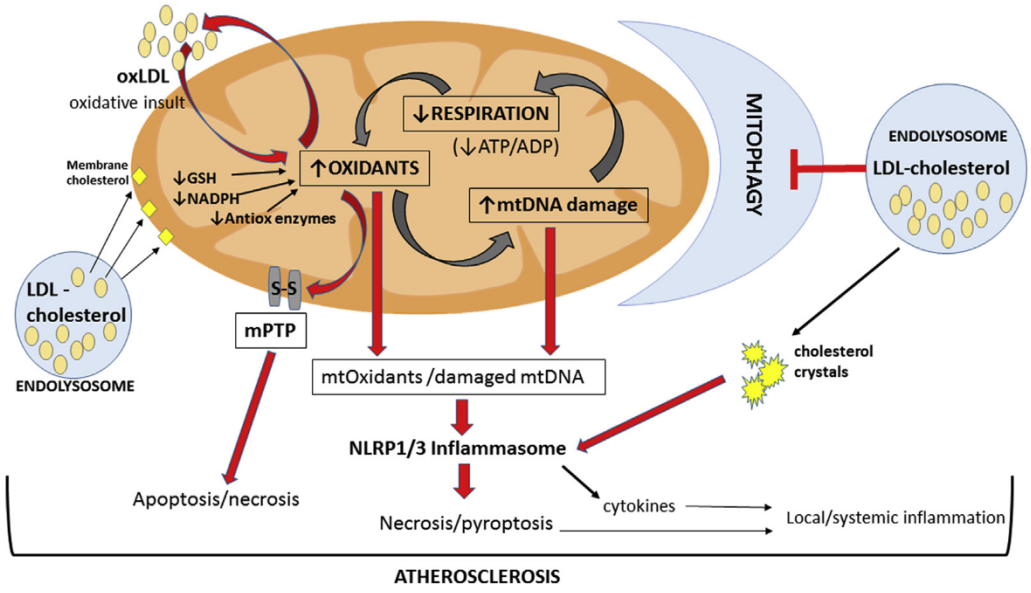

Fig. 2. Multiple mechanisms linking mitochondrial dysfunctions and key events in atherosclerosis. Excess LDL-derived cholesterol and oxysterols (e.g., 7-ketocholesterol) are toxic to mitochondria. On the other hand, mitochondrial oxidants may cause LDL oxidation. Excess free cholesterol enters mitochondria and is metabolized to oxysterol (e.g., 27-hydroxycholesterol) by the P450 enzymes that consumes mitochondrial reducing power (NADPH, GSH) and decrease antioxidant defences. Enrichment of mitochondrial membranes with free cholesterol decreases GSH transport into mitochondria. Oxidant-generating mitochondria become dysfunctional, mediate oxidative damage of mtDNA and exhibit increased susceptibility to MPT that leads to cell death. Cholesterol-loaded cells have impaired autophagy/mitophagy due to the accumulation of cholesterol in lysosomes, preventing the clearance of dysfunctional mitochondria. Oxidant-generating mitochondria, oxidized mtDNA and cholesterol crystals activate inflammasome assembly, cytokine release and cell death.

The mechanisms linking mitochondrial dysfunction, cell death and atherosclerosis are summarized in Fig. 2.

4.3. Mitochondrial-targeted strategies

Since most conventional antioxidant treatments studied in clinical trials have failed to reduce atherosclerosis and cardiovascular disease, specific oxidant scavengers that target mitochondria have been studied mostly in preclinical models and cell culture systems. A wide range of antioxidants are targeted to the mitochondria by conjugation with triphenylphosphonium cation (TPP), such as MitoE, MitoSOD, MitoQ and MitoTempo (Smith et al., 2011; Oyewole and Birch-Machin, 2015). They diminish oxidant formation without affecting mitochondrial oxidative phosphorylation, thus suggesting a therapeutic role for mitochondria-targeted antioxidants. MitoE and MitoQ prevent cell death due to endogenous oxidative stress in cultured fibroblasts from Friedreich ataxia patients in which glutathione synthesis was blocked (Jauslin et al., 2003). In mouse models, MitoQ reduced macrophage content and cell proliferation within atherosclerotic plaques and inhibited multiple features of metabolic syndrome (Mercer et al., 2012). Aged apoE knockout mice treated with MitoTempo had decreased vascular oxidant levels and atherosclerosis (Vendrov et al., 2015). Few clinical trials using MitoQ have been conducted. Chronic treatments with MitoQ showed no effects on Parkinson's disease progression (Snow et al., 2010) but alleviated liver damage in hepatitis C (Gane et al., 2010) and improved vascular function in healthy older adults (Rossman et al., 2018). The latter study showed improved endothelial function, lower aortic stiffness and lower plasma oxLDL after 6 weeks of MitoQ treatment compared with those of placebo (Rossman et al., 2018). It is important to highlight that the mitochondrial antioxidant MitoQ-mediated reduction in circulating oxLDL levels is an impressive outcome, since oxLDL acts on the three vascular wall cell types (endothelial cells, macrophages and smooth muscle cells) to promote atherogenesis. Longer trials are necessary to confirm the benefits of MitoQ in human disease progression.

Another putative strategy to decrease mitochondrial production of oxidants is to accelerate the electron flow through the electron transport chain (respiration), thus reducing the probability of electron leakage and superoxide production (Turrens, 2003). An effective way to do this is by inducing a mild mitochondrial uncoupling of respiration-phosphorylation, causing a minor decrease in mitochondrial inner membrane potential and a potent decrease in superoxide formation, without compromising the intracellular demand on ATP (Skulachev, 1998). As described in section 2, mitochondria possess an endogenous uncoupling mechanism that, once activated, promotes proton leakage through the inner membrane to the matrix and accelerates respiration to re-establish membrane potential. These mechanisms are mediated by the activation of uncoupling proteins (UCPs), adenine nucleotide translocators (ANTs) and mitochondrial ATP-sensitive potassium channels (mitoK$_{ATP}$). Activity of mitoK$_{ATP}$ promotes mild uncoupling and thus regulates mitochondrial redox balance. Activation of the mitoK$_{ATP}$ channel has been widely reported to promote protection against ischaemia-induced tissue injury (reviewed in Cunha et al., 2011). In addition to regulating the mitochondrial redox state, the activity of this channel plays a role in the regulation of energy metabolism (Alberici et al., 2011). We previously found these channels to have increased activity in the liver mitochondria of genetic hypertriglyceridemic mice (Alberici et al., 2003). This condition also predisposes mice to atherosclerosis in a cholesterol-enriched context (Masucci-Magoulas et al., 1997). These mice present elevated plasma and intracellular levels of triglycerides and free fatty acids (Alberici et al., 2006), as well as enhanced levels of oxidants in their cytosol, but mitochondrial oxidant generation was attenuated in a mitoK$_{ATP}$-dependent manner (Alberici et al., 2009). In addition, the enhanced mitoK$_{ATP}$ was linked to decreased energy conversion efficiency, allowing these animals to maintain equal weight gain while eating more (Alberici et al., 2006).

Mild mitochondrial uncoupling may also be induced by several exogenous compounds. Treatment of mice with safe low doses of the protonophore 2,4-dinitrophenol accelerated energy metabolism, improved redox balance and enhanced longevity (Caldeira da Silva et al., 2008). Mild mitochondrial uncoupling has also been shown to be protective in cell and animal models of ischaemia-reperfusion and metabolic syndrome (reviewed in Cunha et al., 2011). In addition, niclosamide ethanolamine, a commercial anthelminthic drug, has been shown to induce mild mitochondrial uncoupling and improve diabetic symptoms in mice (Tao et al., 2014). A novel cationic mitochondrial uncoupler, C4R1 (a derivative of rhodamine 19), is effective in combating obesity in C57Bl/6 mice (Kalinovich and Shabalina, 2015). However, there have been no reports of mitochondrial uncoupler effects on atherosclerosis. Further studies are necessary to translate these findings into safe and applicable therapies.

5. Concluding remarks

The concepts introduced by the Mitchell chemiosmotic theory of coupling respiration to ATP synthesis through the H^+ electrochemical potential included mitochondria serving as the centre of a multitude of essential cellular functions. It is now well recognized that changes in mitochondrial electrochemical potential also regulate relevant cell signalling processes such as superoxide generation, redox balance and mitochondrial Ca^{2+} handling. While mild mitochondrial uncoupling regulates redox signalling and protects against oxidative stress, an intense uncoupling compromises ATP synthesis and other energy-dependent processes and may trigger the mitochondrial cell death machinery.

Regarding atherosclerosis, there is increasing evidence showing that oxidative stress is the common denominator of a variety of traditional risk factors. Research over the past few decades has led to the identification of mitochondria as a major oxidant-generating system that could potentially be modulated in atherosclerosis. Here, we emphasized the role of mitochondrial redox dysfunctions that lead to several death pathways in arterial wall cells and may contribute to the initiation and progression of the disease. We also point to pre-clinical evidence and basic research on strategies targeting mitochondria as promising therapies, such as mitochondria-specific oxidant scavengers, stimulation of the mitochondrial antioxidant system and activation of autophagy.

Declarations of interest

The authors have no competing interests to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.mam.2019.100840.

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