Mitochondrial Bioenergetics and Redox Dysfunctions in Atherosclerosis

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Abstract

In the first part of this review, we summarize mitochondrial bioenergetics basic concepts showing that mitochondria are critical regulators of cell life and death. Until few decades ago, mitochondria were considered to play essential roles in respiration, ATP formation, non-shivering thermogenesis and in a variety of metabolic pathways. However, the Peter Mitchell concept of coupling between electrons flow and ATP synthesis through the intermediacy of a H+ electrochemical potential lead to the recognition that the proton-motive force also regulate a series of relevant cell signaling 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 mitochondria dysfunctions in the particular 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 would be relevant for atherosclerosis.

Key words: mitochondrial membrane potential, mitochondrial permeability transition, mitochondrial uncoupling, hypercholesterolemia, oxidative stress, cell death.

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 (tricarboxylic acid cycle, TCA) or fats (ß-oxidation) flow to molecular oxygen, yielding free energy used to generate ATP from ADP and inorganic phosphate. Four pairs of hydrogen atoms are transferred by specific dehydrogenases from isocitrate, alfa-ketoglutarate, succinate and malate, in each turn around the TCA, to NAD^+ and FAD. These coenzymes are the major carriers of hydrogen atoms in the process of fuel molecules oxidation such as glucose, fatty acids and, at a much lower extent, some amino acids that undergo loss of their amino groups and enter the TCA. From these coenzymes, the hydrogens are separated into H+ and "energy-rich" electrons which are transferred through a sequence of electrons carriers called electron transport chain (ETC) or respiratory chain to the final electron acceptor, the 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 oxidized by molecular oxygen via the respiratory chain, thus, originating the expression P/O ratio equals to 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 (Ochoa, 1943).

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

fine granularity is surrounded by two membranes, an outer and an inner membrane. The outer membrane is smooth and contains proteins (porins) that confer non-specific permeability to solutes with molecular weight 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 as the components of the ETC and also carriers and channels responsible for the flux of ions and metabolites that move in and out of the mitochondrial matrix.

Until few decades ago, mitochondria were considered to play essential roles in respiration, ATP formation, non-shivering thermogenesis (though to occur exclusively in the brown adipose tissue) and in a variety of metabolic pathways such as the TCA, fatty acid beta oxidation, aminoacids metabolism, ketogenesis, gluconeogenesis, ureogenesis and other metabolic activities. By the 60-70's, one of the main challenges in the field of bioenergetics was the understanding of the mechanisms by which the oxidation of substrates by mitochondria could be used to drive ADP phosphorylation. It was already known (Racher, 1976) that the catalysis of oxidative phosphorylation was composed by two distinct protein assemblies. The first is a multi-enzymatic system, the ETC, embedded in the inner mitochondrial membrane where pairs of electrons flow thermodynamically "downhill" from $NADH^{+}H^{+}$ (-0.32 volt) to complex I (NADH dehydrogenase) or from $FADH_2$ to complex II (succinate dehydrogenase) and sequentially to ubiquinone (CoQ10) to generate ubiquinol (CoQH2). Ubiquinol transfers two electrons to complex III (ubiquinol-cytochrome c oxidoreductase) and from complex III via cytochrome c to complex IV (cytochrome c oxidase) and finally to molecular oxygen (+0.82 volt) 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) centers. The second protein assembly, the ATP-synthase (complex V), also characterized as the coupling device, catalyzes the "uphill" ATP formation from ADP and Pi using the free energy released from electrons flow 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 between cytochromes b and c1 and the third 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 protons leakage. Other mechanisms that regulate the rates of respiration will be outlined below.

During a period of at least three decades (50 -70's) there was an intense debate among researchers involved in the investigations on the mechanisms by which redox free energy was conserved in the form of ATP. Three different hypotheses received more 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 (Boyer, 1975) satisfy both chemical and chemiosmotic hypotheses outlined below.

The chemical hypothesis was originally proposed by Slater (Slater, 1953) stating that the oxidative phosphorylation occurred analogously to the glycolytic substrate-level phosphorylation catalyzed by the glyceraldehyde-3-phosphate dehydrogenase and phosphoglicerate kinase. This hypothesis predicted the formation of three "high-energy" intermediates (A~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 by phosphorolysis, would be converted to X~Pi, the putative donor of the phosphate group to ADP to generate ATP. Since the A~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 the recognition of ETC composition and the order of interactions among its components (Racher, 1976). It should be emphasized that, according to the chemical hypothesis, oxidative phosphorylation could be catalyzed by a simple flat fragment of the inner membrane or even by solubilized enzymes (Racher, 1976). In contrast, Peter Mitchel (Mitchell, 1961; Mitchell, 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 the complexes I, III and IV when electrons flow through the respiratory chain generates a negative inside electrical membrane potential ($\Delta P = \Delta$ pH + $\Delta\Psi$) defined by chemical and electrical components of the proton-motive force. According to Peter Mitchel, this proton-motive force provides the energy for ATP synthesis, implying that the ATP synthase functions as a coupling device operating in reverse, that is, the H+ back flow through the proton

channel (Fo) of the ATP synthase provides the energy to catalyze 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, the Peter Mitchell's hypothesis required both (a) compartments that permit the formation of the proton-motive force and (b) an inner membrane asymmetrically organized and highly impermeable to H+ and other ions (Mitchell, 1961; Mitchell, 1976). This hypothesis had previous support from other systems such as the reversible Ca^{2+} pump of sarcoplasmic reticulum (Makinose and Hasselbach, 1971) and the Na^{+}-K^{+} pump of the plasma membrane (Garrahan and Glynn, 1967) that can use ion gradients to generate ATP. Whether the proton-motive force would satisfy quantitative thermodynamic considerations, such as a P/O ratio of 3 was a matter of intense controversy mainly due to difficulties to experimentally determine 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 laureated a Nobel Prize in Chemistry in 1978.

2. Mitochondria after the Chemiosmotic Theory

The main reason we have briefly revisited these hypotheses of energy coupling between respiration and ADP phosphorylation was to draw the attention of students and non-specialists in the field to the reasons by which the Mitchell Theory (the concept of coupling between electrons flow and ATP synthesis through the intermediacy of a H^{+} electrochemical gradient) has generated excitement and caused an exponential growth in the research on mitochondria around the world. In this respect, it is worth to remind that according to the Mitchell Theory, mitochondrial bioenergetics requires various properties of the inner membrane that were neglected by the chemical hypothesis (Slater, 1953; Mitchell, 1961; Mitchell, 1976). Moreover, it is now amply recognized that the proton-motive force, in addition to be the driving force for ADP phosphorylation is also the driving force for several energy requiring processes, such as (***a***) Ca^{2+} and other ions transport across the membrane (Vercesi et al, 2018), (***b***) non-shivering thermogenesis (Nicholls and Locke, 1984; Klingenberg, 2017), (***c***) the reduction of NADP+ by NADH catalyzed by the nicotinamide nucleotide transhydrogenase, the main source of reducing power to the mitochondrial antioxidants enzyme systems (Rydstrom, 2006), (***d***) the 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 nucleotides 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 catalyze the slow return of protons to the matrix (mild uncoupling) are able to promote a fine tune of the proton-motive force. As proposed by Professor Skulachev (1996), this regulated mild uncoupling protects against excess of 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; Jezek 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 the 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 reentry of protons, extruded by the respiratory chain, into the matrix bypassing the ATP-synthase (Klingenberg, 2017; Skulachev, 1996). The discovery of the plant counterpart of UCP in 1995 (Vercesi et al., 1995; Vercesi et al., 2006) initiated a search for UCP homologs. Between 1997 and 2000, several homologs of UCP1 were identified in all mammalians tissues (Ježek et al., 2018). It is now accepted that except for the UCP1, these new UCPs are not thermogenic but are widespread in eukaryotes, and they may have various physiological roles including regulation of cellular redox signaling (Brandalise et al., 2003; 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. These activate a K^+/H^+ antiporter that generate 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 permit only mild uncoupling. Similarly to the case of UCPs, this decreases the reduction state of the respiratory complexes I, II and III and as a consequence decreases the generation of superoxide by the ETC. The

mitoK_{ATP} is sensitive to ATP, glybenclamide, and 5-hydroxydecanoate and is stimulated by diazoxide (Liu et al., 2001). Increased mitoK_{ATP} activity has been shown to be protective against ischemia-reperfusion injury and hyperlipidemia metabolic and redox stress (Facundo et al., 2006; Alberici et al., 2006; Alberici et al., 2009)

Considering the importance of the inner membrane intactness to sustain membrane electrochemical potential, it should be emphasized that any agent able to bind to this membrane altering specifically or nonspecifically the proton gradient may partially or totally disrupt membrane electrochemical potential, thus compromising ATP synthesis and other energy dependent processes. These agents include the traditional uncouplers such as dinitrophenol and FCCP but also a large number of natural, commercial, pharmaceutical and a increasing number of environmental chemicals that affect transiently or irreversibly the mitochondrial functions (Wallace and Starkov, 2000; Meyer et al., 2018).

Since changes in the proton-motive force also regulate relevant cell signaling processes such as superoxide generation (Hamanaka and Chandel, 2010), redox balance and mitochondrial Ca^{2+} handling (Glance and Balaban, 2012; Vercesi et al., 2018), these new concepts brought by the chemiosmotic theory included mitochondria as a center of a multitude of essential cellular functions. Therefore, alterations in ATP synthesis, Ca^{2+} transport and oxidants generation can 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 diagnose is difficult and treatments are largely palliative (Wallace, 2015). The location of mtDNA molecules in the proximity of the sites of oxidants production expose 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 aging and result in complex diseases of great relevance in public health such as cancer, diabetes, neurodegeneration and many others (Wallace, 2015).

Overall, these new concepts developed on the light of the Mitchell Theory increased the general interest in mitochondrial research and new discovered processes comprise key events in the mechanisms of aging 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 oxidants production (Vercesi et al., 1997; Kowaltowski et al., 2009), Ca2+ transporting system (Vercesi et al., 2018) and the membrane permeability transition pore (mPTP) formation (Zoratti and Szabo, 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 cation stimulate oxidants generation (Castilho et al., 1995) that synergistically with Ca^{2+} promote the opening of a proteinaceous mega-channel, the membrane permeability transition pore (mPTP) (Zoratti and Szabo, 1995). At high conductance state this pore permits the flux of solutes up to 1500 Da, thus eliminating all mitochondrial energy-linked functions (Zoratti and Szabo, 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 includes regulation of mitochondrial Ca^{2+} release and mitochondrial volume (Vercesi, 1984; Vercesi, 1985; Bernardes et al., 1986; Ichas et al., 1998). The transition from low to high conductance states of the pore seems to be dependent on the mitochondrial redox balance (Zago et al., 2000). Indeed, mPTP induced 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; Kowaltowski et al., 1998). The demonstration that cyclosporin A (CsA), a pore opening inhibitor (Crompton et al., 1988; Broekemeier et al., 1989) prevent cell death under different pathological conditions (Griffiths and Halestrap, 1993; Bernardi et al., 2006) support the participation of this pore in the pathogenesis of ischemia/reperfusion, heart

and neurodegenerative diseases, traumatic brain injury, muscular dystrophy, inflammation, dyslipidemias, drug toxicity and aging (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 of its opening by several antioxidants (Vercesi et al., 2018) or the absence of molecular oxygen (Castilho et al., 1995a). In addition, evidences have been provided that exogenously added 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 mPTP opening. 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 mPTP opening (Mailloux et al., 2014), thus suggesting that mPTP is not a molecularly defined channel, but rather a permeability transition formed by protein thiol cross-linking (Vercesi et al., 2018). Mitochondrial dysfunctions that lead to mPTP and consequently to cell death have been implicated in the pathogenesis of several metabolic and aging 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 particular emphasis on hyperlipidemia induced atherosclerosis. Atherosclerosis may develop because of predisposing risky circumstances such as primary or secondary dyslipidemias, diabetes, obesity, hypertension, smoking and infections. Except from the genetic hyperlipidemias, all other conditions are complex settings composed of several metabolic, hormonal and immunological disturbances simultaneously. Therefore, genetic dyslipidemia models are useful to investigate whether lipid excess in the circulation and hence 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 proven a causal role for the excess of low-density lipoprotein (LDL) - cholesterol in atherosclerosis. However, as

elegantly demonstrated by Brown and Goldstein in the early 80´s, the disease culprit is not the native LDL particle, since most cell types have effective defense mechanisms against an overflow of LDL-cholesterol. When sufficient amount of LDL-cholesterol is internalized by cells, LDL receptors and de novo cholesterol synthesis are shutdown, thus preventing a cholesterol overload (Goldstein and Brown, 1990) and subsequently 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), the 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) act as inflammatory stimuli within the vessel wall, activating endothelial cells and recruiting circulating monocytes, which avidly 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 atherosclerosis initiation and progression.

There is strong support for oxidation of LDL taking place in vivo as they are present in human and mouse atherosclerotic lesions (Yurdagul et al., 2016). In addition, oxLDL and electronegative LDL have been found elevated in the plasma of patients with hypercholesterolemia and coronary artery disease (Hulthe et al., 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 represent a unifying mechanism of a wide range of risky contexts (metabolic, hemodynamic and immunological), a mitochondrial redox dysfunction became an attractive hypothesis of 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 evidences (**Figure 1**) have associated mitochondrial dysfunctions with atherosclerosis (Madamanchi and Runge 2007; Peng et al., 2019). One pioneer 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 human and from

hypercholesterolemic apoE knockout mice and that, this damage preceded the phenotypic establishment of the disease in these mice. mtDNA may undergo 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 can lead to decreased respiratory subunits formation, impaired mitochondrial respiration and increased oxidants production, stablishing a vicious cycle between mtDNA damage and mitochondrial dysfunction. A more recent proof of concept was shown in hyperlipidemic apoE knockout mice with deficiency for mitochondrial polymerase-γ proofreading activity. These mice exhibited extensive mtDNA damage, defects in oxidative phosphorylation and increased atherosclerosis (Yu et al., 2013).

We have previously investigated the possible contribution of mitochondria to cellular oxidative stress in the familial hypercholesterolemia model, the 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 could indeed be involved in two key events of atherosclerosis: i) as a source of oxidants that oxidize LDL and ii) as the mitochondrial pathway for cell death (Vercesi et al., 2007). We have 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 was later shown in another model, the porcine myocardium of hypercholesterolemic pigs (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). Indeed, mitochondrial NADPH deficiency, oxidant accumulation and MPT could be partially reversed by treatments with isocitrate, catalase (Paim et al., 2008) and with the natural antioxidants Mangiferin and Vimang (Pardo-Andreu et al., 2008; Dorighello et al., 2018). The NADPH deficit in LDL receptor-defective cells can be in part explained by the augmented cholesterol synthesis in these cells (Oliveira et al., 2005), a pathway that consumes large amounts of NADPH (24:1 molar ratio). Using several in vivo treatments of LDLr-/- mice in an attempt to spare mitochondrial NADPH content (citrate,

pravastatin, citrate+pravastatin), we were able to modulate 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 aging (Dorighello et al., 2018). In agreement, increased MPT response to Ca^{2+} in hypercholesterolemic pigs was associated with decreased levels of reduced glutathione (GSH) and of antioxidant enzymes activities (MnSOD, thioredoxin and peroxiredoxin) (McCommis et al., 2011).

Accelerated atherosclerosis and elevated mitochondrial oxidants production were observed in experiments involving the deletion of components of the mitochondrial antioxidant system in atherosclerosis models, reinforcing the connection of mitochondrial oxidants and atherogenesis. Attenuated superoxide dismutase 2 activity enhanced atherogenesis in apoE knockout mice 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 to preserve mitochondrial antioxidant mechanisms such as the mitochondrial ectopic expression of catalase (mCAT), neutralized mitochondrial oxidants and reduced lesion area and inflammatory markers in LDLr-/- mice transplanted with mCAT transgenic mice bone marrow (Wang et al 2014).

4.2 Cell cholesterol content, mitochondria and cell death

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

Regarding cell cholesterol content, loading macrophages with free cholesterol has been associated with widespread mitochondrial dysfunction and activation of the mitochondrial apoptosis pathway (Yao and Tabas, 2001). In addition, oxysterols, and particularly 7-ketocholesterol, present in oxLDL and generated by autoxidation (Zarrouk et al., 2014), are also cytotoxic to the vascular wall cells, by inducing calcium-dependent activation of several pro-apoptotic pathways (Berthier et al., 2005). Although mitochondria exhibit 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 at al., 2017). Mitochondrial sterol 27-hydroxylase (CYP27A1) is widely distributed in numerous tissues. The 27-hydroxylation of cholesterol by CYP27A1 is part of the bile acid synthesis in the liver and, in non-steroidogenic tissues, regulates cholesterol homeostasis (Adams et al., 2004). In macrophages, 25- and 27-hydroxycholesterol down-regulate cholesterol synthesis through the SREBP pathway and enhance the cell efflux of cholesterol via LXR (Fu et al., 2001; Graham, 2015), thus alleviating the threatening cholesterol overload condition. However, mitochondrial cytochrome P450 enzymes consume NADPH to metabolize cholesterol and thus decrease the mitochondrial reducing power content. In addition, mitochondrial membranes enriched with cholesterol have increased membrane order parameter, which affects negatively specific membrane carriers, such as the GSH transport system without effect on others, such as the adenine nucleotide translocator (Garcia-Ruiz et al., 2017). This results in GSH depletion in the mitochondrial matrix, enhancing mitochondrial oxidants induced by different stimuli. Replenishment of GSH, using 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 may be triggered by intracellular excess of cholesterol, named, 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 includes cholesteryl esters and free cholesterol, and alkalization of organelle content (Cox et al., 2007). Autophagy impairment prevents the turnover of organelles such as dysfunctional mitochondria compromising overall cell function (Mizushima and Komatsu, 2011). This process leads to foam cell death and contributes to the development of a more complex atherosclerotic lesion. A recent study supports the hypothesis that autophagy might be useful in vascular disease prevention by stimulating vascular cells 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, can provoke and potentiate inflammatory response, a key event in atherosclerosis. Previous studies indicate that increased oxidants production can induce the assembly of multiprotein inflammatory complexes called the inflammasomes, which

are implicated in induction of regulated cell death modes (de Vasconcelos et al., 2016). Nod-like receptor protein 3 (NLRP3) subset of inflammasome is the major immune sensor for cellular stress signals. NLRP3 activation triggers the caspase-1-mediated maturation of the precursors of IL-1β and IL-18 cytokines (Salminen et al., 2012). Experimental approaches have demonstrated that autophagic uptake capacity can regulate mitochondrial integrity, oxidant production, and subsequent NLRP3 activation (Nakahira et al., 2011; Zhou et al., 2011). Zhou et al. (2011) demonstrated that the 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, can directly activate NLRP3 inflammasomes in human macrophages by causing lysosomal damage and cathepsin B release (Rajamaki et al., 2010; Duewell et al., 2010). It seems that cholesterol crystals can induce inflammation mostly in macrophages but probably also in other endocytotic cells, e.g., endothelial cells. Activation of macrophage inflammasomes promotes atherosclerosis and its complications in both mice and humans (Tall and Westerterp, 2019). The mechanisms linking mitochondrial dysfunction, cell death and atherosclerosis are summarized in the **Figure 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 could be targeted to 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 mice models, mitoQ reduced macrophage content and cell proliferation within the 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), 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 versus placebo (Rossman et al., 2018). It is important to highlight that the mitochondrial antioxidant mitoQ mediated reduction of circulating oxLDL levels is an impressive outcome, since oxLDL acts on the three vascular wall cell types (endothelial, macrophages and smooth muscle cells) to promote atherogenesis. Longer trials are necessary to confirm benefits in human disease progression.

Another putative strategy to decrease mitochondrial production of oxidants is accelerating the electron flow through the electron transport chain (respiration), thus reducing the probability of electron leak and superoxide production (Turrens, 2003). An effective way to do this is inducing a mild mitochondrial uncoupling of respiration-phosphorylation, causing a minor decrease in mitochondrial inner membrane potential, a potent decrease in superoxide formation, without compromising the intracellular demand on ATP (Skulachev, 1998). As described in the previous section 2, mitochondria possess endogenous uncoupling mechanism that, once activated, promote proton leak through the inner membrane to the matrix and accelerate respiration to re-stablish membrane potential. These mechanisms may be mediated by the activation of uncoupling proteins (UCPs), adenine nucleotide translocators (ANT) and mitochondrial ATP sensitive potassium channel (mitoK_{ATP}). mitoK_{ATP} activity promotes a mild uncoupling and thus regulates mitochondrial redox balance. mitoK_{ATP} channel activation has been widely reported to promote protection against ischemia induced tissue injury (reviewed in Cunha et al., 2011). Besides regulating mitochondrial redox state, the activity of this channel play a role in the regulation of energy metabolism (Alberici et al., 2011). We previously found these channels more active in liver mitochondria of genetic hypertriglyceridemic mice (Alberici et al., 2003). This condition also predisposes 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}

were linked to a lower efficiency of energy conversion, allowing these animals to maintain equal weight gains while eating more (Alberici et al., 2006).

Mild mitochondrial uncoupling may be also 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 et al., 2008). Mild mitochondrial uncoupling has also been shown to be protective for cell and animal models of ischemia-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 improvement of diabetic symptoms in mice (Tao et al., 2014). A novel cationic mitochondrial uncoupler, C4R1 (derivative of rhodamine 19) has been shown effective in combating obesity in C57Bl/6 mice (Kalinovich and Shabalina, 2015). However, there has been no reports of mitochondrial uncouplers effects on atherosclerosis. Further studies are necessary to translate these findings into safe and applicable therapies.

5. **Concluding remarks**

The concepts brought up by the Mitchell chemiosmotic theory of coupling respiration to ATP synthesis through the H^+ electrochemical potential included mitochondria as a center of a multitude of essential cellular functions. It is now well recognized that changes in the mitochondrial electrochemical potential also regulate relevant cell signaling processes such as superoxide generation, redox balance and mitochondrial Ca^{2+} handling. While mild mitochondrial uncoupling regulate redox signaling and protects against oxidative stress, an intense uncoupling compromise 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 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 leads to several death pathway in the arterial wall cells and may contribute to the initiation and progression of the disease. We also point to pre-clinical evidences and basic research on strategies

targeting mitochondria as promising therapies, such as mitochondrial specific oxidants scavengers, stimulation of mitochondrial antioxidant system and activation of autophagy.

Declarations of interest

The authors have no competing interests to declare.

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FIGURE LEGENDS

Figure 1. Scientific Publications along the years with the key words: mitochondria* and cholesterol (blue line) and mitochondria* and atherosclerosis (orange line). PubMed searchs on May 9, 2019, limiting the results by the presence of key words in the Title and Abstract.

Figure 2: Multiple mechanisms linking mitochondrial dysfunctions and key events in atherosclerosis**.** Excess of LDL derived cholesterol and oxysterols (e.g. 7-ketocholesterol) are toxic to mitochondria. On the other hand, mitochondrial oxidants may cause LDL oxidation. Excess of free cholesterol enter mitochondria to be metabolized to oxysterol (e.g. 27-hydroxicholesterol) by the P450 enzymes that consumes mitochondrial reducing power (NADPH, GSH) and decrease antioxidant defenses. 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 lead 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, cytokines release and cell death.

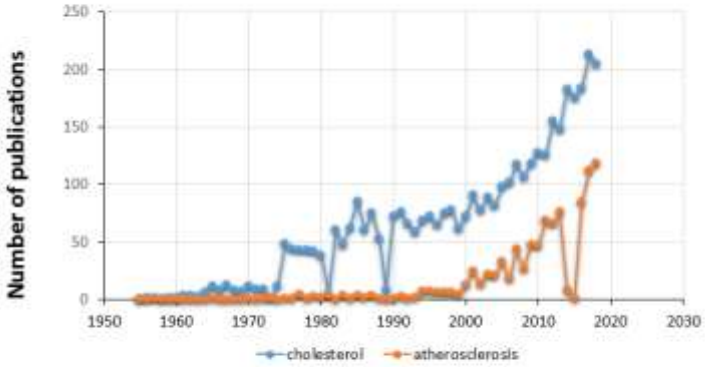
Number of publications
250
200
150
100
50
0
1950
1960
1970
1980
1990
2000
2010
2020
2030
cholesterol
atherosclerosis

Fig 1

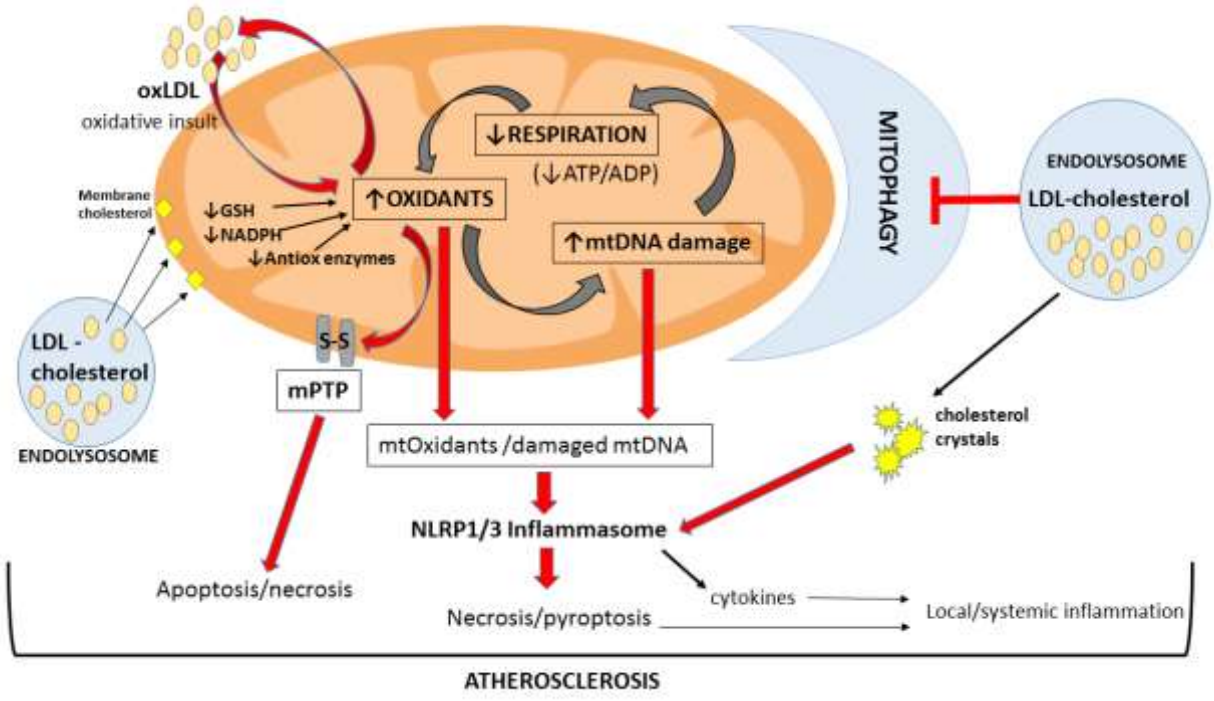
oxLDL
oxidative insult
Membrane cholesterol
↓GSH
↓NADPH
↓Antiox enzymes
↑OXIDANTS
↓RESPIRATION
(↓ATP/ADP)
↑mtDNA damage
MITOPHAGY
ENDOLYSOSOME
LDL-cholesterol
LDL - cholesterol
ENDOLYSOSOME
S-S
mPTP
cholesterol crystals
mtOxidants /damaged mtDNA
NLRP1/3 Inflammasome
Apoptosis/necrosis
cytokines
Necrosis/pyroptosis
Local/systemic inflammation
ATHEROSCLEROSIS

Fig 2

Vitae

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