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Mitochondria as a Biosensor for Drug-Induced Toxicity – Is It Really Relevant?

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1. Introduction

Mitochondria, from the Greek *mito* (thread) and *chondros* (grains) are small organelles that exist as a network in the cytoplasm of eukaryotic cells, performing a variety of important functions including energy production, calcium homeostasis, fatty acid metabolism or heme and pyrimidine biosynthesis (Pereira, Moreira et al., 2009). Moreover, mitochondria play a critical role in programmed cell death (apoptosis) (Jeong & Seol, 2008; Wang & Youle, 2009). Mitochondrial structure comprises two different membranes, the outer (OMM) and the inner membrane (IMM) that functionally separate two distinct compartments, the inter-membrane space (IMS) and the matrix (Jezek & Plecita-Hlavata, 2009) (Fig. 1). The outer membrane encloses mitochondria and it is somewhat identical to other cell membranes, including cholesterol in its composition, and is permeable to a large variety of ions and metabolites. The inner membrane lacks cholesterol, is rich in the tetra fatty acid-containing phospholipid cardiolipin, and basically controls the entry of metabolites and ions into mitochondria, through the action of specific transport proteins (Scatena, Bottoni et al., 2007). Inner membrane invaginations and membrane enclosed structures which can exist connected to the IMM or freely in the mitochondrial matrix are called *cristae*. It is in these latter structures that most of the membrane-bound metabolic proteins and energy-producing respiratory complexes (complexes I–V) exist (Fig. 1) (Zick, Rabl et al., 2009).

1.1 Organization and genomics

Also considered as a *reticulum*, the mitochondrial network continuously moves, fuses and divides in a process tightly regulated by cellular stimuli and disturbances inside this organelle (Detmer & Chan, 2007). The shape greatly varies depending on the tissue, developmental and physiological state. Within a cell, the distribution of mitochondria is unequal depending on the cellular energetic or metabolic demands (Grandemange, Herzig et al., 2009). The overall shape of mitochondrial network results from an equilibrium between fusion and fission events (Wallace & Fan, 2010). These events allow the exchange of organelle contents such as membrane lipids, proteins, solutes, metabolites and even mitochondrial DNA (Detmer & Chan, 2007), as well as to provide a balance of

the electrochemical gradient (Twig, Graf et al., 2006). Balanced mitochondrial fusion and fission is crucial to preserve mitochondrial integrity and functionality (Wallace & Fan, 2010). Three distinct proteins seem to be involved in mitochondrial fusion: Mitofusins 1 and 2 (Mfn1 and Mfn2) and Optic Atrophy-1 (OPA-1). Mfn 1 and 2 are GTPases proteins that are localized in the OMM and form homo- and hetero-oligomeric complexes between themselves and with counterparts in adjacent mitochondria, which mediate their tethering (Arnoult, 2007). OPA-1 is a dynamin-related protein that can be found in a soluble form in the IMS or tightly associated with the IMM, being a key protein for the fusion of this mitochondrial membrane (Arnoult, Grodet et al., 2005). Evidence also suggests that OPA-1 controls *cristae* morphology and is implicated in the complete release of cytochrome c during apoptosis (Jourdain & Martinou, 2009; Perkins, Bossy-Wetzel et al., 2009). Fusion of both membranes is a two-step process that occurs in a coordinate fashion, although the precise mechanism remains unclear (Malka, Guillery et al., 2005). Mitochondrial fission requires the recruitment of dynamin-related protein 1 (Drp1) from the cytosol to the OMM where it forms multimeric rings and spiral-like structures that surround and constrict the organelle in a GTP-dependent manner (Sheridan & Martin, 2010). The mechanism that triggers this recruitment is still unknown, however, Fis1, a small mitochondrial transmembrane protein, seems to be responsible for this mobilization (Sheridan & Martin, 2010).

Mitochondria are the only organelles outside of the nucleus that contain their own genome and replicate itself in an independent manner from the nuclear genome. A single DNA polymerase (polymerase-gamma), with base excision repair activity, ensures the replication of the mitochondrial DNA (mtDNA). Moreover, mtDNA has a particular feature since it is exclusively maternally inherited. Each mitochondrion contains approximately 10-15 copies of a small circular chromosome that are organized into one or more structures called nucleoids. Mitochondrial DNA encodes for 13 proteins that are essential for the electron transport and ATP generation by oxidative phosphorylation (OXPHOS) and 2 rRNA and 22 tRNA (Van Houten, Woshner et al., 2006). The remaining proteins required for mitochondrial activity are encoded by the nucleus, synthesized in the cytosol and translocated to mitochondria (Wallace, 2008). Mitochondrial DNA undergoes a mutation rate that seems to be between 5- to 20- fold higher than what occurs in nuclear DNA mutations, although this is not consensual (Malka, Lombes et al., 2006; Scatena, Bottoni et al., 2007). The high rate of mutations, if indeed real, can be explained for both the lack of mtDNA protective proteins and its proximity to the electron transport chain, where the majority of, free-radical production occurs (Fruehauf & Meyskens, 2007). Furthermore, the repair mechanism of mitochondrial DNA is less efficient than of nuclear DNA (Berneburg, Kamenisch et al., 2006).

1.2 Oxidative phosphorylation and energy production

Production of energy within a living cell is performed by the conversion of dietary fats and carbohydrates into reducing equivalents. Mitochondria are considered the powerhouses of the cell, due to a variety of important energy-producing metabolic pathways in their interior. Pyruvate is formed in the cytosol as an end-product of glucose metabolism (glycolysis) and can undergo lactic acid or alcoholic fermentation in the absence of oxygen (anaerobic conditions). Under aerobic conditions, pyruvate is converted into acetyl coenzyme A (acetyl-CoA) by pyruvate dehydrogenase (PDH) in the mitochondrial matrix (Pereira, Moreira et al., 2009).

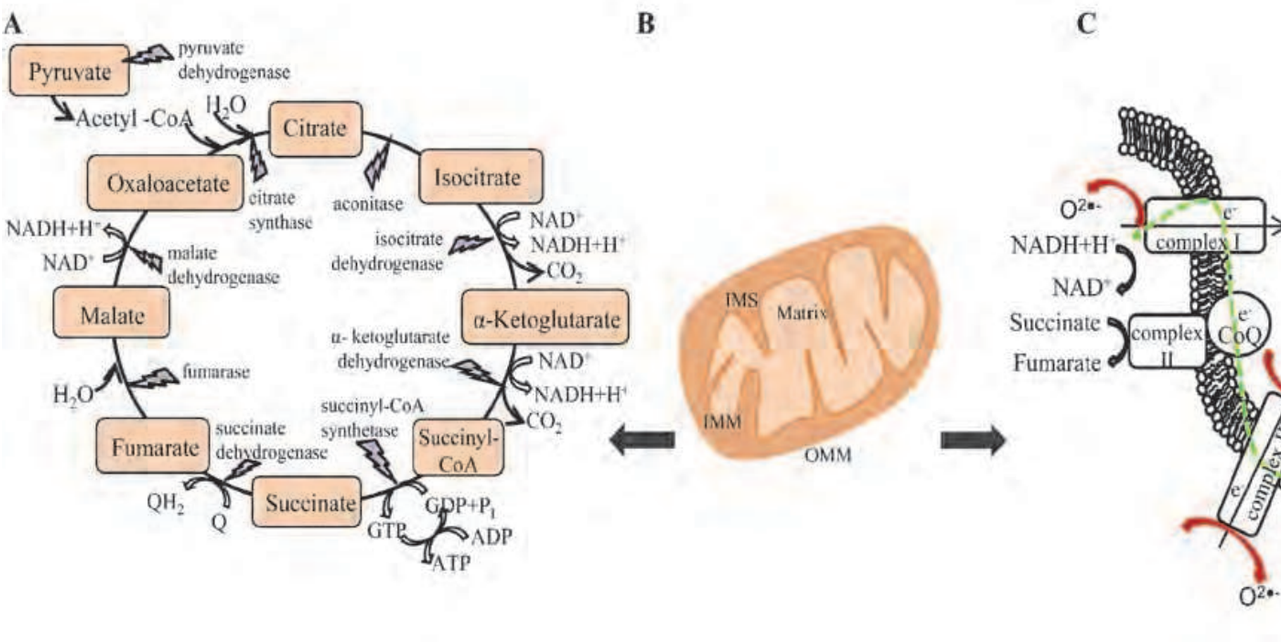

Fig. 1. Mitochondria play a critical role in ATP production, biosynthesis, calcium homeostasis and cell d
some of the functions referred to in the text: (**A)** The Krebs cycle occurs in the matrix and supplies reduc
phosphorylation, besides participating as intermediate in several biosynthetic pathways. (**B**) Overall vie
morphology: The outer mitochondrial membrane (OMM) encloses the organelle within the cell; the inne
(IMM) separates functionally the matrix from the mitochondrial inter-membrane space (IMS). (**C**) Oxida
(OXPHOS): electrons from the Krebs cycle are transferred along the respiratory chain. The energy deriv
used to pump out protons across the inner membrane at complexes I, III and IV, creating a proton electr
both sides of inner membrane. This electrochemical gradient forms a proton-motive force, which is use
protons to the matrix through complex V (ATP synthase). A small amount of electrons can leak towards
and complex III due to an one electron transfer reduction of molecular oxygen forming superoxide anio
(Pereira, Moreira et al., 2009), with permission.

Acetyl-CoA enters the Krebs cycle, being oxidized to generate several intermediates including NADH and succinate (Fig. 1). Other intermediates of the Krebs cycle are also important in several metabolic pathways, including biosynthesis of heme and amino acids (Shadel, 2005). Mitochondria can be involved in the β-oxidation of fatty acids (Vockley & Whiteman, 2002). The end product of this pathway is, once again, Acetyl-CoA, which is used in the Krebs cycle. NADH and succinate, among other intermediates that are produced by different pathways are oxidized by the electron transport chain, ultimately leading to the production of adenosine triphosphate (ATP) in a process known as OXPHOS (Zick, Rabl et al., 2009; Hebert, Lanza et al., 2010) (Fig. 1). Electrons derived from reduced substrates are transferred through several multi-protein complexes (mitochondrial complexes I to IV), down their redox potentials and the energy derived from electron transfer is used to pump out protons across the IMM at complexes I, III and IV which creates an electrochemical gradient between both sides of the IMM. This electrochemical gradient is a proton-motive force driving the re-entry of protons towards the matrix through complex V (ATP synthase), which is coupled to ATP synthesis (Hebert, Lanza et al., 2010). ATP that is produced is exported from the mitochondria by the mitochondrial ADP/ATP translocator (ANT). Molecular oxygen is the final electron acceptor in the mitochondrial respiratory chain, which is reduced via a sequential four-electron transfer into water by complex IV (cytochrome c oxidase, COX). However, some of the electrons that are transferred across the mitochondrial electron transport chain can escape and perform a single electron reduction of molecular oxygen. This phenomenon occurs continuously even in normal conditions leading to formation of superoxide anion ($O_2^{\bullet-}$) and it will be discussed in the next section of this chapter.

1.3 Generation of free radicals

Among the reactive species that are produced within a living cell, reactive oxygen species (ROS) are the most significant. Mitochondrial complexes I and III account for a significant proportion of intracellular ROS formation, although complex I is considered the major contributor (Adam-Vizi & Chinopoulos, 2006; Soubannier & McBride, 2009). The mitochondrial electron transport chain contains several redox centers, which can react with molecular oxygen. As a result, a small amount of electrons leaks from complex I (NADH dehydrogenase) and complex III (CoQ cycle), performing a one-electron reduction of molecular oxygen that gives rise to superoxide anion ($O_2^{\bullet-}$). Approximately 1-2% of the oxygen consumed during OXPHOS under physiological conditions is converted into this product (Solaini, Baracca et al., 2010). Superoxide anion produced by respiratory complex I is released in the mitochondrial matrix and transformed into hydrogen peroxide (H_2O_2) spontaneously or via manganese superoxide dismutase (MnSOD). In turn, $O_2^{\bullet-}$ generated by complex III can be released in both sides of the IMM but in the IMS, the dismutation into H_2O_2 is achieved via Cu/Zn-dependent SOD (Cu/ZnSOD). Hydrogen peroxide can be converted into water in the mitochondrial matrix by catalase or glutathione peroxidase (GSH). Mitochondrial thioredoxin, glutaredoxin and even cytochrome c are other relevant ROS scavengers (for a review see (Fruehauf & Meyskens, 2007)). The H_2O_2 produced can also diffuse to the cytosol and trigger the activation of some transcription factors and various enzymatic cascades (Cadenas, 2004). General oxidative stress arises when an imbalance in the redox steady-state occurs and ROS production exceeds the capacity of the cell for detoxification. If H_2O_2 encounters a reduced transition metal (Fe^{2+} or Cu^{2+}) or $O_2^{\bullet-}$ it

can be further reduced in a highly reactive and toxic hydroxyl radical (•OH) by a Fenton or Haber-Weiss reaction, respectively (Brandon, Baldi et al., 2006), which is the most potent ROS. Although very short-lived, •OH can damage cellular macromolecules including proteins, lipids and nucleic acids. The oxidation of proteins can inactivate and target them for degradation; oxidative damage to DNA causes single and double strand-breaks, cross-link to other molecules and base modifications, while lipid oxidation can generate membrane disturbances. As described above, mtDNA represents a critical target of oxidative damage since it does not contain histones and it is located in proximity to the production site of ROS (Hebert, Lanza et al., 2010). Once damaged, mtDNA can indirectly amplify oxidative stress since transcription of critical mitochondrial proteins is defective, leading to a vicious cycle of ROS production and eventually triggering cell death. Oxidative stress is largely related with aging (Balaban, Nemoto et al., 2005) and is often associated with some disorders such as cancer and diabetes (Van Houten, Woshner et al., 2006). Reactive nitrogen species (RNS), including nitric oxide and peroxynitrite, can also contribute for a regulation of mitochondrial function (especially the former (Brown & Borutaite, 2007)), as well for increased mitochondrial damage during pathological conditions (Poderoso, 2009).

1.4 Cell death

Unlike what was thought during several years, cell death is not a process only observed when cell tissues are injured by external factors. Actually, cell death is an evolutionary conserved and genetically regulated process that is crucial for development, morphogenesis and homeostasis in tissues (Martin & Baehrecke, 2004). Programmed cell death (PCD) was the first designation attributed to this regulated process. Later, Kerr *et al.* introduced the term apoptosis (Kerr, Wyllie et al., 1972) to designate programmed cell death and these designations remain synonymous until now. Cell death was classified into two types: apoptosis (programmed cell death) and necrosis (accidental cell death). Nowadays, other types of cell death have been identified, including autophagy. Although it has become clear that autophagy can work as an adaptive response to nutrient starvation, cell death can occur due to autophagy over-stimulation (Rami, 2009). Autophagy is a spatially restricted phenomenon characterized by the absence of chromatin condensation and in which parts of the cytoplasm are engulfed by specialized double membrane vesicles, so-called autophagosomes, and digested by lysosomal hydrolases (Ulivieri, 2010). Mitophagy is a specific autophagic elimination of mitochondria, identified in yeast and mammals and regulated by PINK-1, among others (Youle & Narendra, 2011). However, if for some reason the clearing of old/damaged mitochondria is insufficient, a malignant transformation may occur (Morselli, Galluzzi et al., 2009). Necrotic cell death is characterized by a moderate or null chromatin condensation and by an increase in cell volume that culminates in loss of plasma membrane integrity and swelling of cytoplasmic organelles (Galluzzi, Maiuri et al., 2007). The disruption of cell membranes leads to the release of cell contents usually resulting in local inflammatory reactions and damage to contiguous cells. Several studies have already demonstrated that mitochondria can be involved in this type of cell death due to a phenomenon called mitochondrial permeability transition (MPT), which results from the opening of unspecific protein pores in the IMM. The MPT results in dissipation of mitochondrial membrane potential ($\Delta\Psi$) and leads to an uncoupling of OXPHOS and

decreased ATP, leading cells to necrosis (Sharaf El Dein, Gallerne et al., 2009; Zorov, Juhaszova et al., 2009). Apoptosis is the best-studied modality of cell death and plays an essential role in the maintenance of homeostasis by eliminating damaged, infected or superfluous cells in a regulated form that minimizes inflammatory reactions and damage to neighboring cells (Jeong & Seol, 2008; Schug & Gottlieb, 2009; Sheridan & Martin, 2010). Apoptotic imbalance may contribute to the development of neurodegenerative disorders, autoimmune disorders, cancer or even viral infections (Arnoult, 2007; Jourdain & Martinou, 2009). Apoptotic cells exhibit specific changes, including chromatin condensation, nuclear fragmentation, and plasma membrane blebbing. The late stages of apoptosis are characterized by fragmentation of the cell-membrane into vesicles called apoptotic bodies which contain intact cytoplasmatic organelles or nuclear fragments. These vesicles are recognized by the immune system macrophages, preventing inflammatory responses (Martin & Baehrecke, 2004; Jeong & Seol, 2008; Tait & Green, 2010). There are two main pathways by which a cell can engage apoptosis: the extrinsic (or cell death receptor-mediated) apoptotic pathway and intrinsic (or mitochondrial-mediated) apoptotic pathway (Tait & Green, 2010) (Fig. 2). In both pathways, the apoptotic process is driven by a family of cysteine proteases that are expressed as pro-enzymes and are activated by proteolysis.

These proteases, known as caspases, specifically cleave their substrates at aspartic residues and are categorized into initiators (such as caspases -8 and -9) and effectors or executioners (such as caspases -3 and -7) (Arnoult, 2007; Jeong & Seol, 2008). Mitochondria are central players in the intrinsic apoptotic pathway; in fact, mitochondria retain a pool of pro-apoptotic factors in the IMS. During the development of the intrinsic pathway, pores are formed in the OMM in a process called outer mitochondrial membrane permeabilization (OMMP, different from the mitochondrial permeability transition). The OMMP results in the release of pro-apoptotic factors, such as cytochrome *c* and the apoptotic-inducing factor, AIF, to the cytosol (Saelens, Festjens et al., 2004; Sheridan & Martin, 2010). Although the effects of pro-apoptotic factors that are released in the cytosol are well characterized, the mechanisms underlying the OMMP remains controversial (Martinou & Green, 2001) and there are currently several mechanisms that have been proposed. One of these mechanisms involves members of Bcl-2 proteins family, which comprises three subgroups; the anti-apoptotic family members such as Bcl-2 and Bcl-xL, the pro-apoptotic Bax/Bak sub-family and the pro-apoptotic BH3-only proteins such as Bim, Bad, Bid, Puma and Noxa. BH3-only proteins links cell death signals to mitochondria, where the interplay between various members of the Bcl-2 family determines the fate of the cell (Martinou & Green, 2001; Wong & Puthalakath, 2008). A mild change in the dynamic balance of these proteins may result either in inhibition or exacerbation of cell death. The intrinsic and extrinsic pathways can interact with each other at the mitochondrial level where signal amplification occurs (Fig. 2) (Saelens, Festjens et al., 2004).

2. Mitochondria and disease

As it was discussed in the previous sections, mitochondria are organelles with crucial importance in cell bioenergetics, signaling and survival, among others. Mitochondrial dysfunction is associated with several diseases, as it will be discussed in the present section.

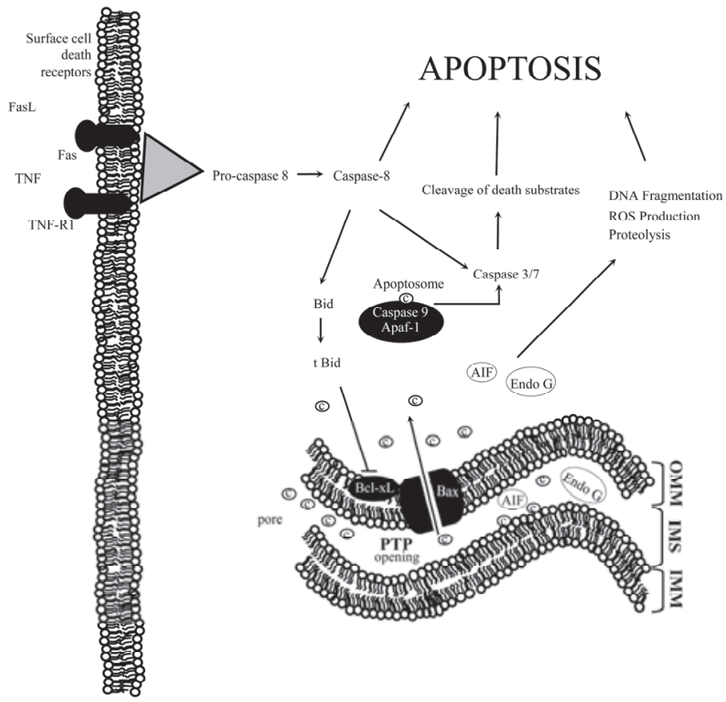

Fig. 2. An overview of the extrinsic and intrinsic pathways of apoptosis. The intrinsic and extrinsic pathways can crossroad in mitochondria, which leads to signal amplification. IMM, inner mitochondrial membrane; IMS, intermembrane space; OMM, outer mitochondrial membrane. Figure adapted from (Pereira, Moreira et al., 2009), with permission.

2.1 Cancer

As described above, the impact of mitochondria on cellular physiology is not limited to ATP production. Due to the importance of mitochondria for cellular functions and cell fate, the role of these small organelles in cancer cell biology is becoming increasingly recognized. The first suggestion about the role of mitochondria in tumor metabolism appeared in 1920's, when Otto Warburg observed increased glycolysis in tumor cells, even in the presence of abundant oxygen. Following this observation, Warburg hypothesized that tumor cells tend to obtain most of their energy through aerobic glycolysis (Warburg, 1930). This phenomenon, known as the Warburg effect, is considered one of the major metabolic alterations observed during cancer development (Warburg, 1956). Since then, several hypotheses have been suggested in order to explain the aerobic glycolysis observed in some (but not all) cancer cells. An irreversible respiratory impairment was first proposed by Warburg (Warburg, 1956). In fact, the author suggested that the origin of cancer cells was in an irreversible damage to the respiration apparatus (Warburg, 1956). However, Warburg results were questioned when Boyland observed an increase in respiration after addition of succinate or fumarate to tumor slices (Boyland & Boyland, 1936). Also, it was described that neoplasias can have a normal oxidative phosphorylation capacity when supplemented with NAD^+ (Wenner & Weinhouse, 1953). More recently, it was demonstrated that oxidative phosphorylation can be improved in cancer cells by changing substrate availability (Rossignol, Gilkerson et al., 2004). Despite all the arguments against the hypotheses raised

by Warburg, the truth is the Warburg effect was an important discovery that allowed for an important progress in cancer research and prognosis (Ak, Stokkel et al., 2000). Being mitochondria the organelle where several cellular metabolic reactions occur and where the majority of cellular energy is produced, the role of mitochondria in cancer development is indubitable. For example, mutations in mitochondrial and nuclear genes encoding proteins involved in oxidative phosphorylation have been observed in several cancers, suggesting a role for defective mitochondrial oxidative phosphorylation in tumorigenesis (for a review see (Chandra & Singh, 2010)). Mutations can be acquired during or after oncogenesis and result in an inhibition of oxidative phosphorylation, increased ROS production, tumor cells proliferation and adaptation to tumor microenvironments (Hung, Wu et al., 2010; Lee, Chang et al., 2010). Also, decreased mtDNA copy number has been associated with resistance to apoptosis and increased invasiveness (Chandra & Singh, 2010). The loss of function of mitochondrial-specific enzymes, such as succinate dehydrogenase and fumarate dehydrogenase, results in the accumulation of specific metabolites in the cytosol, that can favor the activation of transcription factors (eg. hypoxia-inducible factor, HIF), directing the metabolism to aerobic glycolysis (Yeung, Pan et al., 2008; Bellance, Lestienne et al., 2009; Marin-Hernandez, Gallardo-Perez et al., 2009) establishing a possible correlation between mitochondrial alterations and the Warburg effect observed in cancer cells.

2.2 Mitochondrial DNA diseases

Besides the nucleus, mitochondria have their own functional genome (Reich & Luck, 1966). Mutations in mtDNA are associated with the development of different pathologies. Although the mtDNA of an individual is usually identical in all cell types (homoplasmy), variations may occur, causing dissimilarities between wild type and mutant mtDNA (heteroplasmy). Progressive accumulation of mutant mtDNA in affected tissue will increase the severity of the phenotype associated with those mutations. Besides the rate of heteroplasmy, the age, gender and environment clearly contribute for the high diversity of phenotypes (McFarland, Taylor et al., 2002). The so-called mitochondrial diseases are caused by mutations in mtDNA or in nuclear genes that codify for proteins involved in the mitochondrial respiratory chain or in overall mitochondrial biology. For the sake of simplicity, we will focus now in diseases that are the result from mtDNA mutations. The degree of severity of mtDNA alterations and the impact on organ phenotype is determined by the threshold effect, or in other words, the dependency of the organ on the mutated protein, or on the mitochondrial function itself (Dimauro & Davidzon, 2005). Simplifying, this means that organs that are more dependent on energy will be first affected by alterations of mitochondrial function caused by mtDNA mutations (Rossignol, Faustin et al., 2003). Mitochondrial DNA diseases can be divided in two main categories based on the genomic origin of the disorder: 1) syndromes due to mtDNA rearrangements or 2) syndromes based on mtDNA point mutations. Kearns-Sayre (KSS) and Person Marrow-Pancreas Syndromes are classical examples of disorders associated with mtDNA rearrangements. KSS is characterized by external progressive opthalmoplegia and pigmentary retinopathy and is associated with heteroplasmatic mtDNA deletions. Pearson Marrow-Pancreas Syndrome is commonly diagnosed during infancy or postmortem and is caused by deletions or duplications in mtDNA. It is rarely diagnosed during pregnancy, but

should be suspected in the presence of severe anemia or lactic acidosis (Morel, Joris et al., 2009). Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) is a multisystem mitochondrial maternally inherited disease. It is caused by a point mutation characterized by a A to G transition at the position 3260 of the mitochondrial genome. It is normally associated with frequent episodes of migraine and intraventricular conduction disturbances and syncopal episodes based on paroxysmal atrioventricular block have been found already (Connolly, Feigenbaum et al., 2010). Leigh Syndrome is a maternal-inherited point mutation in polypeptide-encoding genes based disorder. Although still largely unknown, it is suggested that the Leigh Syndrome is caused by defects in genes coding for the pyruvate dehydrogenase complex, cytochrome *c* oxidase, ATP synthase subunit 6 or complex I subunits (Quintana, Kruse et al.; Naess, Freyer et al., 2009; Quintana, Mayr et al., 2009).

2.3 Diabetes

Diabetes mellitus (DM) is a metabolic disease characterized by hyperglycemia and alterations in carbohydrate, lipid and protein metabolism due to disturbances in insulin secretion, having as a long-term consequence, the failure in several organs. As in previous cases, mitochondrial multi-tasking suggests an important role of this organelle not only in the pathogenesis of this condition, but also in the development of long-term complications. Several mitochondrial alterations have been described during the progress of diabetes mellitus, including respiratory alterations and altered induction of the MPT (reviewed in (Oliveira, 2005)). Besides the heart (Oliveira, Rolo et al., 2001; Oliveira, Seica et al., 2003; Santos, Palmeira et al., 2003; Bugger & Abel, 2011), alterations of mitochondrial function have been recorded in liver (Ferreira, Seica et al., 2003), kidney (Oliveira, Esteves et al., 2004), brain (Moreira, Santos et al., 2004) and testis mitochondria (Palmeira, Santos et al., 2001; Amaral, Oliveira et al., 2008; Amaral, Mota et al., 2009), which show a multi-organ scope of hyperglycaemia-induced mitochondrial alterations. Oliveira *et al*. demonstrated that streptozotocin (STZ)-induced diabetes results in inhibition of cardiac mitochondrial respiration and increased susceptibility to calcium-induced MPT (Oliveira, Seica et al., 2003). In theory, this means that heart mitochondria from diabetic animals are less able to withstand a metabolic stress, mimicked in this work by the addition of ADP and calcium. Interestingly, heart mitochondria from Goto-Kakizaki (GK) rats have decreased susceptibility to the MPT (Oliveira, Rolo et al., 2001). GK rats are an animal model for non-obese type 2 diabetes, developing hyperglycaemia earlier in life, suggesting that the severity/duration of the hyperglycaemic period is important for cardiac mitochondrial alterations. Interestingly, different alterations in terms of hepatic mitochondrial respiratory activity were found in both STZ-treated and GK rats, such alterations being modulated by the age of the animals (Ferreira, Palmeira et al., 2003; Ferreira, Seica et al., 2003). Alterations in MPT induction are also widespread to other tissues. Lumini-Oliveira *et al*. reported that 18 weeks of STZ treatment lead to a decrease in gastrocnemius mitochondrial respiratory control ratio and to decreased calcium-dependent MPT, which may counteract the negative effects of hyperglycaemia. It is still unclear what may cause mitochondrial alterations during the course of diabetes and why such alterations appear to be organ and age-specific. Increased oxidative stress due to increased mitochondrial generation of ROS and/or depression of mitochondrial antioxidant defenses may be an attractive mechanism

(Kucharska, Braunova et al., 2000; Turko, Li et al., 2003; Kowluru, Atasi et al., 2006; Ren, Li et al., 2008; Munusamy & MacMillan-Crow, 2009). A growing body of evidence also suggests that mitochondrial dysfunction in pancreatic beta-cells may be also one of the initiation factors responsible for depressed insulin release (Mulder & Ling, 2009). In fact, mitochondria in beta-cells have a critical role in the release of insulin. Beta cell mitochondria play a key role in this process, not only by providing ATP to support insulin secretion when required, but also by synthesizing metabolites that can couple glucose sensing to insulin exocytosis. ATP alone or possibly modulated by several coupling factors, triggers closure of the ATP-sensitive potassium channel, resulting in membrane depolarization that increases intracellular calcium and insulin secretion (Liu, Okada et al., 2009; Jitrapakdee, Wutthisathapornchai et al., 2011). In several models for diabetes, mitochondrial defects in beta-cells have been found (reviewed in (Maechler, Li et al., 2011)), including altered expression of the voltage-dependent anion-channel (Ahmed, Muhammed et al., 2011) and altered respiratory activity and oxidative stress (Lu, Koshkin et al., 2011). In beta-cell mitochondria, increased oxidative stress may be critically important in the pathogenesis of the disease (Nishikawa & Araki, 2007), although what exactly leads to that is still a matter of debate. What is interesting is that some forms of diabetes are originated by defects on mitochondrial DNA, present in pancreatic beta-cells (de Andrade, Rubi et al., 2006; Mezghani, Mkaouar-Rebai et al., 2011). Other mitochondrial-relevant alterations in beta-cells include enhanced apoptosis in some forms of auto-immune type I and type II diabetes (Johnson & Luciani, 2011).

3. Drug-induced mitochondrial toxicity

Toxic compounds can interfere and modify physiological mechanisms, leading to cell alterations and ultimately damage. In many cases of drug-induced toxicity, mitochondria are the preferential target for toxic compounds and one important initiator of cell damage. In this section, we will focus on the present knowledge regarding the mechanism of action of some selected drugs, whose mechanism of toxicity has a clear mitochondrial component.

3.1 Anti-cancer drugs

For five decades, anthracycline antibiotics have played an important role in the treatment of a variety of cancer types, due to their efficacy and broad spectrum of activity (Sawyer, Peng et al., 2010). The anti-tumor activity of anthracyclines is based on their ability to intercalate DNA and to inhibit enzymes involved in DNA replication and transcription such as topoisomerase II and RNA polymerases, respectively (Sawyer, Peng et al., 2010). Disturbance of DNA function is thought to be the main responsible for tumor cell death, a typical behavior shared by other anti-cancer drugs (Singal, Iliskovic et al., 1997). However, anthracycline therapy is associated with significant side effects, including cardiotoxicity (Chen, Peng et al., 2007; Sawyer, Peng et al., 2010). A particular leading drug of this group, Doxorubicin (DOX), has been intensively studied and rapidly stood out from other analog molecules due to its efficacy. Unfortunately, its cardiotoxicity also stood out, although the molecular mechanisms are still far of being completely understood (Arola, Saraste et al., 2000; Horenstein, Vander Heide et al., 2000). The onset of DOX-induced cardiomyopathy is characterized by several forms of tachycardia (Bristow, Minobe et al., 1981), altered left ventricular function (Hrdina, Gersl et al., 2000), and severe histological changes such as

vacuolization of the cytoplasm, loss of myofibrils, altered sarcoplasmic reticulum, deposition of lipid droplets, and mitochondrial swelling (Lefrak, Pitha et al., 1973; Olson & Capen, 1978; Iwasaki & Suzuki, 1991; Sardao, Oliveira et al., 2009). More evidence suggests that mitochondria are a critical target in the development of DOX-induced cardiomyopathy (Yoon, Kajiyama et al., 1983; Praet & Ruysschaert, 1993; Jung & Reszka, 2001; Wallace, 2003; Berthiaume & Wallace, 2007). Numerous mechanisms for the toxicity of DOX on cardiac mitochondrial function have been proposed, such as generation of free radicals (Muraoka & Miura, 2003), interaction with mitochondrial DNA (L'Ecuyer, Sanjeev et al., 2006), disruption of cardiac gene expression (Berthiaume & Wallace, 2007), alteration of calcium homeostasis (Lebrecht, Kirschner et al.), lipid peroxidation mediating disturbance of mitochondrial membranes (Mimnaugh, Trush et al., 1985), and inhibition of mitochondrial respiration chain, decreasing both intracellular ATP and phosphocreatine (PCr) (Tokarska-Schlattner, Zaugg et al., 2006). DOX can also interfere with mitochondrial function in other targets, including by inhibiting phosphorylation steps (Marcillat, Zhang et al., 1989) or by exerting partial uncoupling (Bugger, Guzman et al.). Although several hypotheses have been proposed to explain cardiac DOX toxicity, oxidative stress is the most widely accepted; in fact, data from the literature indicate that the cardiac tissue is particularly susceptible to free radicals due to reduced levels of enzymatic antioxidants defenses when compared with other tissues (Hrdina, Gersl et al., 2000). DOX is able to increase ROS through both an enzymatic mechanism involving a redox cycle and cellular oxidoreductases such as NADH dehydrogenase of complex I or cytochrome P-450 reductase, and through a non-enzymatic pathway involving complexes with iron (Fe^{3+}) (Davies & Doroshow, 1986; Doroshow & Davies, 1986; Jung & Reszka, 2001; Minotti, Recalcati et al., 2004). DOX-induced oxidative stress can also be related with induction of the MPT (Ascensao, Lumini-Oliveira et al.; Zhou, Starkov et al., 2001; Oliveira, Santos et al., 2006; Oliveira & Wallace, 2006), which is observed in both *in vivo* and *in vitro* studies (Pereira & Oliveira, 2010). *In vitro*, DOX-induced MPT pore opening results in mitochondrial depolarization, respiratory inhibition, matrix swelling, pyridine nucleotides depletion and release of intermembrane proteins, including cytochrome c (Oliveira, Bjork et al., 2004; Berthiaume, Oliveira et al., 2005; Oliveira, Santos et al., 2006).

3.2 Nucleoside-analog reverse transcriptase inhibitors

Nucleoside reverse transcriptase inhibitors (NRTIs), a class of anti-retroviral drugs, are specifically prescribed as a therapy to Acquired Immune Deficiency Syndrome (AIDS). Several studies indicate that these drugs induce mitochondrial toxicity by interfering with mitochondrial DNA (mtDNA) synthesis (Lund & Wallace, 2004; Lewis, Kohler et al., 2006). The targets of NRTIs are reverse transcriptase enzymes but due to the similarities with substrates for the mitochondrial enzyme DNA polymerase-gamma, NRTIs also inhibit this mitochondrial enzyme, affecting mtDNA copy number (Lewis, Simpson et al., 1994). As described above, mitochondrial DNA depletion may be clinically manifested in one or several main targets tissues, depending on the energy requirements of that same tissue (Rossignol, Faustin et al., 2003). Liver mitochondrial complications as hepatomegaly and increased lipid deposits have been primarily observed with dideoxynucleosidesdidanosine, stavudine, and zalcitabin. mtDNA depletion has been demonstrated in the liver of HIV patients, with each of dideoxynucleosides inducing a time- and concentration-dependent mtDNA depletion (Walker, Bauerle et al., 2004). Several NRTIs were shown to directly interfere with cardiac mitochondrial respiratory chain decreasing membrane potential and

decreasing mitochondrial calcium buffer capacity (Lund & Wallace, 2004). Zidovudine (AZT) is the most well-known antiviral and its side effects have been subject of several studies focused on studying mitochondrial interactions (Lewis, Simpson et al., 1994). Competitive inhibition of thymidine phosphorylation (Lynx, Bentley et al., 2006; Lynx & McKee, 2006), induction of superoxide anion formation (Szabados, Fischer et al., 1999; de la Asuncion, Del Olmo et al., 2004), inhibition of adenylate kinase activity (Barile, Valenti et al., 1994), and inhibition of the ANT both in heart (Valenti, Barile et al., 2000) and liver (Barile, Valenti et al., 1997) are some of the effects observed in isolated mitochondria incubated with AZT and other NRTIs. Inhibition of phosphate transport in rat heart mitochondria by AZT was found to be related with increased superoxide anion production, as shown by the protective effects of several ROS scavengers (Valenti, Atlante et al., 2002). Oxidative stress probably plays the most important role in AZT-induced mitochondrial dysfunction. Indeed, a 2-week treatment of rats with AZT leads to increased ROS and peroxynitrite production and induced single-strand DNA breaks (Szabados, Fischer et al., 1999). Lipid peroxidation and oxidation of cell proteins, determined from protein carbonyl content, increased as a consequence of AZT treatment (Szabados, Fischer et al., 1999). Depletion of mitochondrial glutathione was also found in mitochondria isolated from the hearts of AZT-treated rats (de la Asuncion, Del Olmo et al., 2004). Furthermore, NRTIs are able to indirectly inhibit the regulation of mitochondrial complex I by cyclic adenosine monophosphate (cAMP). This type of inhibition may explain disturbances observed in many patients regarding ROS production, NADH/NAD^+ ratio, and high lactate levels (Lund & Wallace, 2008).

3.3 Anti-diabetic agents

Treatment of hyperglycemia during diabetes involves the use of hypoglycemic drugs. Initially, biguanide agents such as metformin, phenformin and buformin were used for the management of hyperglycemia in type 2 diabetes mellitus (T2D). However, these anti-diabetic drugs rapidly resulted into a number of serious adverse effects, which made the pharmacological management of hyperglycemia still a challenge to the clinic.

Both buformin and phenformin were withdrawn from the market in the 1970´s due to high incidence of lactic-acidosis-associated mortality and gastrointestinal symptoms, although phenformin is still available in some countries. Metformin is now believed to be the most widely prescribed anti-diabetic drug in the world (Correia, Carvalho et al., 2008). The anti-diabetic effect of metformin and phenformin and increased lactic acidosis observed during treatment are suggested to result from a single mechanism, the inhibition of mitochondrial complex I (El-Mir, Nogueira et al., 2000; Correia, Carvalho et al., 2008). Other investigators described that inhibition of hepatocyte complex I not only caused not only a reduction of blood glucose levels in human subjects but also a complete inhibition of hepatic gluconeogenesis, a metabolic process that is significantly increased in T2D contributing to the observed fasting hyperglycemia (Hundal, Krssak et al., 2000). In intact cells, metformin increases AMP-activated protein kinase (AMPK) activity, resulting in increased fatty acid oxidation, down-regulation of lipogenic genes, decreased hepatic glucose production and stimulation of glucose uptake (Zhou, Myers et al., 2001). Beyond biguanides, thiazolidinediones (TZD) is a class of oral antihyperglycemic drugs also known as glitazones that have been used as an auxiliary therapy for diabetes mellitus (Petersen, Krssak et al., 2000; Mudaliar & Henry, 2001). Glitazones includes troglitazone, rosiglitazone, and pioglitazone, which are used to ameliorate hyperglycemia by increasing insulin-

stimulated glucose removal by skeletal muscle (Petersen, Krssak et al., 2000; Mudaliar & Henry, 2001). Indeed, TZDs can also be considered insulin sensitizers because they are able to lower glucose levels in models of insulin resistance without increasing pancreatic insulin production (Kliewer, Xu et al., 2001). The ability of TZD to lower serum glucose levels and promote an increase in glucose utilization by accelerating glycolytic flux, can lead to excessive lactic acid production. Although lowering glucose efficiently is considered a desired effect of TZD, lactic acidosis seems to be a compensatory mechanism to a decrease in mitochondrial generated ATP, something that is often observed in diabetic individuals. These drugs are known to bind and activate the nuclear peroxisome proliferation receptor γ (PPARγ), and interestingly to also inhibit mitochondrial complex I. The efficacy of TZD to inhibit complex I or to cause lactate release in skeletal muscle or rat liver homogenates follows the sequence troglitazone, rosiglitazone, and metformin, being the latter less efficient (Brunmair, Staniek et al., 2004). Several studies reveal that TZDs may increase the risk of heart failure (Delea, Edelsberg et al., 2003; Karter, Ahmed et al., 2004), which limits their clinical application. The risk for heart failure may lie on mitochondrial impairment as consequence of TZD toxicity. In this case, disruption of NADH oxidation by mitochondrial complex I tends to occur, although the toxicity effect may also be the mechanism for the pharmacological benefits observed (Scatena, Martorana et al., 2004). This means the border line between a desired pharmacological effect and a toxic consequence is very blurred, and in fact, long-term and/or large-scale inhibition of complex I activity can lead to ATP depletion, oxidative burst and ultimately cell death (Li, Ragheb et al., 2003). An example of TZD which had high impact in the clinic is troglitazone (TRO), introduced in 1997 but soon withdrawn from the market because of reports of serious hepatotoxicity, receiving a black box warning from the U.S. Food and Drug Administration (FDA). In fact, TRO, when incubated with HepG2 cells, decreased cellular ATP and $\Delta\Psi$ (Tirmenstein, Hu et al., 2002; Bova, Tam et al., 2005). Lim *et al.* also demonstrated that TRO increases intramitochondrial oxidative stress that activates the Trx2/Ask1 pathway, leading to mitochondrial permeabilization (Lim, Liu et al., 2008). More recently, data indicate that significant mtDNA damage caused by TRO is a prime initiator of the hepatoxicity caused by this drug (Rachek, Yuzefovych et al., 2009). Overall, the data suggest that the reported mitochondrial effects of anti-diabetic drugs, especially complex I inhibition are worth of further attention, not only to explain some of its pharmacological effects but also to predict safety during drug development.

3.4 Anti-depressant agents

Tricyclic antidepressants (TCAs) are heterocyclic chemicals discovered in the early 1950s and which have been primarily used to relieve depressive symptoms. Fluoxetine (Prozac), an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class, presents some cardiovascular side effects and drug-drug interactions. Interestingly, some studies show that fluoxetine indirectly affects electron transport and F_1F_o-ATPase activity inhibiting OXPHOS in isolated rat brain and liver mitochondria (Souza, Polizello et al., 1994; Curti, Mingatto et al., 1999). The results obtained by Curti *et al.*, suggested that these effects are mediated by the drug interference with the physical state of lipid bilayer of the IMM (Curti, Mingatto et al., 1999). In turn, nefazodone is a TCA with a more favorable side effect profile when compared to fluoxetine and even with other drugs commonly used to mitigate depressive

conditions. Nefazodone was initially considered very advantageous among several other TCAs (Davis, Whittington et al., 1997). Initially, the incidence of specific organ toxicity was considered very low, and related fatalities by severe toxicity were non-existent on several hundred of patients during long periods of treatment (Lader, 1996; Robinson, Roberts et al., 1996; Davis, Whittington et al., 1997). Among other physiological advantages, nefazodone had the ability to treat some patients who did not respond to other TCAs (Ellingrod & Perry, 1995; Robinson, Roberts et al., 1996). However, some cardiovascular complications such as asymptomatic reduced systolic blood pressure and asymptomatic sinus bradycardia, started to be detected and considered as markers for cardiotoxicity (Robinson, Roberts et al., 1996). Despite the possible therapeutic advantages, the drug was withdrawn from the U.S. market in 2004, based on cardiotoxicity and later on some severe cases of adverse hepatoxicity as well. Indeed, more recent data show that when compared to buspirone, nefazodone is more toxic to hepatic mitochondrial function (Dykens, Jamieson et al., 2008). Dykens *et al*. demonstrated that nefazodone promoted inhibition of mitochondrial respiration and increased glycolysis in isolated rat liver mitochondria and in intact HepG2 cells, respectively (Dykens, Jamieson et al., 2008). Two other anti-depressant drugs, amineptine and tianeptine, can also lead to hepatitis associated with microvesicular steatosis, in fact, their heptanoic acid side chain may be responsible for reversibly inhibiting mitochondrial fatty acid oxidation by a competitive mechanism (Fromenty, Freneaux et al., 1989).

3.5 Statins and fibrates

Statins (or HMG-CoA reductase inhibitors) are a class of drugs used to decrease cholesterol levels by inhibiting the enzyme HMG-CoA reductase, which plays a central role in the production of cholesterol in the liver. Statins are generally safe and well tolerated, but the major side effect, which occurs in about 1% of patients, is skeletal myopathy (Davidson, 2001). Interestingly, many congenital myopathies are associated with defects in mitochondrial enzymes (Cornelio & Di Donato, 1985; Wallace, 2000) and bio-accumulation of statins by fast twitch skeletal muscle cells can increase the risk of mitochondrially-induced rhabdomyolysis (Westwood, Bigley et al., 2005). Several reports describe acute effects of statins on skeletal muscle mitochondria. Lovastatin and simvastatin were reported to induce the MPT *in vitro* and decrease the content of total membrane thiol groups in mitochondria isolated from mouse hind limb (Velho, Okanobo et al., 2006). Mitochondrial degeneration was observed on rat skeletal muscle fibers treated with cerivastatin (Seachrist, Loi et al., 2005). A variety of other statins are known to induce the MPT leading to irreversible collapse of the transmembrane potential and release of pro-apoptotic factors (Cafforio, Dammacco et al., 2005; Kaufmann, Torok et al., 2006), in a Bcl-xL-preventable manner (Blanco-Colio, Justo et al., 2003). Kaufman and colleagues also reported inhibition of β-oxidation and swelling of isolated skeletal muscle mitochondria by statins (Kaufmann, Torok et al., 2006). Ubiquinone coenzyme Q10 (CoQ_{10}) depletion is another hypothetic contributor to statin-induced myopathy (Folkers, Langsjoen et al., 1990). Thus, CoQ_{10} depletion can contribute to mitochondrial dysfunction leading to statin-induced myopathy since CoQ_{10} acts as an electron carrier in the mitochondrial respiratory chain (Schaars & Stalenhoef, 2008). Besides the effects on skeletal muscle, lovastatin and simvastatin inhibit mitochondrial respiration of isolated liver mitochondria by a direct effect on complexes II, III, IV and V (Nadanaciva, Dykens et al., 2007). Fibrates, in turn, are used as accessory therapy in many forms of hypercholesterolemia, usually in combination with statins

(Steiner, 2007). Fibrates are structurally related to the thiazolidinediones, and pharmacologically act on PPARγ, impairing mitochondrial function (Barter & Rye, 2006). In an *ex vivo* experiment with isolated mitochondria, fenofibrate inhibits complex I activity and disturbs rat mitochondrial function (Brunmair, Lest et al., 2004). The fibrates ciglitizone, bezafibrate, gemfibrozil, and clofibric acid were reported to increase lactate and acetate levels due to increase anaerobic glycolysis and fatty acid beta-oxidation, to inhibit NADH-cytochrome *c* reductase activity, and show a correlation between mitochondrial toxicity and inhibition of HL-60 cell growth (Scatena, Martorana et al., 2004). In opposition, Scatena *et al.* argued that fibrates induce toxicity by disrupting mitochondrial function through a mechanism partly independent on PPARs (Scatena, Bottoni et al., 2004).

4. Environmental pollutants

Humans are daily exposed to a variety of molecules, which can be present in food, beverages and even in the atmosphere. Although most are harmless, either due to their intrinsic safety or to the decreased exposure levels, the truth is that some of those molecules disturb several biological systems, including mitochondria, leading to short or long-term organ toxicity (Wallenborn, Schladweiler et al., 2009).

Heavy metal toxicity is widespread in the world due to the very large amount of industrial activities that release these compounds in nature. Heavy metal toxicity can have different aspects and result into different pathologies, including carcinogenesis and vascular diseases (Nash, 2005). As expected, the toxicity of heavy metals also impacts mitochondria. Cadmium, for example, which has been associated with learning impairments and neurological disorders, has been described to cause mitochondrial-dependent apoptosis in oligodendrocytes (Hossain, Liu et al., 2009) and in a skin cell line (Son, Lee et al., 2011). Cadmium accumulation in the kidney involves alteration of mitochondrial function, which results into increased generation of mitochondrial free radicals (Gobe & Crane, 2011), similarly to what occurs in other target organs (Cannino, Ferruggia et al., 2009). As expected, cadmium, similarly to as mercury and copper, induces the MPT, resulting in mitochondrial swelling and activation of basal respiration, as well as in membrane depolarization (Belyaeva, Glazunov et al., 2004). Mercury also caused apoptosis in several biological models by interfering with mitochondrial function (Shenker, Guo et al., 1998). In fact, low concentrations of methylmercury cause inhibition of mitochondrial function, which progresses to apoptotic cell death (Carranza-Rosales, Said-Fernandez et al., 2005). Mitochondrial respiration in hepatoma AS-30D cells is initially uncoupled for lower concentrations and progressively inhibited for higher concentrations, resulting also in increased generation of ROS (Belyaeva, Dymkowska et al., 2008). Although in this same model, copper (Cu^{2+}) was not as toxic (Belyaeva, Dymkowska et al., 2008), other works have shown that copper causes toxicity in astrocytes, due to increased MPT induction and oxidative stress (Reddy, Rao et al., 2008). Also, copper decreased $\Delta\Psi$, followed by apoptosis in MES23.5 dopaminergic cells (Shi, Jiang et al., 2008). Interestingly, at least a significant part of copper toxicity in non-human species can also be explained by inhibition of mitochondrial function, including activation of the MPT, as observed in trout hepatocytes (Krumschnabel, Manzl et al., 2005). Iron has been considered a significant pro-oxidant metal due to its role in the formation of hydroxyl radical via Fenton reactions (Stohs & Bagchi, 1995). Although iron is essential for life, it can pose serious health risks with the liver being

the most relevant target. Heavy iron overload, as described during primary (hereditary) or secondary forms of hemochromatosis, may cause cirrhosis, liver failure, and hepatocellular carcinoma (Bonkovsky & Lambrecht, 2000). In addition, iron can contribute to the development or progression of alcoholic liver disease, nonalcoholic liver steatohepatitis, chronic viral hepatitis and prophyria cutanea tarda, among other diseases (Bonkovsky & Lambrecht, 2000). In thalassemia major, one of the clinical end-points is an iron overload resulting from diverse factors. The excess of iron results in ROS formation, damaging several intracellular organelles, including mitochondria (Hershko, 2011). The observed effects are very close to what has been observed in rats subjected to a single injection of a massive dose of iron-dextran. In this case, mitochondria from treated rats showed decreased respiratory control ratio (Pardo Andreu, Inada et al., 2009). In another different model, rats diet-supplemented with iron lactate showed decreased ATP content in the liver and spleen, which was suggested to occur due to mitochondrial alterations (Fujimori, Ozaki et al., 2004). An interesting hypothesis is drawn from the work of Liang *et al*. The authors suggest that mitochondrial aconitase may be an important early source of mitochondrial iron accumulation in a model for experimental Parkinson's disease, with an oxidative inactivation of that enzyme occurring due to iron-mediated oxidative stress (Liang & Patel, 2004). The role of iron in exacerbating the toxic effects of clinically used drugs is demonstrated, among other examples, by the fact that the iron chelator dexrazoxane protects cardiac myocytes against the toxicity of DOX (see above), via a mitochondrial mechanism (Hasinoff, Schnabl et al., 2003).

Dioxins are environmental pollutants with a large impact on human health, being by-products of incineration processes and of production of several chloro-organic chemicals (Sweeney & Mocarelli, 2000; Parzefall, 2002). 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is the best studied and the most toxic dioxin and data are vast describing clear direct effects of this compound on mitochondria. Several works identified the inhibition of the mitochondrial electron chain and increased generation of ROS as one mechanism by which TCDD exerts its toxicity in the heart (Nohl, de Silva et al., 1989) and liver (Stohs, Alsharif et al., 1991; Latchoumycandane, Chitra et al., 2002; Senft, Dalton et al., 2002). Senft *et al.* demonstrated that mitochondria are the source of TCDD-induced ROS, although the exact mechanism was still not clearly identified (Senft, Dalton et al., 2002). TCDD treatment resulted in an increased hydrogen peroxide release by the respiratory chain, although no alterations in mitochondrial superoxide dismutase or glutathione peroxidase were observed (Senft, Dalton et al., 2002). Interestingly, one week after treating mice with TCDD, coenzyme Q levels in the liver decreased, while activities of some of the mitochondrial complexes were increased (Shertzer, Genter et al., 2006). These and other results, led to the proposal that TCDD causes a defect on the ATP synthase in the liver, resulting in decreased ATP levels in the liver (Shertzer, Genter et al., 2006). Results in isolated rat hepatocytes confirmed the mitochondrial role on oxidative stress caused by TCDD (Aly & Domenech, 2009). It was also demonstrated by using a knock-out model that mitochondrial reactive oxygen production is dependent on the aromatic hydrocarbon receptor (Senft, Dalton et al., 2002) and causes direct damage to mtDNA (Shen, Dalton et al., 2005). Interestingly, TCDD induces apoptosis of human lymphoblastic T-cells, which do not express the aromatic hydrocarbon receptor; the mechanism being the triggering of mitochondrial-mediated intrinsic apoptotic pathway, mediated by calcium/calmodulin (Kobayashi, Ahmed et al., 2009). Another interesting possibility regarding the linkage between mitochondria and TCDD toxicity is the

perturbation of reproductive function by that dioxin (Wu, Li et al., 2001). Reported data indicate that low doses of TCDD cause increased oxidative stress, including depletion of antioxidant enzymes, in mitochondria and microsomal fractions from rat testis, which can alter the mitochondrial ability to supply energy to male germ cells (Latchoumycandane, Chitra et al., 2002). Mitochondrial interactions of TCDD and the possible carcinogenesis associated with dioxin exposure (Knerr & Schrenk, 2006; Jenkins, Rowell et al., 2007) (although others disagree, (Cole, Trichopoulos et al., 2003)) were also demonstrated to be related since TCDD causes mitochondrial depolarization, stress signaling and tumor invasion, besides altering calcium homeostasis (Biswas, Srinivasan et al., 2008). Besides, TCDD directly targets mitochondrial transcription and causes a mitochondrial phenotype which is similar to what is observed in rho0 cells (Biswas, Srinivasan et al., 2008).

5. Mitochondrial liability in drug development and safety assessment

Mitochondria are indeed, the crossroad for many cellular pathways, which explains the growing number of publications dealing with the mitochondrial role in cell life and death (Pereira, Moreira et al., 2009). As a result of the increased efforts focused on the role of mitochondria on a variety of human disorders as cancer, neurodegenerative, cardiovascular diseases, obesity, and diabetes, "mitochondrial medicine" emerged as a whole new field of biomedical research. Based on the recent developments in this field, a large effort is underway to understand how different molecules regulate or damage mitochondrial function, with the ultimate goal to improve human health. Two distinct and important mechanisms/endpoints by which drugs may inhibit mitochondrial function, can be considered (Fig. 3): a) direct interference with mitochondrial respiration/ATP synthesis (inhibition of respiratory complex activity, damage by ROS production, uncoupling activity, MPT induction) and b) inhibition of mtDNA synthesis. Regardless of the initial trigger, inhibition of ATP synthesis and bioenergetic failure of the tissue are severe manifestations of mitochondrial impairment. Several drugs or other xenobiotics can drive mitochondrial to an irreversible collapse via formation of the MPT pore leading to release of pro-apoptotic factors such as cytochrome *c*. Drugs that alter the normal equilibrium between pro-apoptotic and anti-apoptotic proteins, such as Bak/Bax and Bcl-2, can also induce mitochondrial failure and eventually cell death. Additional information for drug development and safety, as well for toxicity assessments may be achieved by the use of targeted approaches, affinity for overexpressed/subexpressed mitochondrial proteins during different diseases types, or selective mitochondrial accumulation of delocalized lipophilic molecules with positive charge and with different redox actions. Nevertheless, further investigation in these endpoints or guidelines of the molecular mechanisms of mitochondria-drug interaction will be needed for a better understanding of the mechanism of action involved in mitochondrial toxicity, allowing an improvement in the design of safer drugs or hazard assessment of xenobiotics with relevant human exposure. Notwithstanding these concerns, until now, several high-throughput techniques have been used to test and screen drug safety on mitochondrial function and could easily be studied to improve basic knowledge in drug development and associated toxicity.

6. High throughput methods – the faster the better?

High throughput methods have been developed with the ultimate objective of allowing company and research laboratories to perform large-scale screening or biochemical

analyses for a certain research or commercial objective. During many decades, low throughput methods were used in most research laboratories, including the Clark-type electrode or the tetraphenylphosphonium electrode to measure mitochondrial membrane potential or $\Delta\Psi$, respectively (Pereira, Moreira et al., 2009; Pereira, Pereira et al., 2009). Other low-throughput methods to investigate mitochondrial toxicity of several agents involved the measurement of activities of components of the mitochondrial respiratory chain by using polarographic, spectrophotometric or blue-native gel techniques (Barrientos, Fontanesi et al., 2009; Diaz, Barrientos et al., 2009). Although such methods are still in use in many laboratories worldwide (and in our own as well), profit-thirsty pharmaceutical companies require faster and cheaper methods to screen thousands of compounds per month in an attempt to uncover mitochondrial liabilities. For example, in the context of mitochondrial toxicity screening in drug development and safety, a fluorescence-based oxygen consumption assay was developed to analyze the ability of certain compounds to cause mitochondrial dysfunction. This approach provides detailed and specific information about the possible mechanisms of toxicity based on measurements of respiratory states 3 and 4 by means of oxygen-sensitive probes. The advantages of this particular fluorescence method are the simplicity and large-scale of measurement, since it can be adapted to a plate reader system. The results can be visualized in real time and quantified in plate reader software (Hynes, Marroquin et al., 2006). A later development included a combination of five high-throughput assays adding important information by identifying enzymes which can be target of the test compounds (Nadanaciva, Bernal et al., 2007). A set of immunocapture-based assays to identify compounds that directly inhibit oxidative phosphorylation can be used in the early evaluation of compound for clinical trials (Nadanaciva, Bernal et al., 2007). The same research group improved a method based on fluorescent probes for the study of oxygen consumption. The advantage is the possibility of screening several compounds simultaneously, being further up-scaled, automated and adapted for other enzyme- and cell-based screening applications (Will, Hynes et al., 2006). To test compounds that interfere with the synthesis of mitochondrial DNA or mtDNA-encoded proteins, a 96-well plate format method, that measures complex IV subunit 1, a protein encoded by mtDNA and complex V subunit 1, an nuclear DNA- encoded protein was developed (Nadanaciva, Dillman et al., 2010).

The literature is getting richer in terms of new methods for high-throughput methods to evaluate mitochondrial function in different applications. When comparing fibroblasts from patients with mtDNA diseases with control subjects, a decrease in ATP production rate in muscle with normal OXPHOS enzyme activities was observed (Jonckheere, Huigsloot et al., 2010). This and other types of assays allow finding primary and secondary mitochondrial dysfunction, which can facilitate the search for genetic defects that can lead to mitochondrial diseases (Jonckheere, Huigsloot et al., 2010). Although in a smaller scale, the Seahorse Bioscience analyzer can be used for a multi-end point of cell and mitochondrial metabolism. In one particular study, the authors measured the mitochondrial function of renal proximal tubular cells observing that several nephrotoxicants alter mitochondria function before altering the basal respiration (Beeson, Beeson et al., 2010). The future will no doubt yield new fast and cost-effective high-throughput methods to quickly investigate mitochondrial toxicity of xenobiotics in order not only to produce safer drugs but also to perform safety screenings on many compounds that humans are daily exposed to.

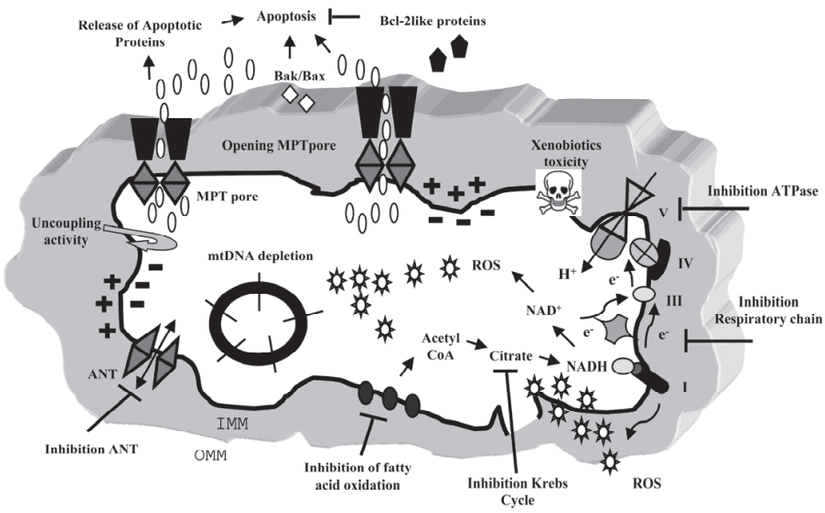

Fig. 3. Drugs or environmental xenobiotics can impair mitochondrial function through affecting different targets, including mitochondrial oxidative phosphorylation and ATP production. Oxidative stress and calcium overload increase the probability of irreversible mitochondrial failure via MPT pore, leading to release of pro-apoptotic factors such as cytochrome *c*. In addition, drugs that alter the ratio pro-apoptotic and anti-apoptotic proteins, such as Bak/Bax and Bcl-2, can also induce mitochondrial failure. OMM, Outer mitochondrial membrane; IMM, Inner mitochondrial membrane; NADH, Nicotinamide adenine dinucleotide reduced form; NAD^+, Nicotinamide adenine dinucleotide oxidized form.

7. Concluding remarks

The question in the title suggests that doubts would still exist regarding the use of mitochondria as a biosensor for drug-induced toxicity. Hopefully, the present chapter provides enough evidence that mitochondria are a critical target in the toxicity of a wide variety of agents, ranging from clinically-relevant drugs, to environmental poisons. Moreover, it has been here demonstrated that failure of mitochondrial function originates several pathologies, which by its turn, contribute to amplify mitochondrial damage. Idiosyncratic drug reactions have also been proposed to involve mitochondria as well (Lucena, Garcia-Martin et al.). In fact, an individual who has a lower mitochondrial power may succumb first to the toxicity of mitochondrial-directed toxicants, even if the original mild mitochondrial alterations are asymptomatic. This is extremely critical for patients with diagnosed mitochondrial DNA diseases, who are in a high risk of suffering mitochondrial failure upon a second hit with a toxicant, either a clinically used drug or an environmental pollutant.

The large number of mitochondrial targets, some of which were not even explored in this chapter, and the growing list of compounds presenting mitochondrial liabilities, clearly

answers our initial question. The field of mitochondrial pharmacotoxicology (Scatena, Bottoni et al., 2007) is now critical for many pharmaceutical companies and for a large number of research laboratories which work on basic toxicology (Chan, Truong et al., 2005; Dykens, Marroquin et al., 2007; Wallace, 2008; Nadanaciva & Will, 2009; Pereira, Moreira et al., 2009; Pereira, Pereira et al., 2009). Some for the sake of profit, others for the good of science itself, but all focusing on that little organelle that is in the spotlight right now.

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A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 16 different countries. The book consists of 24 chapters written by 76 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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