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The role of mitochondrial DNA damage in the citotoxicity of reactive oxygen species

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Abstract Mitochondria contain their own genome, a small circular molecule of around 16.5 kbases. The mitochondrial DNA (mtDNA) encodes for only 13 polypeptides, but its integrity is essential for mitochondrial function, as all 13 proteins are regulatory subunits of the oxidative phosphorylation complexes. Nonetheless, the mtDNA is physically associated with the inner mitochondrial membrane, where the majority of the cellular reactive oxygen species are generated. In fact, the mitochondrial DNA accumulates high levels of oxidized lesions, which have been associated with several pathological and degenerative processes. The cellular responses to nuclear DNA damage have been extensively studied, but so far little is known about the functional outcome and cellular responses to mtDNA damage. In this review we will discuss the mechanisms that lead to damage accumulation and the *in vitro* models we are establishing to dissect the cellular responses to oxidative damage in the mtDNA and to sort out the differential cellular consequences of accumulation of damage in each cellular genome, the nuclear and the mitochondrial genome.

Keywords Mitochondrial DNA · Reactive oxygen species · Oxidative DNA damage · Methylene blue · Singlet oxygen

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Introduction

In eukaryotic cells, mitochondria play a central role in energy metabolism and control cellular fate after stress through their role in apoptosis and necrosis. A unique feature of these organelles is that they contain their own genome, reminiscent of the bacteria from which they originated. In mammals, the mitochondrial genome is quite small and encodes for only 13 proteins and the ribosomal and transfer RNAs necessary for intramitochondrial translation. But all of these 13 proteins are regulatory components of the mitochondrial respiratory complexes, and mutations in these genes can lead to severe bioenergetic consequences. In fact, over 150 pathogenic mutations have been identified in the mtDNA which cause human diseases (Wallace 2010).

Thus, the stability of the mtDNA is very important for maintenance of cellular function. This is particularly relevant considering that the mtDNA is closely associated with the inner mitochondrial membrane, where reactive oxygen species (ROS) are generated in abundance. These species are extremely reactive with biomolecules and readily oxidize DNA. Moreover, due to the charge difference across the membranes mitochondria can accumulate several lipophilic cationic molecules, some of which can damage DNA as well.

The preferential accumulation of damage in the mitochondrial DNA has been demonstrated in a plethora of pathological and degenerative conditions, including cancer, neurodegenerative diseases and normal aging. These results raised the question whether the differential efficiency in DNA repair activities could be responsible for the accumulation of lesions in mtDNA. In this regard, the existence of DNA repair mechanisms in mammalian mitochondria was initially investigated by Clayton and Friedberg, in 1974,

using UV as a DNA damaging agent (Clayton et al. 1974). They observed that mammalian cells did not remove UV lesions from their mitochondrial genomes, which led to the wide-spread notion that mitochondria lacked DNA repair pathways. While it still holds true that mitochondria from mammalian cells do not have a nucleotide excision repair pathway, the pathway which removes UV-induced lesions, we and others have demonstrated that mitochondria have a proficient base excision repair pathway, and have non-homologous end joining and mismatch repair activities. These topics have been comprehensively reviewed elsewhere (Maynard et al. 2009) and will not be addressed here. Conversely, this review will focus on the relationship between mitochondrial function and mtDNA damage accumulation, and on our efforts to establish models to dissociate the cellular responses induced by damage to the mtDNA from those induced by damage to the nuclear DNA.

Mitochondrial reactive oxygen species production

The energy required to phosphorylate ADP to ATP during the oxidative phosphorylation process comes from the electrochemical proton gradient generated by the electron transport chain (ETC) (Mitchell 1966). The electrons flowing through the ETC are finally accepted by molecular oxygen, at the active site of cytochrome oxidase, generating H_2O. However, this process has an important side effect to the cells, which is a constant generation of ROS through monoeletronic reduction of O_2, upstream of the cytochrome oxidase complex.

It is estimated that between 1–5% of the oxygen consumed during oxidative phosphorylation is partially reduced to generate the radical anion superoxide ($O_2^{\bullet-}$) (Boveris and Chance 1973; Liu 1997; Turrens 1997), mostly at Complexes I and III of the ETC (reviewed in (Kowaltowski et al. 2009)). However, due to the continuous flow of electrons through the ETC, even this small percentage of electron leak is sufficient to make the mitochondrial $O_2^{\bullet-}$generation the largest cellular source of ROS in most tissues (Liu 1997; Turrens 1997).

Superoxide anion can be generated primarily by the NADH dehydrogenase (Complex I) (Boveris and Chance 1973; Turrens and Boveris 1980) and by coenzyme Q (Boveris and Chance 1973; Cadenas et al. 1977; Turrens et al. 1985). The production of $O_2^{\bullet-}$in complex I is promoted by NAD-linked substrates, such as malate, glutamate, and pyruvate, and stimulated by rotenone, an inhibitor of electron transfer from complex I to coenzyme Q (Turrens 1997; Turrens and Boveris 1980). The electron leakage at coenzyme Q is probably due to the direct oxidation of the semiquinone anion formed during the electron transfer cycle by O_2. Superoxide anion generation at this point is stimulated by succinate, which donates electrons directly into complex II, and by ETC inhibitors, such as cyanide and antimycin A (Boveris et al. 1976; Cadenas et al. 1977; Kowaltowski et al. 1998; Turrens et al. 1985; Turrens 1997).

Mitochondrial antioxidant system

As the generation of mitochondrial $O_2^{\bullet-}$ is an ongoing process, mitochondria have an efficient antioxidant defense system, comprised of enzymatic and non-enzymatic components. Mammalian mitochondria contain Mn-superoxide dismutase (Doonan et al. 1984), which dismutate $O_2^{\bullet-}$ to H_2O_2; glutathione peroxidase (Sies and Moss 1978; Zakowski and Tappel 1978), a thiol peroxidase that removes H_2O_2 using glutathione as substrate; and glutathione reductase, which regenerates the reduced glutathione pool at expense of NADPH. Moreover, the energy-dependent $NAD(P)^+$ transhydrogenase, at the inner mitochondrial membrane, also contributes to the mitochondrial antioxidant defense (Hoek and Rydstrom 1988), as it helps to maintain the NADPH pool in the reduced state by catalyzing the reversible hydride transfer between NADH and $NADP^+$, according to the reaction: $NADH+NADP^+ \rightleftharpoons NAD^+ + NADPH$.

Mitochondria also contain lipid-soluble low molecular weight antioxidants, such as α- tocopherol (vitamin E) and UQH_2, both potent inhibitors of mitochondrial lipid peroxidation (Ernster et al. 1992). Cytochrome c, a component of the ETC, can also act as an antioxidant (Skulachev 1998), as the oxidized form of cytochrome c can oxidize $O_2^{\bullet-}$ back to O_2. It is noteworthy that the cytochrome oxidase complex, another component of the ETC, promotes the catabolism of $NO^{\bullet}$ to $NO_2^{\bullet-}$ and NO_3^-. This process not only removes $NO^{\bullet}$, but also prevents the formation of the potent oxidant peroxynitrite ($ONOO^-$) (Giulivi 1998).

Oxidative stress and mitochondrial permeability transition (MPT)

When the mitochondrial generation of ROS exceeds its antioxidant capacity, mitochondrial proteins, lipids and nucleic acids become targets of oxidation by these species. Under these conditions, mitochondrial membrane proteins suffer extensive oxidation (Castilho et al. 1995; Castilho et al. 1996; Fagian et al. 1990; Kowaltowski et al. 1996a, b; Valle et al. 1993). Reactive oxygen species, especially hydroxyl radical, can oxidize cysteine and methionine residues of proteins, leading to the formation

of dithiol crosslinks and methionine sulfoxide crosslinks, respectively.

The oxidation of inner mitochondrial membrane proteins leads to a nonspecific mitochondrial permeabilization, known as mitochondrial permeability transition (MPT) (Vercesi et al. 1997; Zoratti and Szabo 1995). This phenomenon is characterized by a progressive permeabilization of the inner mitochondrial membrane, which gradually becomes permeable to protons, ions, osmotic support and even small proteins. The MPT is dependent on the presence of Ca^{2+} in the intramitochondrial compartment, and is inhibited by submicromolar concentrations of the immunosuppressant cyclosporin A (Broekemeier et al. 1989; Crompton et al. 1988), probably due to its binding to inner mitochondrial membrane cyclophilins, which are required for MPT opening (Connern and Halestrap 1994; Nicolli et al. 1996). This process is referred to as a "permeability transition" because it can be partially reversed by the addition of Ca^{2+} chelators or dithiol reducing agents, immediately after induction of permeabilization (Castilho et al. 1996; Hunter and Haworth 1979; Valle et al. 1993). The molecular mechanisms leading to MPT in conditions of oxidative stress are extensively discussed elsewhere in this issue (Alberici et al. 2011).

A consequence of MPT can be an increase in the mitochondrial generation of ROS, likely associated with increased intramitochondrial Ca^{2+} and impaired respiration, as demonstrated in intact cells (Zorov et al. 2000) and isolated mitochondria (Batandier et al. 2004; Maciel et al. 2001). Thus, MPT can contribute to the accumulation of oxidative damage in mitochondria, including DNA lesions.

Mitochondrial DNA damage accumulation

Reactive oxygen species, like the hydroxyl radical (·OH), can abstract protons or attack molecules at electron-dense sites, generating secondary radical species which undergo intramolecular rearrangements resulting in more stable products. In nucleic acids, both the bases as well as the sugar are subjected to radical attack, leading to base modifications and abasic sites and strand breaks, respectively (Cooke et al. 2003). The ·OH can add to the double bonds of the bases with a reaction rate limited by diffusion, of around 4.5×10^9 to 9×10^9 $M^{-1}s^{-1}$ (Evans et al. 2004). These attacks result mostly in hydroxylation at C5 and C6 of pyrimidines and C4, C5 and C8 of purines. To date, over 80 different oxidized bases have been identified and characterized in DNA exposed to various oxidants, including H_2O_2 and singlet oxygen (Evans et al. 2004).

The mtDNA is associated with the inner mitochondrial membrane, as part of a large nucleoproteic complex known as nucleoid. This supramolecular arrangement has been recently identified, and the protein components and the dynamics of nucleoids in mammalian mitochondria are still under intense investigation (reviewed in Spelbrink 2010). Nonetheless, while this organization may provide some protection against DNA damage, the fact that the mtDNA is in close proximity with the ETC, where most ROS are generated, implies that the mtDNA is an important target for oxidative damage. In fact, mtDNA accumulates more 8-hydroxyguanosine, the most abundant oxidized base in DNA, than the nuclear DNA in several conditions, including aging and neurodegenerative diseases (for review, see Gredilla et al. 2010).

In vitro models to study the effects of mtDNA oxidative damage accumulation

DNA lesions, such as those introduced by ROS, can be either mutagenic or cytotoxic, depending on whether they block DNA and/or RNA polymerases or not. The biological consequences of oxidative DNA damage have been extensively studied, and range from induction of apoptotic and necrotic cell death to cellular transformation (Maynard et al. 2009). However, most of the experimental models used in these studies are such that DNA lesions are induced both in the nucleus as well as in the mitochondrial DNA, and thus it is difficult to sort out whether the responses are specific to lesions in either genome. Cellular responses to nuclear DNA damage have been extensively characterized in the last decade, and include the activation of a signaling cascade that leads to cell cycle arrest and induction of DNA repair pathways (Huen and Chen 2010). On the other hand, little is known about how mammalian cells respond to mtDNA damage, but since mtDNA repair enzymes are all encoded by nuclear genes and imported into mitochondria after being transcribed in the cytoplasm, it is likely that mammalian cells have specific signaling pathways that control these processes. Yeast cells respond to changes in mtDNA content and mitochondrial function via a pathway know as the retrograde response (Butow and Avadhani 2004), in which nuclear and mitochondrial gene expression are modulated concomitantly to adjust the cellular demand for energy, but no mammalian counterpart of such a signaling pathway has been described yet.

A cellular model in which lesions are introduced exclusively in the mitochondrial genome is required in order to sort out the pathways activated only in response to mtDNA damage. Because mitochondria are known to accumulate positively charged molecules (Murphy 1997; Ross et al. 2005), we have chosen to use photosensitizers which are charged under physiological pH. Methylene blue (MB) and crystal violet (CV) are both positively charged at pH 7.0 (Fig. 1) and have been reported to localize in

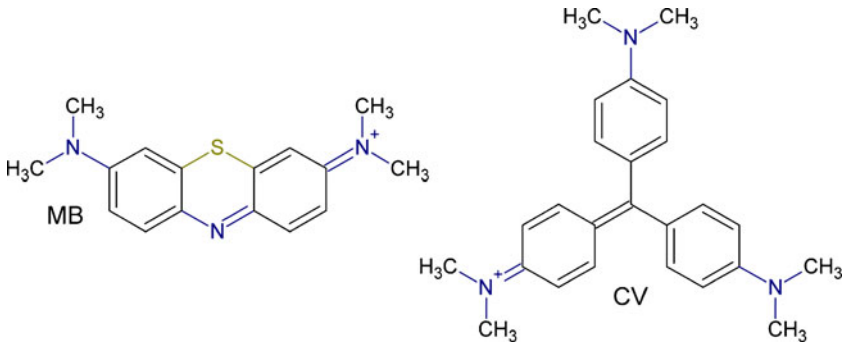

Fig. 1 Chemical structure of methylene blue (MB) and crystal violet (CV), showing the net positive charge at pH 7

mitochondria (Atamna et al. 2008; Huang et al. 2005; Indig et al. 2000) as well as in other subcellular compartments (Atamna et al. 2008; Blazquez-Castro et al. 2009; Mellish et al. 2002). These molecules constitute an interesting model system, as they generate singlet oxygen ($^1O_2$) upon photo-excitation with a well established photochemistry (Moore et al. 1972; Zhu and Finlay 2008). Singlet oxygen can attack the DNA bases forming several oxidized products, of which 8-hydroxy-guanosine is a major one (Devasagayam et al. 1991; Di Mascio et al. 1990).

Thus, we have employed these two compounds to introduce oxidative lesions in the mtDNA and evaluated cell survival after incubation and photoactivation of the dyes. For this, HeLa cells were plated at 500 cells per dish and incubated with increasing concentrations (from 1 to 50 μM) of MB and CV in the dark for 30 min. After this, the cultures were extensively washed in phosphate-buffered saline (PBS) and irradiated in PBS with the appropriate LEDs for each dye (red for MB and yellow for CV) for 0, 2 or 10 min. The buffer was then replaced by culture medium and the cells incubated for 7–10 days, for colony formation assessment, as described in (de Souza-Pinto et al. 1996). Figure 2 shows the results obtained. Surprisingly, MB showed no citotoxic effect with any concentration used, even with the highest dose of light, 1.25 J/cm^2. On the other hand, CV showed a significant, dose-dependent cytotoxic effect in the same concentration range (1–25 μM), even with a lower light dose (0.32 J/cm^2) than used for MB. This result suggests that even though these compounds have similar photochemical properties in solution, they may behave differently in cells, as the biological effect is quite diverse. We speculate that the different effects were due to the formation of dimers by MB intracellular. It has been previously shown that MB can dimerize after a certain concentration threshold and that these dimers do not generate $^1O_2$ upon photo-excitation (Gabrielli et al. 2004; Severino et al. 2003). On the other hand, CV photochemistry is not altered by the formation of dye aggregates (Jones et al. 1991). Thus, if the active accumulation of MB inside the mitochondria reached levels high enough to induce dimer formation, the absence of a cytotoxic effect could be due to the fact that under these conditions no oxidative damage had been formed. In these experiments, the dye/cell ratio was 6×10^{12} molecules of dye/cell, at the 1 μM concentration. We are now testing this hypothesis by exposing the cells to a much lower dye/cell ratio, and our preliminary results support the hypothesis that dimer formation contributed to lack of cytotoxicity in cells incubated with MB (not shown).

It is important to point out here that a very careful and thorough characterization of these cellular models are needed to support any conclusion as to the specificity of the cellular responses to mtDNA oxidative damage. For that purpose, we are also quantifying the induction of oxidized DNA lesions in the nuclear and mitochondrial DNA of cells exposed to MB and CV, as well as to another photosensitizer, RO19-8022

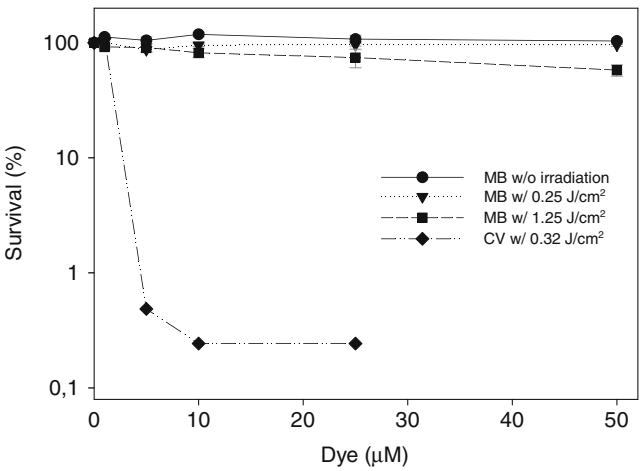

Fig. 2 Clonogenic survival of Hela cells treated with MB or CV. Five hundred cells were plated in 10 cm dishes and allowed to fix. The medium was then replaced by PBS containing increasing concentrations of MB or CV, as shown in the figure, and the cultures incubated for 30 min, at 37 °C. The treatments were removed, the cultures washed twice with PBS and irradiated with the doses in the figure with light-emitting diodes at 700 nm (for MB) or 560 nm (for CV). The PBS was then replaced with culture medium and the plates incubated for 7–10 days for colony formation. Survival was calculated relative to the plating efficiency of control cultures (treated with PBS alone). The results shown are average±error of two independent experiments, performed in triplicate, except for CV in which one typical experiment is presented

(Will et al. 1999) which does not seem to have a specific subcellular localization, and induces oxidative damage in the nuclear DNA (Thorslund et al. 2002; Will et al. 1999). These models will allow for a precise characterization of the cellular responses induced after oxidative damage in mitochondria, and to the identification of the relative contribution of mtDNA damage and damage to other macromolecules in the molecular events that lead to oxidative stress-induced mitochondrial and cellular dysfunction.

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