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The “fast” and the “slow” modes of mitochondrial DNA degradation

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Abstract

In a living cell, oxidative stress resulting from an external or internal insult can result in mitochondrial DNA (mtDNA) damage and degradation. Here, we show that in HeLa cells, mtDNA can withstand relatively high levels of extracellular oxidant H₂O₂ before it is damaged to a point of degradation, and that mtDNA levels in these cells quickly recover after removal of the stressor. In contrast, mtDNA degradation in mouse fibroblast cells is induced at eight-fold lower concentrations of H₂O₂, and restoration of the lost mtDNA proceeds much slower. Importantly, mtDNA levels in HeLa cells continue to decline even after withdrawal of the stressor thus marking the “slow” mode of mtDNA degradation. Conversely, in mouse fibroblasts maximal loss of mtDNA is achieved during treatment, and is already detectable at 5 min after exposure, indicating the “fast” mode. These differences may modulate susceptibility to oxidative stress of those organs, which consist of multiple cell types.

Keywords

Circulating mtDNA; mtDNA repair; mtDNA degradation; oxidative mtDNA damage

Introduction

In mammalian cells, both the nucleus and mitochondria serve as repositories for genetic information (DNA). Mitochondrial DNA (mtDNA) is a circular DNA molecule that encodes 37 genes: 22 tRNA, 2 rRNA required for proper functioning of the mitochondria's own translational apparatus and 13 polypeptide components of respiratory complexes I, III, IV, and V, which are translated on mitochondrial ribosomes (Anderson et al., 1981).

In the cell, mitochondria perform numerous functions including, but not limited to producing the bulk of the ATP (Zu & Guppy, 2004), generating reactive oxygen species, which can be both damaging and play a role in normal cellular signaling (Alexeyev, 2009; Murphy, 2009; Al-Mehdi et al., 2012), and regulation of cell death (Carlsen, 1990; Wang & Youle, 2009).

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Declaration of interest

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All these functions, directly or indirectly, are affected by the state of mitochondrial respiratory complexes, which in turn depends on mtDNA-encoded subunits, whose supply critically depends on mtDNA integrity. As a consequence, alterations in mtDNA integrity have been associated with various human pathologies, such as mitochondrial diseases (Schapira, 2012; Ylikallio & Suomalainen, 2012; Zheng et al., 2012), diabetes (Bannwarth et al., 2011; Maassen et al., 2005; Supale et al., 2012), cancer (Kurelac et al., 2013; Larman et al., 2012; Wallace, 2012; Yu, 2012) and neurodegenerative disorders (Milone, 2012).

While significant progress has been achieved in understanding the mechanisms involved in the maintenance of nuclear genome integrity, our understanding of corresponding mitochondrial mechanisms remains incomplete. The mitochondrial genome accumulates mutations at a faster rate than nuclear genome (Ballard & Whitlock, 2004; Brown et al., 1979; Tatarenkov & Avise, 2007). However, the exact mechanism behind this observation remains controversial. Three hypotheses have been put forward over the years: (1) the lack of “protective” histones in mitochondria; (2) a close proximity of the mtDNA to the electron transport chain, which is a major cellular source of reactive oxygen species (ROS); and (3) a smaller repertoire of DNA-repair pathways in mitochondria. However: (1) depending on the experimental conditions, histone proteins may either protect DNA from oxidative damage or promote it (Liang & Dedon, 2001; Liang et al., 1999). Also, mitochondrial nucleoid proteins have been shown to be just as “protective” as histones (Guliaeva et al., 2006); (2) a close proximity to the source of ROS may only contribute to mtDNA mutagenesis, if it is not efficiently counteracted by a combination of antioxidant defenses and DNA repair. Also, actual rates of mitochondrial ROS production *in vivo* remain controversial (Alexeyev, 2009); (3) while the full complement of mitochondrial DNA repair pathways remains to be elucidated, there is evidence for the presence of many nuclear pathways in mitochondria (Alexeyev et al., 2013; Gredilla et al., 2010; Kazak et al., 2012; Liu & Demple, 2010). Mitochondria are proficient in Base Excision Repair, the main pathway for the repair of oxidative base lesions and single-strand breaks, and at least one oxidative DNA lesion, 8-oxoguanine, is repaired more efficiently in mitochondria than it is in the nucleus (Thorslund et al., 2002). Moreover, mitochondria possess a unique mechanism for the degradation of damaged mtDNA molecules, which co-exists with DNA repair and may be activated by excessive mtDNA damage (Furda et al., 2012; Shokolenko et al., 2009, 2013b). This pathway, together with the high-redundancy of organellar genomes may enable effective management of even relatively high levels of mtDNA damage in both mitochondria and chloroplasts (Bendich, 2013).

Recently, we have demonstrated that in several cell lines of epithelial origin, mtDNA degradation coincides with repair and occurs predominantly after withdrawal of the stressor during the recovery phase (Shokolenko et al., 2009). mtDNA degradation is of particular interest because it may contribute to both the etiology of mtDNA depletion syndromes (Clay Montier et al., 2009; Rotig & Poulton, 2009) and to the activation of the innate immune system by circulating mtDNA (Oka et al., 2012; Zhang et al., 2010). Here, we investigated mtDNA degradation patterns in mouse fibroblasts and HeLa cells, and report that among the studied cell lines, fibroblasts are more sensitive to hydrogen peroxide (H₂O₂)-induced damage, that mtDNA degradation in these cells proceeds faster, and that mtDNA

degradation process in these cells is largely completed during 30 min treatment with the stressor.

Methods

Cell lines, media and treatments

Unless specified otherwise, all cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 10% Fetal Bovine Serum, 50 µg/ml gentamycin, 50 µg/ml uridine, and 1 mM sodium pyruvate in a humidified atmosphere containing 5% CO₂ at 37 ° C. Cells were treated with H₂O₂ in Hank's Balanced Salt Solution (HBSS) under the same conditions. HeLa (cervical epithelial cell line) and L929 (areolar connective tissue cell line) were from laboratory collection. SV40 large T-antigen immortalized mouse embryonic fibroblast (MEF) cell lines Cre2 and 4B6 were derived in our lab (Shokolenko et al., 2013a), and 92TAg (Sobol et al., 2003) was kindly provided by Dr. R. Sobol.

Quantitative Southern Blotting

Quantitative Southern Blotting under non-denaturing conditions (QSBN) was performed as described earlier (Shokolenko et al., 2009), except mouse total DNA was digested with EcoRI. When blotting BamHI-digested total human DNA, the membrane was cut at the level of the 9 kb band of lambda/HindIII marker after transfer. The upper portion was then hybridized with the mtDNA probe (detects 16,569 bp fragment), and the lower portion was hybridized with the 18S rDNA probe (5102 bp fragment). Similarly, for mouse DNA the membrane was cut at the same level, and the upper portion was hybridized with a probe encompassing 6615–8053 bp of the mouse mtDNA (GenBank NC006914, detects 14,037 bp fragment), while the lower portion was hybridized with rDNA probe encompassing 12,949–13,738 bp of mouse rDNA (GenBank GU372691, detects 6627 bp fragment). After hybridization, membranes were exposed to an imaging screen to measure band intensities. The number of pixels per band was determined by encompassing bands with identical rectangular regions of interest and subtracting the background. It is important to note that both nuclear DNA (nDNA) and mtDNA are subjected to oxidative damage with H₂O₂, and therefore nDNA can not serve as true loading control in these experiments. However, it has been reported that nDNA is less susceptible to oxidative damage (Shokolenko et al., 2009; Yakes & Van Houten, 1997), and therefore it can serve as a useful reference in Southern hybridizations of oxidatively damaged total cellular DNA.

The percent mtDNA remaining was determined by means of QSBN as $\% = T/C \times 100\%$, where C is intensity of the band in the control lane and T is intensity of the band in the lane corresponding to a given time point.

Western blotting

Protein extracts from treated and control cells were prepared using lysis solution containing 10 mM Tris-HCl, 1% SDS, 1 × EDTA-free protease inhibitor cocktail (Roche, Indianapolis, IN). Protein concentrations were measured using the BCA assay (Pierce, Rockford, IL). Proteins were separated by PAAG electrophoresis and transferred to PVDF membranes, blocked and incubated with primary and secondary antibodies using standard techniques

(Sambrook & Russel, 2001). Blots were developed with SuperSignal West Pico and exposed to CL-Xposure film (both Pierce). Primary antibodies were α -myc tag (Cell Signaling), α -HSP60 (mitochondrial, BD Biosciences).

Microscopy

For phase contrast imaging, cells were plated into 35 mm glass bottom MaTek dishes, allowed to attach overnight, and imaged with a Nikon TE200U microscope (10 $\times$ objective).

Results

The extent and pattern of mtDNA degradation in HeLa cells depends on the level of oxidative stress

Our previous studies indicate that in the human adenocarcinoma cell line HCT116 (epithelial origin) the mtDNA was largely (>90%) preserved during a 30-min treatment with exogenous H₂O₂ despite the fact that during this period a substantial amount of lesions was accumulated. Instead, mtDNA degradation occurred after H₂O₂ withdrawal, during the recovery period (Shokolenko et al., 2009). During this period, both mtDNA repair and degradation were observed to occur simultaneously. In contrast, when oxidative stress was induced by the generation of H₂O₂ through enzymatic oxidation of hypoxanthine with xanthine oxidase, a substantial loss of mtDNA was observed at the end of the treatment (Shokolenko et al., 2009). One possible explanation for this discrepancy is in the dynamics of the H₂O₂ concentrations. When cells were exposed to pure H₂O₂, its concentration steadily decreased over the time, so that at the end of treatment most of the H₂O₂ has decomposed (Yakes & Van Houten, 1997). In contrast, when H₂O₂ was generated enzymatically, its concentration in the treatment medium rose steadily and was the highest at the end of treatment. This suggests that the pattern of mtDNA degradation may depend on the concentration of H₂O₂. To directly test this hypothesis, we exposed HeLa cells to different concentrations of H₂O₂ for 30 min, and followed mtDNA content in the exposed cells for up to 3 days after withdrawal. At the lowest concentration of H₂O₂ used, 100 μ M, no mtDNA degradation was observed (Figure 1A and B), whereas higher concentrations led to a substantial mtDNA loss immediately after the treatment. This loss became more prominent after 6 h recovery period (Figure 1C–H). Importantly, there was a clear dose response in mtDNA degradation as measured both immediately after the treatment and after a 6 h recovery period (Figure 1I and J). Interestingly, at all concentrations of H₂O₂ used, a substantial increase in mtDNA content was observed in treated cells as compared to control at 48 h after the treatment. This increase became even more prominent at 72 h after the treatment when cells were treated with 40 μ M or 800 μ M of H₂O₂ (Figure 1F and H). This increase is reminiscent of the “overshoot repair” reported by us previously (Driggers et al., 1997).

The exposure of HeLa cells to H₂O₂ was accompanied by growth arrest, cell swelling (especially at 400 and 800 μ M H₂O₂), cell death and detachment (Figure 2). The detachment was most profound at 60–90 h after the treatment.

mtDNA in mouse fibroblast cell lines is highly susceptible to oxidative stress-induced degradation

Our initial attempts to characterize mtDNA degradation in cell types other than human epithelial cell lines led to the observation that, in the immortalized MEF cell line 4B6, mtDNA loss was dramatic not only at 200 μM H_2O_2 , which is a threshold concentration for mtDNA degradation in HeLa (Figure 3A and B), but also at 75 μM H_2O_2 , a concentration which does not induce mtDNA degradation in HeLa cells (Figure 3C and D). Similar rapid and extensive mtDNA loss was also observed in another mouse fibroblast cell line, 92 TAG (Figure 3E and F). In sharp contrast to HeLa cells, mtDNA recovery after degradation in both 4B6 and 92Tag was much slower. Whereas in HeLa cells mtDNA “overshot” its baseline 48 h after treatment, mtDNA recovery in mouse fibroblasts just became noticeable at this time point (Figure 3G–J), and was only around 50% at 120 h after the treatment in 92TAG (Figure 3I and J).

To obtain a better quantitative insight into the differences in susceptibility to mtDNA degradation between HeLa and MEFs, we conducted a dose response study of mtDNA degradation to H_2O_2 concentration in 4B6. In these cells, mtDNA degradation was detectable at 25 μM H_2O_2 compared to 200 μM H_2O_2 in HeLa cells (Figure 4A and B). Therefore, it appears that mtDNA in MEFs is about 8 times more sensitive to H_2O_2 than in HeLa cells. We then determined whether the extent of mtDNA degradation was dependent of the duration of exposure to H_2O_2 . As expected, a 15 min incubation generally resulted in lesser mtDNA degradation as compared to a 30-min incubation (Figure 4C and D). Finally, a time course of mtDNA degradation in response to the treatment with 100 μM H_2O_2 was determined in 92TAG and L929 cells. In both cell lines, mtDNA loss was detectable within 5–10 min upon exposure to the stressor (Figure 4E–H).

Similar to HeLa cells, exposure of 92TAG cells to H_2O_2 was accompanied by cell death and growth arrest (Figure 5), although to a much lesser extent. No cell swelling or significant detachment was observed. The experiment was terminated at 48 h after the treatment when cells reached confluency.

Discussion

MtDNA depletion has been observed in a variety of pathological conditions such as biliary atresia (Tiao et al., 2007), viral infections (Corcoran et al., 2009; Wiedmer et al., 2008), statin-induced myopathy (Stringer et al., 2013), anti-retroviral drug toxicity (Feeney et al., 2012; Koczor & Lewis, 2010; Nasi et al., 2011; Stankov et al., 2009), mtDNA depletion syndromes (Barthelemy et al., 2001; El-Hattab & Scaglia, 2013; Rotig & Poulton, 2009), metabolic syndrome (Huang et al., 2011), diabetes (Monickaraj et al., 2012), metastatic cancer (Cloos et al., 2009; Guo et al., 2011; Koochekpour et al., 2013; Moro et al., 2008; Potenza et al., 2011; Yu et al., 2010), and aging (Barazzoni et al., 2000; Cree et al., 2008; Kaaman et al., 2007). Also, mtDNA content strongly correlates with lipogenesis in adipocytes (Kaaman et al., 2007). However, the detailed mechanism behind this phenomenon remains poorly understood.

In mice, intragastric administration of ethanol induced oxidative stress, which was accompanied by a reversible loss of mtDNA (Mansouri et al., 1999). The loss of mtDNA was approximately 50% in all organs studied. It could be partially prevented by the antioxidants melatonin, vitamin E and coenzyme Q and was followed by adaptive mtDNA resynthesis (Mansouri et al., 2001). Lipopolysaccharide, a known inducer of *in vivo* oxidative stress, also induced mtDNA depletion (Suliman et al., 2003). Angiotensin II induced mitochondrial ROS production and decreased skeletal muscle mtDNA content in mice (Mitsuishi et al., 2008). Degradation of mtDNA was observed in the rat model of cerebral ischemia/reperfusion (Chen et al., 2001). Similar to mtDNA depletion induced by intragastric ethanol administration, mtDNA levels returned to normal within 24 h of cerebral ischemia/reperfusion (Chen et al., 2001). Finally, H₂O₂-induced oxidative stress in hamster fibroblasts was accompanied by a Ca²⁺-dependent degradation of mtDNA (Crawford et al., 1998). Taken together, these findings strongly suggested a link between oxidative stress (which may result in oxidative mtDNA damage) and mtDNA degradation.

In this study, we observed that mtDNA degradation in response to oxidative stress proceeds with different kinetics in different cell types. In terms of mtDNA degradation, the human epithelial cell line HeLa, similar to other human epithelial cell lines studied previously (Shokolenko et al., 2009), was relatively resistant to oxidative stress, and underwent mtDNA degradation only at H₂O₂ concentrations in excess of 100 μ M. In contrast, under the same experimental conditions the threshold H₂O₂ concentration was eight times lower in fibroblasts, which are connective tissue cells. Moreover, in HeLa cells mtDNA degradation continued after the removal of H₂O₂, whereas in connective tissue mouse fibroblasts, degradation reached its maximum during 30-min treatment. We designate patterns of mtDNA loss observed in HeLa and connective tissue cells as “slow” and “fast” modes of mtDNA degradation, respectively. Recovery of mtDNA levels occurred faster in HeLa cells, and original levels of mtDNA per cell were achieved and even exceeded within 48 h of recovery in complete medium even at H₂O₂ concentrations as high as 800 μ M. In contrast, in connective tissue cells, recovery of mtDNA levels only became noticeable at 48 h after the treatment, and was still incomplete at 120 h. Previous studies have demonstrated that other epithelial cell lines like HCT116 and A549 behave in a way similar to HeLa and lose their mtDNA by a “slow” mechanism (Shokolenko et al., 2009). Similar to HeLa cells, exposure of connective tissue cells to H₂O₂ was accompanied by cell death and growth arrest. However, no cell swelling or massive detachment was observed in connective tissue cells even though mtDNA loss, under the experimental conditions employed, was much more extensive in these cells. This suggests that mtDNA retention is not likely to be the main determinant of cell swelling, growth arrest or survival in our experiments. Since H₂O₂ has access to both nDNA and mtDNA, it was likely that the nDNA damage was responsible for growth arrest in our experiments, even though nDNA was less susceptible to oxidative damage (Yakes & Van Houten, 1997).

This study demonstrates that mtDNA degradation can proceed with either “fast” or “slow” kinetics as extreme examples, and that mode of degradation may be determined by the cell type. It is likely that future studies will uncover other cell type-specific intermediate modes. The “fast” mode which is described here for the first time may play a regulatory or signaling role, e.g. it may contribute to the pool of extracellular fragmented mtDNA (damage-

associated molecular patterns or DAMPs), which was shown to play a leading role in sterile inflammation (Zhang et al., 2010). It is also tempting to speculate that this “fast” mode contributes to the raise in the pool of circulating mtDNA observed after myocardial ischemiareperfusion injury, a pathological condition associated with oxidative stress (Bliksoen et al., 2012).

Conclusions

Overall, this study provides mechanistic insight into how oxidative stress in a particular organ composed of different tissues and cell types can lead to predominant mtDNA damage in some cell type(s), while relatively sparing other cell type(s).

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Figure 1.

mtDNA degradation in HeLa cells. HeLa cells were exposed to indicated concentrations of H_2O_2 for 30 min in HBSS as described in “Methods” Section, and mtDNA degradation and recovery were assessed over 72 h period by QSB. Mito, mtDNA band; 18S, 18S (nDNA) band. A, C, E, and G, representative gel images; B, D, F, and H, quantitation of band intensities. I, per cent of mtDNA remaining after 30 min treatment with various concentrations of H_2O_2 . J, the same as I, except after 6 h recovery from H_2O_2 treatment. Note direct correlation between H_2O_2 concentration and the extent of mtDNA degradation.

Figure 2.

Morphological changes in HeLa cells following oxidative stress. HeLa cells were treated with the indicated concentrations of H_2O_2 for 30 min in HBSS as described in “Methods” Section, and morphological changes in population were followed with phase contrast microscopy. Black arrows, detached apoptotic cells; open triangles, growth arrested cells; white arrows, swollen cells.

Figure 3.

mtDNA degradation in connective tissue cells. 4B6 or 92TAg cells were exposed to the indicated concentrations of H₂O₂ for 30 min in HBSS as described in “Methods”, and mtDNA degradation and recovery were assessed by QSB. Mito, mtDNA band; 18S, 18S (nDNA) band. A, C, and E, representative gel images; B, D, and F, quantitation of band intensities. G, H, I and J, time course of mtDNA recovery in 92TAg cells after exposure to 100 μM H₂O₂ for 30 min.

Figure 4.

Time course and dose response of "fast" mtDNA degradation in connective tissue cells. 4B6 or 92TA cells were exposed to the indicated concentrations of H_2O_2 for indicated periods of time in HBSS as described in "Methods" Section, and mtDNA degradation was assessed by QSNB. A–D, dose response of mtDNA degradation in 4B6 cells exposed to various concentrations of H_2O_2 for either 30 min (A and B) or 15 min (C and D). Mito, mtDNA band; 18S, 18S (nDNA) band. A, C, E, and G, representative gel images; B, D, F, and H, quantitation of band intensities. E–H, time course of the "fast" mtDNA degradation in

92TAg (E and F) and L929 (G and H). Note that mtDNA degradation is noticeable at the earliest time point taken, 5 min.

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Figure 5. Morphological changes in 92TAg cells following oxidative stress. 92TAg cells were treated with the indicated concentrations of H_2O_2 for 30 min in HBSS as described in “Methods” Section, and morphological changes in population were followed with phase contrast microscopy. Black arrows, apoptotic cells.