Published in final edited form as:

Free Radic Biol Med. 2009 October 15; 47(8): 1190–1198. doi:10.1016/j.freeradbiomed.2009.07.032.

Contribution of glutathione status to oxidant-induced mitochondrial DNA damage in colonic epithelial cells

Magdalena L. Circu¹, Mary P. Moyer², Lynn Harrison¹, and Tak Yee Aw^{1,*}

¹ Department of Molecular & Cellular Physiology, Louisiana State University Health Science Center, Shreveport, LA 71130

² INCELL Corporation, San Antonio, TX 78249

Abstract

While oxidative stress induces mitochondrial DNA (mtDNA) damage, a role for redox in modulating mtDNA oxidation and repair is relatively unexplored. The current study examines the contribution of cellular glutathione (GSH) redox status to menadione (MQ)-induced mtDNA damage and post-oxidant mtDNA recovery in a non-transformed NCM460 colonic cell line. We show that MQ caused dose-dependent increases in mtDNA damage that was blunted by N-acetylcysteine, a thiol antioxidant. Damage to mtDNA paralleled mitochondrial protein disulfide formation and glutathione disulfide (GSSG) increases in the cytosol and mitochondria, and was exacerbated by inhibition of GSH synthesis in accordance with decreased cytosolic and mitochondrial GSH. Blockade of mitochondrial GSH (mtGSH) transport potentiated mtDNA damage which was prevented by overexpression of the oxoglutarate mtGSH carrier, underscoring a link between mtGSH and mtDNA responsiveness to oxidative stress. The removal of MQ post treatment elicited mtDNA recovery to basal levels by 4h, indicating complete repair. Notably, mtDNA recovery was preceded by restored cytosolic and mtGSH levels at 2h, suggesting a connection between the maintenance of cell GSH and effective mtDNA repair. MQ-induced dose-dependent increase in mtDNA damage was attenuated by overexpressing mitochondrial 8-oxoguanine DNA-glycosylase (Ogg1), consistent with 8oxoG being a major oxidative mtDNA lesion. Collectively, the results show that oxidative mtDNA damage in colonic cells is highly responsive to the mtGSH status and that post-oxidant mtDNA recovery may also be GSH sensitive.

INTRODUCTION

Mitochondria are major players in the regulation of cellular processes, including oxidant mediated apoptotic signaling. An important mitochondrial component that is highly vulnerable to oxidative damage is the mitochondrial DNA (mtDNA) [1–3] due to its open circular structure, lack of histone protection, and proximity to the mitochondrial electron transport chain, a main source of reactive oxygen species (ROS) [2,3]. The mitochondrial genome encodes several hydrophobic components of the respiratory chain belonging to complexes I, II and IV, and ATP synthase that is essential for oxidative phosphorylation. MtDNA damage will decrease gene expression of these key respiratory proteins [4]. An enhanced ROS production from consequent disruption in electron flow creates a vicious cycle of mitochondrial

*Corresponding author: Tak Yee Aw, PhD, Department of Molecular & Cellular Physiology, LSU Health Sciences Center-Shreveport, 1501 Kings Highway, Shreveport, LA 71130-3932, Tel: 318-675-6032, Fax: 318-675-4217, taw@lsuhsc.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

dysfunction that ultimately results in cell apoptosis [5]. The findings that oxidative mtDNA damage can induce cell apoptosis [6] have spurred much interest in understanding the factors that influence oxidative mtDNA damage and its repair. At steady state, the extent of mtDNA damage would be determined by the efficiency of its repair. The mitochondrial base excision repair (mtBER) system plays a central role in the removal of oxidized base damages. Specific DNA glycosylases release the damaged bases generating abasic (AP) sites that are further cleaved by AP endonucleases [7,8]. Of importance, the 8-oxoguanine DNA glycosylase (Ogg1) recognizes and removes 7,8-dihydro-8-oxoguanine (8-oxoG) [9–11], a biologically relevant oxidative lesion induced by mitochondrially derived ROS [12]. A decrease in mitochondrial Ogg1 has been shown to correlate with mitochondria-initiated apoptosis [13].

The mitochondrial GSH (mtGSH) status is expected to play a quantitative role in the degree of mtDNA damage, given that ROS-induced oxidative stress is typically associated with altered redox balance. MtGSH maintains mitochondrial integrity via its versatile role in oxidant reduction, electrophile conjugation, and preservation of protein-SH, and is an integral component of the mitochondrial redox cycle in the elimination of ROS that arise as by-products of aerobic respiration and enhanced mitochondrial dysfunction. Early studies have shown that loss of mtGSH is an important factor in oxidative vulnerability [14], implicating the significance of this redox compartment in cell survival. A link between loss of mtGSH and cytotoxicity was previously observed for aromatic hydrocarbons [15], hypoxia [16], *tert*-butylhydroperoxide (*t*BH, [17], ethanol intoxication [18], and acetaminophen [19]. Our recent studies have demonstrated that decreased mtGSH is a key contributor to the sensitization of colonic cells to menadione-induced apoptosis [20]. Within the mitochondrial matrix, steady-state GSH concentration can be maintained by the reduction of GSSG as catalyzed by the flavoenzyme, glutathione reductase, or achieved by uptake from the cytosol through specific carriers located in the inner mitochondrial membrane. In liver and kidney, the organic anion carriers, 2-oxoglutarate (OGC) and dicarboxylate (DIC) have been characterized to be effective transporters of mtGSH [21–23]. Chemical or genetic modulation of these transporter functions were shown to alter the intra-mtGSH status [23].

In the current study, we have investigated the relationship between mtGSH and oxidative mtDNA damage caused by menadione (2-methyl-1,4-naphthoquinone, MQ), a redox cycling quinone which we recently found to induce significant mtGSH oxidation and colonic cell apoptosis [20]. The contribution of mtGSH to MQ-mediated mtDNA damage was examined in the nonmalignant NCM460 or malignant HT-29 human colonic epithelial cell lines, using pharmacologic and genetic approaches to manipulate mtGSH transport and mitochondrial matrix (mt-matrix) GSH status. Our results demonstrate that MQ dose-dependently elicited mtDNA damage that was exacerbated by inhibition of cellular GSH synthesis and prevented by N-acetylcysteine. Significantly, oxidative mtDNA damage was exaggerated by attenuated mtGSH transport in association with increased mtGSSG, and post oxidant recovery of mtDNA was preceded by restored cytosolic and mtGSH status. Taken together, these results show that the extent of mtDNA damage and the capacity for repair are influenced by the cellular GSH redox state, particularly that of the mtGSH status.

MATERIALS AND METHODS

The following chemicals were obtained from Sigma Chemicals (St. Louis, MO, USA): menadione sodium bisulphite, N-acetylcysteine, EDTA, EGTA, sucrose, dithiothreitol, 2,4-dinitrofluorobenzene, iodoacetic acid, glutathione (GSH and GSSG), butylmalonic acid, phenylsuccinic acid, calf thymus DNA, Tris base, sodium dodecyl sulphate, sodium chloride, sodium hydroxide, sodium citrate. M3:10 media was acquired from INCELL Corporation (San Antonio, TX, USA). Antibiotic/antimycotic, McCoy's media, L-glutamine and trypsin were obtained from GIBCO Corporation (Grand Isle, NY, USA). Fetal bovine serum was acquired

from Atlanta Biologicals (Norcross, GA, USA). Protein dye assay kit and zeta-probe GT blotting membranes were obtained from BIORAD Corporation (Hercules, CA, USA). DNA extraction kit was obtained from QIAGEN (Valencia, CA, USA). DNase-free RNase and XhoI enzymes were purchased from Roche Applied Bioscience (Indianapolis, IN, USA). Lambda DNA *Hind* III marker, Taq polymerase, deoxynucleotides and PCR buffer were from Promega (Madison, WI, USA). All other reagents were of analytical grade and were purchased from local sources.

Cell culture and nucleofection

Cell lines—NCM460 is an immortalized and nonmalignant human colonic epithelial cell line [24] that was generated by Dr. Mary Moyer, INCELL Corporation. The NCM460 cell line was derived from the transverse colon of a human donor and exhibits similar characteristics as primary cultures of cells isolated from the same region [25]. NCM460 cells were cultured in M3:10 media in a 5% CO₂/95% air humidified environment at 37°C. A single stock of NCM460 cells were obtained from INCELL and expanded which we have designated as passage 0; subsequent cell expansions for either freezing back or experiments were designated consecutively as passage 1, 2, 3, etc. In this study, NCM460 cell passages 75–85 were used in all experiments. The human colon epithelial cell line HT-29, originally isolated from a colon adenocarcinoma of a female Caucasian, was purchased from American Type Culture Collection (ATCC, Manassas, VA). HT-29 cells were grown in McCoy's medium supplemented with 10% fetal bovine serum and 2mM glutamine. In recent studies, we found that responses of cellular GSH/GSSG to oxidants and non-oxidants were similar in HT-29 and NCM460 cells [20,26].

Nucleofection and generation of stable colon cell lines—As compared to NCM460 cells, HT-29 cells were easy to transfect, and exhibited high percent and yield of transfection. Hence, HT-29 cells were used to generate stable cell lines that overexpress specific mitochondria targeted proteins. To overexpress the mitochondrial oxoglutarate transporter (OGC), cells were nucleofected with either pcDNA 3.1 (vector control) or with the expression vector containing wild-type rat OGC (pcDNA 3.1/rOGC-WT) (gifts from Dr. Lawrence Lash, Wayne State University School of Medicine, Detroit, Michigan). To overexpress the mitochondrial 8-oxoguanine DNA glycosylase (OGG1), cells were nucleofected with either pcDNA3.neo (vector control) or with pcDNA3 plasmid carrying the mitochondrial targeting sequence (MTS)-OGG1 cDNA (a gift from Dr. Susan LeDoux, University of South Alabama, Mobile, Alabama). Nucleofection was performed with the Nucleofector II system (Amaxa GmbH, Cologne, Germany) according to the manufacturer's protocol (Amaxa Biosystems). Briefly, cells were passaged 3 days before nucleofection and grown to 70% confluency. Cells were harvested by trypsinization and 1×10^6 cells were mixed with 100µl of nucleofector solution and 2µg plasmid DNA. Samples were transferred into cuvettes, and nucleofected using program W-17. Cells were resuspended in growth media and allowed to grow for 2–3 days. After re-plating at a population density of 5×10^3 per cm², cells were grown in culture media containing 800µg/ml G418 (Invitrogen, Grand Island, NY, USA) to select for cells that had integrated the plasmid DNA into their genome. Cells were medium changed every 2 days until single colonies formed. The colonies were isolated, maintained in 500µg/ml G418, and individually expanded to form the respective cell lines. Genomic DNA was prepared using the Qiaamp DNA blood mini kit (Qiagen) and tested for vector DNA incorporation in the genome by PCR. The expression of the recombinant proteins were verified in mitochondrial protein extracts by Western blot analyses.

Cell incubation and digitonin fractionation

Incubation protocols

Preincubation with chemical agents: Colonic cells (5×10^6 /ml) were plated in T25 flasks in complete media one day before the experiment. The next day, the media was changed to FBS- and phenol red-free DMEM. Preincubation with BM/PS or NAC were according to the following conditions: BM/PS, 20mM each, 1h; or NAC, 2mM, 30min. For BSO experiments, cells were seeded at 3×10^6 /ml, and the next day were pretreated with 5 or 20 μ M BSO for another 24h in complete media. Thereafter, the media was replaced by FBS- and phenol red-free DMEM.

Cell incubations with MQ: At the end of the respective pre-exposure times, cell incubations with MQ were performed in one of two ways. In studies of mitochondrial and cytosolic GSH redox status, cells were harvested by trypsinization and resuspended in Dulbecco PBS at a concentration of 2×10^6 /ml. Incubations were then performed in cells in suspension with 100 or 200 μ M MQ at 37°C for different times in rotating round bottom flasks in a rotavap system [27]. In experiments to test the effect of NAC, BM/PS or BSO, these agents were present throughout the incubation. Studies on mtDNA and GSH recovery were conducted using 200 μ M MQ; this 200 μ M dose was selected to ensure a higher level of mtDNA damage such that a larger difference between mtDNA damage and recovery can be quantified. In dose-dependent experiments, MQ concentrations of 50, 100, 200 and 400 μ M were used. At designated times, cell aliquots were removed and subjected to digitonin fractionation (see below) for determination of GSH contents in the cytosol and mitochondria.

Digitonin fractionation: The cytosolic and mitochondrial fractions were obtained using digitonin fractionation [28]. Briefly, digitonin fractionation was performed in 1.5ml Eppendorf tubes containing, from the bottom: 100 μ l 10% trichloroacetic acid (TCA), 500 μ l of silicone oil/mineral oil mixture (4:1, v/v) and 100 μ l of digitonin solution (1.2mg/ml PBS). A 500 μ l aliquot of cell suspension was added to the digitonin top layer and then centrifuged at 10,000g for 5min. The supernatant was removed and the silicone-mineral oil mixture was aspirated. The supernatant and TCA layers were collected and stored at -80°C until analyses. Samples of these fractions, representing the cytosolic and mitochondrial compartments, respectively, were assayed for GSH and GSSG, as well as protein disulfides (protein-SSG). To verify clean separation of the mitochondrial and cytosolic compartments, aliquots of each fractions were routinely assayed for activities of their respective enzyme markers, viz., glutamate dehydrogenase and lactate dehydrogenase as previously described [20].

Determination of GSH, GSSG and protein disulfides (protein-SSG)

GSH/GSSG measurements—Soluble GSH and GSSG were determined in TCA supernatants by high-performance liquid chromatography (HPLC) according to a modified method of Reed *et al*, as we previously described [29,30]. Experimentally, samples were derivatized with 6mM iodoacetic acid and 1% 2,4-dinitrofluoro-benzene to yield the S-carboxymethyl and 2,4-dinitrophenyl derivatives, respectively. Separation of GSH and GSSG derivatives was performed on a 250mm $\times$ 4.6mm Alltech Lichrosorb NH₂ 10micron column using a Shimadzu HPLC system. GSH and GSSG contents were determined in mitochondrial and cytosolic compartments collected by digitonin fractionation (see above) and were expressed as nmol/mg protein.

Protein-SSG—Protein-bound disulfide (protein-SSG) was measured in TCA-insoluble proteins as we previously described [29]. Briefly, the mitochondrial insoluble proteins obtained from the TCA layer after digitonin fractionation were dissolved in 0.1M NaOH and neutralized to pH 8. Samples were mixed with phosphate buffer (1.5mM KH₂PO₄, 6.36mM K₂HPO₄,

1.57mM EDTA) containing 2mM DTNB and incubated for 15min at room temperature. Total protein-SH and protein-SSG were determined colorimetrically at 410nm; protein-SSG was calculated from the difference of absorbance before and after addition of saturated N-ethylmaleimide solution. Protein-SSG concentrations were expressed as nmol/mg protein.

Protein assay—Protein concentrations were determined using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's protocol.

DNA extraction and quantitative Southern analyses of mtDNA damage

Following treatment with the various chemical agents, cells were washed twice with cold PBS, harvested by scraping and processed for quantitative Southern analyses. Total DNA was extracted with a QIAamp DNA mini kit according to the manufacturer's protocol (QIAGEN) and the mtDNA was visualized using a modified quantitative Southern blot method of Dobson *et al* [31]. In brief, DNA was ethanol precipitated, treated with DNase-free RNase (1µg/ml) and digested with restriction endonuclease XhoI (5U/µg DNA) overnight at 37°C to linearize the mtDNA. DNA samples were ethanol precipitated, dissolved in TE buffer (10mM Tris, 1mM EDTA, pH8) and quantified fluorometrically in a Hoefer DyNA Quant200 fluorometer. Restriction digested DNA samples (2µg) were heated at 70°C for 15min, cooled, and treated with 0.1N NaOH at 37°C for 20min to generate single stranded DNA (ssDNA). The digested DNA samples were resolved on 0.6% agarose alkaline gels at 1.5V for 12–16h in an alkaline buffer containing 23mM NaOH and 1mM EDTA. The next day, DNA was transferred under vacuum to a Zeta-Probe GT nylon membrane and cross-linked using a Model 2400 Stratalinker (Stratagene, La Jolla, CA) at 120,000 µJ/cm². The membrane was hybridized with a ³²P-labeled human mtDNA specific probe at 56°C in a Model 131000 hybridization oven (Boekel Scientific, Feasterville, PA). The mtDNA probe was a 672bp PCR DNA fragment of the mitochondrial ATP synthase F0 subunit 6 generated from HeLa genomic DNA. The following primers were used to generate the probe: 5'-CACAACTAACCTCCTCG-3' and 5'-CTTTTGGACAGGTGGTG-3'. The mtDNA band intensity for each sample was quantified using a phosphorimager (Molecular Dynamics phosphorimager, Sunny Vale, CA) and mtDNA damage was expressed as strand break frequency (B_f) determined from the negative natural log (–ln) of the band intensities of treated and control samples as previously described [32]. The B_f value is an arbitrary number calculated using a Poisson distribution and reflects the extent of oxidant-induced mtDNA damage over control [32]. In our studies, we found that B_f values between 0.09 and 1 are essentially linearly correlated with percent fragmentation of total mitochondrial genomes, calculated as 1–(E/C) × 100] where E and C represented band intensities of treated and control DNA samples, respectively (R²=0.99; Figure 1B). From this relationship, a B_f of 1 corresponded to ~60% fragmentation of total mitochondrial genome, consistent with substantial damage.

Statistical analysis—Results are expressed as mean ± SE. Data were analyzed using a one-way ANOVA with Bonferroni corrections for multiple comparisons. P values of <0.05 were considered statistically significant.

RESULTS

MQ-induced mtDNA damage and post-MQ mtDNA recovery: Relationship to cytosolic and mitochondrial GSH redox and protein disulfide status

Figure 1 shows effect of MQ exposure on mtDNA damage in NCM460 cells. Treatment of cells with MQ ranging from 50 to 400 µM caused dose-dependent increases in small DNA fragments that paralleled the decreases in intact mtDNA, consistent with mtDNA damage. DNA break frequencies (B_f) of 0.1, 1.4, 1.9 and 1.8, corresponding to 9%, 76%, 86%, and 83% fragmentation of total mitochondrial genomes (Figure 1A&B), were elicited by 50, 100, 200,

and 400 μ M MQ respectively. Since MQ exhibits redox cycling properties, the results suggest that MQ induces colonic mtDNA damage by an oxidative, redox-dependent mechanism. To examine the extent that cellular redox status was altered, we quantified GSH and GSSG contents in the cytosolic and mitochondrial compartments. Figure 2A shows that MQ caused significant time-dependent decreases in cytosolic GSH (10–30min), with parallel increases in GSSG beginning at as early as 5min. This resulted in a shift in the GSH-to-GSSG ratio in favor of GSSG at all time points. MQ similarly elicited time-dependent decreases in mtGSH (10–30min), increases in mtGSSG (30min) and overall decreases in the mtGSH-to-mtGSSG ratio (10–30min) (Figure 2B). These results indicate that MQ-induced mtDNA damage in NCM460 cells is associated with both cytosolic and mitochondrial GSH redox imbalance. In contrast, MQ caused significant protein disulfide (protein-SSG) formation only in the mitochondria, but not in the cytosol (Figure 2C), indicating that MQ mediated preferential oxidation of mitochondrial protein thiols, and suggests that overall losses of mtGSH and protein thiol redox balance could be important contributors to oxidative mtDNA damage.

The kinetic relationship between cytosolic and mitochondrial GSH and post-MQ recovery of mtDNA integrity was determined after 1h exposure to 200 μ M MQ followed by 2 to 24h MQ wash out. This level of MQ exposure resulted in mtDNA break frequency (B_f) of 1.9 (86% fragmentation, Figure 1). The results in Figure 3A show that MQ-induced mtDNA damage remained significant at 2h after removal of MQ. By 4h, levels of mtDNA returned to pre-MQ values and were maintained at 24h. This finding suggests that removal of the oxidative challenge resulted in full recovery of baseline mtDNA within hours, consistent with an efficient repair mechanism following oxidative stress [31,33]. GSH levels were determined in parallel and the results show that 1h treatment with MQ induced significant GSH decreases in the mitochondrial and cytosolic pools, and removal of MQ restored GSH to pre-oxidant levels at 2h in both compartments (Figure 3B). Notably, the kinetics of GSH restoration preceded full recovery of mtDNA by approximately 2h (Figure 3A), suggesting the interesting possibility that a restored GSH status could facilitate the repair of oxidative mtDNA lesions.

Attenuation of MQ-induced mtDNA damage by N-acetylcysteine (NAC) and overexpression of mtOgg1

To further explore the relationship between cellular GSH redox status and oxidative mtDNA damage, cells were pre-treated with NAC, a thiol antioxidant. Figure 4A shows that at 100 μ M, MQ induced significant damage to mtDNA (B_f of 0.7, corresponding to 51% fragmentation, Figure 1B) that was attenuated by 2mM NAC pretreatment (B_f of 0.09, corresponding to 9% fragmentation, Figure 1B), validating that mtDNA susceptibility to oxidative challenge is redox sensitive. The same dose of NAC was equally effective in protecting against greater oxidative mtDNA damage induced by 200 μ M MQ (B_f of 1.3 versus 0.02; Figure 4A). Measurements of GSH/GSSG revealed that, regardless of dose, MQ induced similar mtGSH decreases and mtGSSG increases at 30min with resultant severe mitochondrial GSH/GSSG redox imbalance (Figure 4B). Cells pretreated with NAC exhibited higher baseline mtGSH levels that were decreased only at 200 μ M MQ. Importantly, NAC pretreatment significantly attenuated MQ-induced mtGSSG increases (Figure 4B, top right panel), but did not fully restore baseline mitochondrial GSH/GSSG balance (Figure 4B, bottom panel), suggesting that the blunting of mtDNA damage by NAC was associated with its capacity to decrease mtGSSG *per se*.

To test whether 8-oxoG is a biologically important mtDNA lesion [34] induced by MQ, HT-29 cells were stably transfected with mitochondria-targeted DNA glycosylase, Ogg1, and exposed to MQ. The results show that the extent of mtDNA damage induced by MQ at 100 μ M and 200 μ M in WT (B_f of 0.7, 0.8, respectively, Figure 5A) and vector control (data not shown) were markedly attenuated in mtOgg1 overexpressing cells (1.5 fold over WT and vector

control) (B_f of 0.5, 0.4, respectively, Figure 5A), consistent with 8-oxoG being a major oxidized lesion. Moreover, a higher mtOgg1 overexpression (2.7 fold over vector control) resulted in essentially complete protection against MQ-induced mtDNA damage (B_f of 0.5 and 0.01 in vector control and Ogg1 over-expressors, respectively, Figure 5B). This correspondence of attenuated mtDNA damage with mtOgg1 overexpression suggests that elevated mtOgg1 likely prevented further mtDNA damage and allowed for complete repair.

Inhibition of GSH synthesis and mtGSH transport exacerbates MQ-induced mtDNA damage

Given the role of cytosolic GSH in controlling mtGSH homeostasis [35], we tested the influence of altered cytosolic GSH pool in MQ-induced mtDNA damage by inhibiting cytosolic GSH synthesis with BSO. The results in Figure 6A show that BSO at 5 and 20 μ M dose-dependently decreased cytosolic GSH without significantly altering GSSG levels, consistent with diminished GSH synthesis. In comparison, only 20 μ M BSO induced significant decreases in mtGSH (Figure 6B), indicating that mild reduction in cytosolic GSH synthesis with 5 μ M BSO did not adversely alter mtGSH status. As it was with cytosolic GSSG, BSO at 5 and 20 μ M exerted minimal effects on mtGSSG (Figure 6B). In accordance with BSO-induced decreases in cytosolic and mitochondrial GSH, we found an exacerbation of mtDNA damage in BSO-treated cells caused by 30min exposure to 100 μ M MQ (B_f of 1.1 and 1.4, for 5 μ M and 20 μ M BSO compared to B_f of 0.4 in the absence of BSO) (Figure 7A). Cytosolic GSH was dose-dependently lower in BSO-treated cells challenged with MQ as compared to non-BSO treated cells (Figure 7B, left panel). Total cytosolic GSSG levels were also decreased in these cells (Figure 7B, right panel), consistent with overall reduction in cellular GSH due to inhibition of synthesis. MQ induced significant mtGSSG formation (Figure 7C, right panel) and a 60% decrease in mtGSH (Figure 7C, left panel), a greater loss than cytosolic GSH under these conditions. Interestingly, BSO treatment did not result in further decrease in mtGSH (Figure 7C, left panel) suggesting that maximal mtGSH depletion was already achieved by MQ challenge. Consistent with the inhibition of GSH synthesis and overall decrease in total cytosolic GSH, the mitochondrial GSH pool (GSH plus GSSG) was lower than non-BSO treated cells regardless of MQ exposure (Figure 7C). Collectively, these results indicate that maintenance of the cytosolic GSH pool is important in mtDNA protection. The protective mechanism is consistent with the role of cytosolic GSH in maintaining mitochondrial GSH supply.

Since the cytosolic GSH pool is critical in supporting normal mtGSH status through mtGSH uptake, we investigated the contribution of mtGSH transport and mitochondrial matrix GSH to MQ-induced mtDNA damage. MtGSH uptake was manipulated by pharmacological or genetic means. Combined inhibition of DIC and OGC carriers by BM and PS, respectively, enhanced mtDNA damage induced by 30min exposure to 100 μ M MQ (lanes 2 and 4, Figure 8A). Interestingly, treatment of cells with combined BM/PS alone caused some mtDNA damage which was exacerbated by MQ (lanes 3 and 4, Figure 8A). The small extent of damage to mtDNA by BM/PS alone suggests some perturbation of basal mitochondrial function, consistent with our recent finding that BM/PS caused a measurable, albeit insignificant decrease in cellular ATP; however BM/PS *per se* did not induce NCM460 cell apoptosis in the absence of MQ [20]. Collectively, the results demonstrate that compromised mtGSH transport enhances mtDNA susceptibility which can be exacerbated by oxidative challenge.

To further define a role for mtGSH transport, MQ-induced mtDNA damage was examined in HT-29 cells that stably overexpress the wildtype OGC carrier. HT-29 cells were treated with 100 μ M MQ for 1h, since these cells were more resistant to MQ [20]. The results in Figure 8B show that MQ induced substantial mtDNA damage in parent (WT) HT-29 cells (B_f , 0.4) which was exacerbated by inhibition of mtGSH transport with BM/PS (B_f , 0.7), responses that were similar to those of NCM460 cells (Figure 8A). In contrast, mtDNA was protected against MQ

challenge in HT-29 clones that overexpress the WT-OGC transporter (B_f , 0.1; Figure 8C), indicating that upregulation of the OGC carrier for increased mtGSH uptake effectively conferred protection of mtDNA against oxidative stress. Taken together, these results support our hypothesis that mtGSH transport is an important contributor to mtDNA preservation during oxidative challenge.

DISCUSSION

The results in the current study provide evidence that oxidative damage to mtDNA and its recovery are sensitive to cell GSH; notably the mtGSH pool plays an important role in preserving mtDNA integrity during oxidative stress. Our conclusion is supported by several lines of evidence. First, MQ elicited oxidative mtDNA damage that was associated with increased mitochondrial and cytosolic GSSG and mitochondrial protein disulfide, and was prevented by NAC, indicating that mtDNA damage is redox sensitive. Second, inhibition of GSH synthesis with BSO exacerbated mtDNA damage in accordance with decreased cytosolic GSH and mtGSH, which supports a role for cytosolic GSH in preserving mtGSH and mtDNA. Significantly, the blockade of mtGSH transport by pharmacologic agents exaggerated mtDNA damage that was prevented by overexpression of the mtGSH transporter OGC, thus underscoring the importance of cytosol-to-mitochondria GSH import in mtDNA integrity. These collective findings support our central hypothesis that mtGSH is a pivotal player in MQ-mediated mtDNA damage. Finally, post oxidative recovery of mtDNA was preceded by restored baseline GSH levels, suggesting that cellular GSH status not only impact oxidant-mediated mtDNA damage, but additionally could influence its repair.

Our finding that the oxidation of the cytosolic and mitochondrial GSH compartments preceded MQ-induced mtDNA damage is consistent with a temporal link between disruption of cellular GSH homeostasis with vulnerability of mtDNA to oxidative challenge. However, preferential oxidation of mitochondrial proteins by MQ suggests that impairment of mitochondrial redox balance could have a greater impact on the level of mtDNA damage during oxidative stress. A direct relationship between mtGSH and mtDNA damage was supported by the findings that blockade of mtGSH uptake potentiated, while overexpression of mtGSH OGC carrier nullified the effect of MQ on mtDNA damage (Figure 8). Since mitochondria are implicated as major sites for quinone redox cycling [20], mitochondria-derived ROS would elicit mtGSH redox imbalance and consequent oxidative injury to mtDNA. Previous studies have shown that the depletion of mtGSH by diethyl maleate to 70–80% of the control value potentiated radiation-induced mtDNA damage [36], and that oxidation of mtGSH increased age-associated levels of 8-oxoG in mice and rats [37]. In hepatocytes isolated from hemin-treated rats, a decrease in mtGSH following exposure to *t*BH was associated with a rapid increase in mtDNA deletion, a marker of oxidative damage [38]. Interestingly, in isolated rat mitochondria, Giulive and Cadenas (1998) demonstrated that an increased in mtDNA oxidation as measured by 8-oxoG, was associated with elevated mtGSH [39]. The authors concluded that mtGSH can function as an electron donor in DNA oxidation because of the presence Cu^{2+} in the incubation reaction [39]. Thus, the significance of the findings in isolated mitochondria regarding the central role of mtGSH in oxidative mtDNA damage or mtDNA protection in cells is unclear. However, it is clear from the current study that in colonic epithelial cells, mtGSH is critical for the protection of mtDNA against oxidative stress.

The result that NAC was highly effective in protection against oxidative mtDNA damage underscores a redox mechanism in mtDNA damage. The mechanism of how mtGSH influences oxidative mtDNA damage is unresolved. Our results suggest that mtGSH oxidation is likely to be a key contributor to the formation of mtDNA oxidative lesions. Consistent with this interpretation, NAC protection against mtDNA damage was correlated with significant attenuation in mtGSSG; interestingly, NAC did not completely restore mitochondrial GSH/

GSSG redox imbalance (Figure 4B), indicating a non-linear relationship between oxidative mtDNA damage and the mtGSH redox state. It is possible that oxidative damage to mtDNA occurred when the mtGSH/GSSG redox status fell below a critical threshold level, and that NAC, by attenuating mtGSSG formation, maintained the redox status above this threshold. Alternately, the extent of oxidative mtDNA damage may also be a direct result of the increase in mtGSSG, which NAC effectively prevented. Thus, by attenuating mtGSSG, NAC could function to lower the oxidative burden on the mitochondria and thereby preserves mtDNA integrity. Interestingly, GSH oxidation was not correlated with basal (steady-state) levels of DNA base modification in a variety of mammalian cell types in the absence of oxidative challenge [40]. Indeed, in AS52 Chinese hamster cells the administration of NAC or cysteine ester not only did not prevent basal damage to DNA, but appear to promote base oxidation [40].

Generally, the maintenance of a reduced mtGSH redox environment would favor mtDNA protection, given the role of mtGSH in quenching mitochondria-derived ROS. Additionally, mtGSH could influence mtDNA repair. A possible connection between mtGSH and post-MQ repair of mtDNA is suggested by the rapid recovery of cytosolic and mitochondrial GSH to pre-oxidant levels that preceded mtDNA recovery (Figure 3). Precisely what role mtGSH plays in the repair process is unclear. One possibility is that mtGSH modulates the function of mtDNA repair enzymes. Oxidative damaged mtDNA are recognized and removed by mitochondrial BER enzymes. Among the main mammalian DNA glycosylases [41] Ogg1 cleaves 8-oxoG [9]. Our results show that the overexpression of mtOgg1 afforded significant protection against MQ-induced oxidative mtDNA damage, indicating that 8-oxoG was, in fact, a major oxidative byproduct. Previous studies have similarly demonstrated the protection of mtDNA from damage induced by high levels of MQ (400 μ M) in mtOgg1 overexpressing HeLa cells [31] that was associated with increased mtOgg1 repair activity [34]. Age-associated decrease in mtOgg1 activity [42] and its mitochondrial localization [43] suggests that increased oxidative stress would comprise both the efficiency and capacity of the repair of oxidized mtDNA. Ongoing studies are underway to examine the effect of mtGSH redox status on mtOgg1 activity and if GSH-dependent S-glutathionylation is important as a post translational mechanism for the regulation of mtOgg1 and other important repair enzymes.

The importance of the cytosolic GSH compartment in mtDNA preservation is notable as reflected in its capacity to preserve the mt-matrix GSH pool. The exacerbation of MQ-induced mtDNA damage by inhibition of *de novo* cytosolic GSH synthesis (Figure 7) is consistent with a crucial role of cytosolic GSH in mtGSH homeostasis and the protection of mtDNA against oxidant challenge. In agreement, previous studies have reported an exacerbation of oxidative DNA damage after GSH depletion with BSO [44,45]. Moreover, Hollins *et al.* demonstrated that decreased cellular GSH levels in lymphocytes exposed to *t*BH were correlated with increases in ROS and mtDNA modification, measured as changes in mtDNA copy number and a 4977-bp deletion from the mitochondrial genome [46]. In other studies in 3Y1 fibroblasts *in vitro* and in rat mammary gland *in vivo*, the oxidation of cellular GSH was found to precede nuclear DNA fragmentation, suggesting a possible link between cellular GSH and nuclear DNA damage.

The significance of mtGSH in colonic cell survival is underscored by our recent findings that the preservation of mtGSH transport and hence mt-matrix GSH is critical to protecting NCM460 cells against MQ challenge and promoting cell survival [20]. Specifically, the maintenance of a reduced mtGSH redox environment was essential in preserving mitochondrial ATP production and the mitochondrial membrane potential [20]. Importantly, the current study demonstrates that mtGSH also functions in the protection of mtDNA integrity, particularly during oxidative challenge. The biological consequence of oxidative mtDNA damage in cell apoptosis has been demonstrated by LeDoux and coworkers [31]. Precisely how damaged

mtDNA signals cell apoptosis during acute oxidative challenge is not completely understood. One possibility may be related to apoptotic signaling via enhanced superoxide generation secondary to mtDNA damage and mitochondrial failure resulting from decreased expression of mitochondrial genome-encoded respiratory proteins. In support of this scenario, recent studies demonstrated that ROS-mediated oxidative mtDNA damage can signal cellular apoptosis by causing impairment of mitochondrial protein expression, complex I dysfunction and increased ROS production that ultimately triggered the collapse of mitochondrial membrane potential [48]. Previous studies have also implicated enhanced ROS formation in apoptotic signaling induced by oxidative damaged mtDNA [49], but its quantitative importance as a major mechanism in oxidative cell killing remains to be validated.

Acknowledgments

This study was supported by a grant, DK44510 from the National Institutes of Health.

References

1. Yakes FM, Van Houten B. Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc Natl Acad Sci U S A* 1997;94:514–9. [PubMed: 9012815]
2. Richter C, Park JW, Ames BN. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc Natl Acad Sci U S A* 1988;85:6465–7. [PubMed: 3413108]
3. Klungland A, Rosewell I, Hollenbach S, Larsen E, Daly G, Epe B, Seeberg E, Lindahl T, Barnes DE. Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. *Proc Natl Acad Sci U S A* 1999;96:13300–5. [PubMed: 10557315]
4. Ballinger SW, Patterson C, Yan CN, Doan R, Burow DL, Young CG, Yakes FM, Van Houten B, Ballinger CA, Freeman BA, Runge MS. Hydrogen peroxide- and peroxynitrite-induced mitochondrial DNA damage and dysfunction in vascular endothelial and smooth muscle cells. *Circ Res* 2000;86:960–6. [PubMed: 10807868]
5. Ide T, Tsutsui H, Hayashidani S, Kang D, Suematsu N, Nakamura K, Utsumi H, Hamasaki N, Takeshita A. Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. *Circ Res* 2001;88:529–35. [PubMed: 11249877]
6. Ozawa T. Oxidative damage and fragmentation of mitochondrial DNA in cellular apoptosis. *Biosci Rep* 1997;17:237–50. [PubMed: 9337479]
7. LeDoux SP, Wilson GL, Beecham EJ, Stevnsner T, Wassermann K, Bohr VA. Repair of mitochondrial DNA after various types of DNA damage in Chinese hamster ovary cells. *Carcinogenesis* 1992;13:1967–73. [PubMed: 1423864]
8. LeDoux SP, Wilson GL. Base excision repair of mitochondrial DNA damage in mammalian cells. *Prog Nucleic Acid Res Mol Biol* 2001;68:273–84. [PubMed: 11554303]
9. Nishimura S. Involvement of mammalian OGG1(MMH) in excision of the 8- hydroxyguanine residue in DNA. *Free Radic Biol Med* 2002;32:813–21. [PubMed: 11978483]
10. Henle ES, Linn S. Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. *J Biol Chem* 1997;272:19095–8. [PubMed: 9235895]
11. Hamilton ML, Van Remmen H, Drake JA, Yang H, Guo ZM, Kewitt K, Walter CA, Richardson A. Does oxidative damage to DNA increase with age? *Proc Natl Acad Sci U S A* 2001;98:10469–74. [PubMed: 11517304]
12. Stuart JA, Brown MF. Mitochondrial DNA maintenance and bioenergetics. *Biochim Biophys Acta* 2006;1757:79–89. [PubMed: 16473322]
13. Harrison JF, Hollensworth SB, Spitz DR, Copeland WC, Wilson GL, LeDoux SP. Oxidative stress-induced apoptosis in neurons correlates with mitochondrial DNA base excision repair pathway imbalance. *Nucleic Acids Res* 2005;33:4660–71. [PubMed: 16107556]
14. Meredith MJ, Reed DJ. Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J Biol Chem* 1982;257:3747–53. [PubMed: 7061508]

15. Hallberg E, Rydstrom J. Selective oxidation of mitochondrial glutathione in cultured rat adrenal cells and its relation to polycyclic aromatic hydrocarbon-induced cytotoxicity. *Arch Biochem Biophys* 1989;270:662–71. [PubMed: 2539778]
16. Lluís JM, Morales A, Blasco C, Colell A, Mari M, Garcia-Ruiz C, Fernandez-Checa JC. Critical role of mitochondrial glutathione in the survival of hepatocytes during hypoxia. *J Biol Chem* 2005;280:3224–32. [PubMed: 15548523]
17. Olafsdottir K, Reed DJ. Retention of oxidized glutathione by isolated rat liver mitochondria during hydroperoxide treatment. *Biochim Biophys Acta* 1988;964:377–82. [PubMed: 3349102]
18. Fernandez-Checa JC, Garcia-Ruiz C, Ookhtens M, Kaplowitz N. Impaired uptake of glutathione by hepatic mitochondria from chronic ethanol-fed rats. Tracer kinetic studies in vitro and in vivo and susceptibility to oxidant stress. *J Clin Invest* 1991;87:397–405. [PubMed: 1991826]
19. Zhao P, Kahlhorn TF, Slattery JT. Selective mitochondrial glutathione depletion by ethanol enhances acetaminophen toxicity in rat liver. *Hepatology* 2002;36:326–35. [PubMed: 12143040]
20. Circu MLRC, Maloney R, Moyer PM, Aw TY. Contribution of mitochondrial GSH transport to matrix GSH status and colonic epithelial cell apoptosis. *Free Rad Biol Med* 2008;44:768–778. [PubMed: 18267208]
21. Chen Z, Lash LH. Evidence for mitochondrial uptake of glutathione by dicarboxylate and 2-oxoglutarate carriers. *J Pharmacol Exp Ther* 1998;285:608–18. [PubMed: 9580605]
22. Chen Z, Putt DA, Lash LH. Enrichment and functional reconstitution of glutathione transport activity from rabbit kidney mitochondria: further evidence for the role of the dicarboxylate and 2-oxoglutarate carriers in mitochondrial glutathione transport. *Arch Biochem Biophys* 2000;373:193–202. [PubMed: 10620338]
23. Lash LH. Mitochondrial glutathione transport: physiological, pathological and toxicological implications. *Chem Biol Interact* 2006;163:54–67. [PubMed: 16600197]
24. Moyer MP, Manzano LA, Merriman RL, Stauffer JS, Tanzer LR. NCM460, a normal human colon mucosal epithelial cell line. *In Vitro Cell Dev Biol Anim* 1996;32:315–7. [PubMed: 8842743]
25. Sahi J, Nataraja SG, Layden TJ, Goldstein JL, Moyer MP, Rao MC. Cl⁻ transport in an immortalized human epithelial cell line (NCM460) derived from the normal transverse colon. *Am J Physiol* 1998;275:C1048–57. [PubMed: 9755058]
26. Circu ML, Stringer S, Rhoads CA, Moyer MP, Aw TY. The role of GSH efflux in staurosporine-induced apoptosis in colonic epithelial cells. *Biochem Pharmacol* 2009;77:76–85. [PubMed: 18840413]
27. Aw TY, Andersson BS, Jones DP. Mitochondrial transmembrane ion distribution during anoxia. *Am J Physiol* 1987;252:C356–61. [PubMed: 3565556]
28. Andersson BS, Jones DP. Use of digitonin fractionation to determine mitochondrial transmembrane ion distribution in cells during anoxia. *Anal Biochem* 1985;146:164–72. [PubMed: 3993928]
29. Okouchi M, Okayama N, Aw TY. Differential susceptibility of naive and differentiated PC-12 cells to methylglyoxal-induced apoptosis: influence of cellular redox. *Curr Neurovasc Res* 2005;2:13–22. [PubMed: 16181096]
30. Reed DJ, Babson JR, Beatty PW, Brodie AE, Ellis WW, Potter DW. High-performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide, and related thiols and disulfides. *Anal Biochem* 1980;106:55–62. [PubMed: 7416469]
31. Dobson AW, Xu Y, Kelley MR, LeDoux SP, Wilson GL. Enhanced mitochondrial DNA repair and cellular survival after oxidative stress by targeting the human 8-oxoguanine glycosylase repair enzyme to mitochondria. *J Biol Chem* 2000;275:37518–23. [PubMed: 10982789]
32. Pettepher CC, LeDoux SP, Bohr VA, Wilson GL. Repair of alkali-labile sites within the mitochondrial DNA of RINr 38 cells after exposure to the nitrosourea streptozotocin. *J Biol Chem* 1991;266:3113–7. [PubMed: 1825207]
33. Rachek LI, Grishko VI, Musiyenko SI, Kelley MR, LeDoux SP, Wilson GL. Conditional targeting of the DNA repair enzyme hOGG1 into mitochondria. *J Biol Chem* 2002;277:44932–7. [PubMed: 12244119]
34. Dobson AW, Grishko V, LeDoux SP, Kelley MR, Wilson GL, Gillespie MN. Enhanced mtDNA repair capacity protects pulmonary artery endothelial cells from oxidant-mediated death. *Am J Physiol Lung Cell Mol Physiol* 2002;283:L205–10. [PubMed: 12060578]

35. Schnellmann RG. Renal mitochondrial glutathione transport. *Life Sci* 1991;49:393–8. [PubMed: 1857187]
36. Morales A, Miranda M, Sanchez-Reyes A, Biete A, Fernandez-Checa JC. Oxidative damage of mitochondrial and nuclear DNA induced by ionizing radiation in human hepatoblastoma cells. *Int J Radiat Oncol Biol Phys* 1998;42:191–203. [PubMed: 9747838]
37. de la Asuncion JG, Millan A, Pla R, Bruseghini L, Esteras A, Pallardo FV, Sastre J, Vina J. Mitochondrial glutathione oxidation correlates with age-associated oxidative damage to mitochondrial DNA. *Faseb J* 1996;10:333–8. [PubMed: 8641567]
38. Suliman HB, Carraway MS, Velsor LW, Day BJ, Ghio AJ, Piantadosi CA. Rapid mtDNA deletion by oxidants in rat liver mitochondria after hemin exposure. *Free Radic Biol Med* 2002;32:246–56. [PubMed: 11827750]
39. Giulivi C, Cadenas E. The role of mitochondrial glutathione in DNA base oxidation. *Biochim Biophys Acta* 1998;1366:265–74. [PubMed: 9814840]
40. Will O, Mahler HC, Arrigo AP, Epe B. Influence of glutathione levels and heat-shock on the steady-state levels of oxidative DNA base modifications in mammalian cells. *Carcinogenesis* 1999;20:333–7. [PubMed: 10069473]
41. Scharer OD, Jiricny J. Recent progress in the biology, chemistry and structural biology of DNA glycosylases. *Bioessays* 2001;23:270–81. [PubMed: 11223884]
42. Imam SZ, Karahalil B, Hogue BA, Souza-Pinto NC, Bohr VA. Mitochondrial and nuclear DNA-repair capacity of various brain regions in mouse is altered in an age-dependent manner. *Neurobiol Aging* 2006;27:1129–36. [PubMed: 16005114]
43. Szczesny B, Hazra TK, Papaconstantinou J, Mitra S, Boldogh I. Age-dependent deficiency in import of mitochondrial DNA glycosylases required for repair of oxidatively damaged bases. *Proc Natl Acad Sci U S A* 2003;100:10670–5. [PubMed: 12960370]
44. Green RM, Graham M, O'Donovan MR, Chipman JK, Hodges NJ. Subcellular compartmentalization of glutathione: correlations with parameters of oxidative stress related to genotoxicity. *Mutagenesis* 2006;21:383–90. [PubMed: 17012304]
45. Hodges NJ, Green RM, Chipman JK, Graham M. Induction of DNA strand breaks and oxidative stress in HeLa cells by ethanol is dependent on CYP2E1 expression. *Mutagenesis* 2007;22:189–94. [PubMed: 17284772]
46. Hollins DL, Suliman HB, Piantadosi CA, Carraway MS. Glutathione regulates susceptibility to oxidant-induced mitochondrial DNA damage in human lymphocytes. *Free Radic Biol Med* 2006;40:1220–6. [PubMed: 16545690]
47. Garrido N, Perez-Martos A, Faro M, Lou-Bonafonte JM, Fernandez-Silva P, Lopez-Perez MJ, Montoya J, Enriquez JA. Cisplatin-mediated impairment of mitochondrial DNA metabolism inversely correlates with glutathione levels. *Biochem J* 2008;414:93–102. [PubMed: 18426391]
48. Ricci C, Pastukh V, Leonard J, Turrens J, Wilson G, Schaffer D, Schaffer SW. Mitochondrial DNA damage triggers mitochondrial-superoxide generation and apoptosis. *Am J Physiol Cell Physiol* 2008;294:C413–22. [PubMed: 18077603]
49. Ho R, Racheck LI, Xu Y, Kelley MR, LeDoux SP, Wilson GL. Yeast apurinic/aprimidinic endonuclease Apn1 protects mammalian neuronal cell line from oxidative stress. *J Neurochem* 2007;102:13–24. [PubMed: 17506861]

Figure 1.

MQ dose-dependently increase mtDNA damage. **A:** NCM460 cells were exposed to 50, 100, 200 and 400 μM MQ for 1h; thereafter, cells were harvested and processed for quantitative Southern analyses using a human mtDNA specific probe as described in Methods. Intact mtDNA (arrow) ran at the top of the 0.6% agarose alkaline gels while damaged mtDNA was represented by small DNA fragments and is expressed as strand break frequency (B_f) [31]. DNA samples were run in duplicates for untreated controls and each MQ dose. One representative of 3 gels is shown. **B:** Relationship between % fragmentation of mitochondrial genomes and break frequency, B_f generated using Southern analyses from Figures 1, 3, 4, 5, 7, and 8.

Figure 2.

Kinetics of MQ-induced changes in cytosolic and mitochondrial GSH, GSSG and protein disulfide (Pr-SSG). NCM460 cells were treated with 100 μ M MQ for 0–30 min and cytosolic and mitochondrial fractions were separated by digitonin fractionation. The contents of GSH, GSSG and protein-SSG in each compartment were determined as described in Materials and Methods. **A, B:** cytosolic, and mitochondrial fractions, respectively. Panels are respectively: left, GSH; middle, GSSG; right, GSH-to-GSSG ratio. Concentrations of GSH and GSSG are expressed as nmol/mg protein and presented as mean $\pm$ S.E. for 4 separate experiments performed in duplicates. Statistical differences between 0 min and other time points are: * p < 0.05; ** p < 0.01; *** p < 0.001. **C,** Protein-SSG (Pr-SSG) content. Left and right panels are cytosolic and mitochondrial Pr-SSG, respectively. Results are expressed as nmol/mg protein and presented as mean $\pm$ SE for 3 separate experiments performed in duplicates. * p < 0.05 versus untreated control.

Figure 3.

Restoration of mitochondrial and cytosolic GSH precedes post-MQ recovery of mtDNA integrity. **A:** NCM460 cells were exposed to 200 μ M MQ for 1h, and thereafter the incubation media was replaced with fresh MQ-free media. At 2, 4, and 24h, cells were harvested for genomic DNA extraction and mtDNA was determination by quantitative Southern blot analyses as described in Methods. DNA samples were run in duplicates and one representative of 3 gels is shown. mtDNA damage is expressed as strand break frequency (B_f). **B:** In parallel experiments, GSH contents in cytosolic (left panel) and mitochondrial (right panel) compartments were determined. GSH concentrations are expressed as nmol/mg protein and presented as mean $\pm$ SE of 3 separate experiments performed in duplicates. * $p < 0.05$ versus untreated control.

Figure 4.

Effect of N-acetylcysteine (NAC) on MQ induced mtDNA damage and the relationship to mitochondrial GSH/GSSG redox status. **A:** NCM460 cells were plated in T25 flasks and pretreated with 2 mM NAC (30 min) prior to MQ exposure at 100 and 200 μ M for 30 min. Total DNA was extracted and mtDNA was quantified by Southern blot analysis as described in Methods. mtDNA damage is expressed as strand break frequency (B_f). One representative of 3 gels is shown. **B:** In parallel experiments, mitochondrial fractions were obtained after selective permeabilization of plasma membrane with digitonin as described in Methods. Mt-matrix GSH and GSSG concentrations were determined. Panels are respectively: left, mtGSH; right, mtGSSG; bottom, mtGSH-to-mtGSSG ratio. Results are expressed as means $\pm$ SE of three separate experiments performed in duplicates. * $p < 0.001$ versus controls (i.e., respective 0 μ M MQ minus or plus NAC); # $p < 0.001$ versus 0 μ M MQ minus NAC.

Figure 5.

Overexpression of mtOgg1 protects against MQ-induced mtDNA damage. HT-29 WT cells as well as clones overexpressing the empty pcDNA3 vector or mtOgg1 (1.5-fold above WT and vector control [A] and 2.7 fold above vector control [B]) were exposed to MQ, (100 μ M [A, B] and 200 μ M [A] for 1h. Genomic DNA was extracted and mtDNA was determined by quantitative Southern blot analyses. DNA samples were run in duplicates and mtDNA damage is expressed as strand break frequency (B_f). One representative of 3 gels is shown. In [A], band intensities of intact mtDNA were similar for WT and pcDNA3 vector controls (data not shown). Similarly, intact mtDNA band intensities of WT (data not shown) were similar to pcDNA3 vector controls in [B].

Figure 6.

Effect of BSO inhibition of GSH synthesis on cytosolic and mitochondrial GSH and GSSG status. NCM460 cells were exposed to 100µM MQ for 30min without or with pretreatment for 24h with BSO (5 or 20µM). GSH and GSSG concentrations in the cytosolic and mitochondrial compartments were determined following digitonin fractionation as described in Methods. Results are expressed as nmol/mg protein and presented as mean $\pm$ SE of 3 separate experiments performed in duplicates. (A) Cytosolic GSH and GSSG; (B) mitochondrial GSH and GSSG. In each instance, * p <0.05 versus untreated control.

Figure 7.

Inhibition of GSH synthesis exacerbates MQ-induced mtDNA damage in association with decreased cytosolic and mitochondrial GSH. NCM460 cells were exposed to 100 μ M MQ for 30min without or with pretreatment for 24h with BSO (5 or 20 μ M). **A**, Total DNA was extracted and probed for mtDNA by Southern blot. mtDNA damage is expressed as strand break frequency (B_f) and one representative of 3 gels is shown. GSH and GSSG concentrations were determined in the cytosol (**B**) and mitochondria (**C**) were determined following digitonin permeabilization. Results are expressed as nmol/mg protein and presented as mean $\pm$ SE of 3 separate experiments performed in duplicates. In each instance, * p <0.05 versus untreated control; # p <0.05 versus MQ alone.

Figure 8.

MQ-induced mtDNA damage correlates with mtGSH transport. NCM460 (A) or HT-29 (B) cells exposed to 100 μ M MQ for 30min without or with pretreatment for 1h with BM/PS (20mM). C: HT-29 cells overexpressing the OGC carrier were exposed to 100 μ M MQ for 30min. In each instance, total DNA was extracted and mtDNA was quantified by Southern blot analysis as described in Methods. mtDNA damage is expressed as strand break frequency (B_f). One representative of 3 gels is shown.