A catalytic antioxidant metalloporphyrin blocks hydrogen peroxide-induced mitochondrial DNA damage

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ABSTRACT

Reactive oxygen species (ROS) have been implicated as the cause of cumulative damage to DNA, proteins and lipids that can ultimately result in cell death. A common problem when measuring oxidative DNA damage has been the introduction of modifications in the native state of the molecule by many DNA isolation methods. We circumvented this problem by employing direct PCR (DPCR) of whole cell lysates. DPCR of mouse lung fibroblasts performed better than PCRs containing template acquired by phenol/chloroform extraction or a commercially available genomic DNA isolation kit. We investigated the direct use of whole cell preparations in the polymerase chain reaction (PCR) to detect hydrogen peroxide (H$_2$O$_2$)-mediated DNA damage. We observed a concentration-dependent decrease in amplification efficiency of a 4.3 kb mitochondrial (mt)DNA target in H$_2$O$_2$-treated mouse lung fibroblasts (MLFs). At low doses the efficiency of amplification returns to control levels over 24 h. We detected no change in amplification efficiency of a plasmid control containing our mtDNA target under any of the culture conditions employed in these studies. Treatment of MLFs with the catalytic antioxidant manganese(III) meso-tetrakis(4-benzoic acid)porphyrin (MnTBAP) attenuates the effects of H$_2$O$_2$ exposure. When quantitated with an external standard the use of DPCR in tandem with a PCR amplification efficiency assay provides a powerful approach to assess oxidative mtDNA damage.

INTRODUCTION

One consequence of aerobic metabolism is the production of reactive oxygen species (ROS) that can damage proteins, lipids and DNA. To abate the deleterious effects of ROS, cells employ defensive enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. Under certain conditions, these defenses can be overwhelmed, allowing ROS to oxidize nucleic acids, proteins and lipids (1–3). Oxidative stress will often create lesions in DNA that are readily repaired by cells (4,5). However, when these repair mechanisms fail, mutations may be introduced (6,7). Indeed, oxidative DNA damage has been implicated as the cause of some forms of cancer (8). Oxidative damage to DNA is most evident in the mitochondrial genome. DNA damage seems to persist longer and occur at a higher frequency in mitochondrial (mt)DNA than in nuclear (n)DNA (9,10). The reasons for this may include fewer mtDNA repair enzymes, the lack of histones protecting the DNA and the proximity of mtDNA to a strong source of ROS. Various aberrations of DNA have been observed at a high level in the mitochondrial genome of aged animals (11).

The role of ROS in some disease states has led to the development of synthetic catalytic SOD mimetics. Manganese(III) meso-tetakis(4-benzoic acid)porphyrin (MnTBAP) is a stable metalloporphyrin which catalyzes the dismutation of superoxide (12,13), hydrogen peroxide (14) and scavenging of peroxynitrite (15). MnTBAP is also known to substitute in vivo in transgenic mice lacking mitochondrial MnSOD (16). Given the broad range of antioxidant activities of metalloporphyrins it was of interest to examine their activity in a model of ROS-induced DNA damage.

The polymerase chain reaction (PCR) enables the detection of rare molecular species from a complex, heterogeneous population. Many lesions such as DNA deletions and substitutions often occur in small subsets of cells that would make them impossible to characterize without enrichment by PCR (17). However, aberrations that directly affect the chemical nature of the molecular subunits of DNA must be assayed by other methods (18). A recently developed technique takes advantage of chemical modifications to DNA that are not recognized by thermostable DNA polymerases and therefore reduce the total amplification efficiency of the PCR (19). This phenomenon has been used as an indirect measure of the overall quality of DNA templates (10,20). Unfortunately, the techniques employed to assess these DNA modifications may be skewed by oxidative damage that occurs during DNA isolation (21,22).

The purpose of our studies is to investigate the H$_2$O$_2$ scavenging effect of MnTBAP, a compound that is known to mimic the cells own antioxidant defenses (14). We also present data acquired by a modified application of a PCR-based method to detect mtDNA damage induced by ROS (23). We applied direct PCR (DPCR) which incorporates the addition of whole mouse lung fibroblasts (MLFs) to the reaction mixture and successfully amplified both mtDNA and nDNA target sequences. The application of DPCR precludes the damaging

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manipulation of DNA during isolation. We used DPCR in tandem with a PCR-based method to detect mtDNA damage and assess the protective effect of a novel catalytic antioxidant.

MATERIALS AND METHODS

Cell isolation and culture

MLFs were cultured from adult male C57BL/6 mice. Mice were first anesthetized with pentobarbital. An incision through their abdomen was made and their lungs were collapsed by puncturing the diaphragm. The lungs were then perfused through the pulmonary artery with sterile phosphate-buffered saline (PBS) and resected. The lungs were minced and then suspended in 50 ml of PBS containing 0.5% trypsin. The cell suspension was incubated for 30 min at 37°C. Cells were separated by filtration through a 250 μm nylon mesh. The cells were then centrifuged at 1000 g for 10 min at room temperature. The supernatant was discarded and the pellet was washed once with PBS, then repelleted and washed in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS). This suspension was centrifuged and the cells were suspended in DMEM with 10% FBS and transferred to a 75 cm² tissue culture flask. Unattached cells were removed by washing with fresh medium after a 24 h incubation in a humidified atmosphere of 5% CO₂ at 37°C.

MLFs were passaged by first washing the cell monolayer with PBS, then suspended in DMEM containing 10% FBS and centrifuged to a 75 cm² tissue culture flask. Unattached cells were removed by washing with fresh medium after a 24 h incubation in a humidified atmosphere of 5% CO₂ at 37°C.

H₂O₂ and MnTBAP treatment

MLFs were cultured in a 24-well plate and grown to ~90% confluence. Culture medium was removed prior to H₂O₂ treatment and the cells washed with PBS. Cells were treated in duplicate by first adding serum-free medium to the culture then adding H₂O₂ to final concentrations of 0, 200, 400, 600 and 800 μM. H₂O₂ concentration was determined by absorbance at 240 nm using a molar coefficient of Ε = 44 M⁻¹ cm⁻¹ (24). The MLFs were then incubated for 1 h at 37°C in an atmosphere of 5% CO₂ and then the medium was removed and the cells washed with PBS. The cells were cultured in DMEM with 10% FBS. At this point cells were either harvested for PCR (0 h) or fresh DMEM + 10% FBS was placed on the cells and they were incubated for a further 24 h.

The effect of MnTBAP was ascertained in two independent experiments using a concentration of 400 μM H₂O₂. The concentration of MnTBAP was determined using an extinction coefficient of Ε₄₆₅ nm = 9.3 × 10⁴ M⁻¹ cm⁻¹ (13). MLFs were incubated with serum-free medium containing 0, 25, 50 and 100 μM MnTBAP for 1 h at room temperature. The cultures were then washed twice with PBS prior to introduction of 400 μM H₂O₂, then incubated as previously described.

Cell preparation and DNA isolation

DNA isolation using two different methods were compared to DPCR of cells. DNA isolated by phenol/chloroform extraction was obtained from 2 × 10⁵ cells using a standard protocol (19). First, the samples were incubated overnight at 50°C in 300 μl of digestion buffer containing 100 mM NaCl, 10 mM Tris–HCl pH 8.0, 25 mM EDTA pH 8.0, 0.5% (w/v) SDS and 0.1 mg/ml proteinase K. Samples were then extracted with an equal volume of phenol/chloroform/isomylalcohol (25:24:1) and centrifuged for 10 min at 1700 g. The aqueous layer was transferred to a new tube where 0.5 vol of 7.5 M ammonium acetate and 2 vol of absolute ethanol were added and vortexed. The samples were centrifuged for 5 min at 1700 g. The DNA was washed in 500 μl of 70% ethanol, air dried and resuspended in dH₂O. DNA isolation using a QiaAmp DNeasy DNA Isolation Kit (Qiagen, Chatsworth, CA) was done according to the manufacturer’s protocol for DNA isolation from tissue. The entire DNA isolate was used in the PCR.

For DPCR whole MLFs were washed with PBS then removed from the monolayer by treatment with trypsin–Puck’s EDTA. The cells were suspended in 1 ml of serum-containing medium and centrifuged at 2000 g for 5 min. The supernatant was completely removed and the pellet was washed twice by resuspending the cells with 1 ml of PBS and centrifuging at 2000 g for 5 min. After the second wash, the cell pellet was resuspended in 20 μl of PBS. The MLFs were quantitated in a Z1 Coulter Counter (Coulter Corp., Bedfordshire, UK) using 1 μl of the cell suspension. The volume of the suspension was adjusted to facilitate the acquisition of 2 × 10⁵ cells in 0.5–2.0 μl, which was added to the PCR mixture.

PCR

Amplification products were obtained by PCR of purified DNA or 2 × 10⁵ whole MLFs lysed by heating in the PCR mixture, using an Expand High Fidelity PCR Kit (Roche Molecular Biochemicals, Indianapolis, IN). Primer pairs that targeted the mouse mitochondrial genome were L15322 (5′-CTG TGT TCT TGT AAA CCT G-3′) and H3266 (5′-CAG GCT GGC AGA AGT AAT CAT-3′), which yielded a product of 4277 bp (Fig. 1; 25–27). Primers βglobin957 (5′-CGG GTG AGA GAT ACA TCC ATC G-3′) and βglobin5638 (5′-GAT CCA GAG AGC AAC TTT CGA-3′) targeted the β-globin gene cluster and yielded a product of 4681 bp. Template, primers, 5 μl 10× buffer without MgCl₂ and dH₂O were mixed in a 0.2 ml thin-walled PCR tube and heated in a PTC 200 DNA Engine (MJ Research, Watertown, MA) for 5 min at 99.9°C. The mixture was immediately placed on ice, at which time the remaining reagents were added. The final concentrations in the 50 μl amplification cocktail were 1× Expand High Fidelity PCR buffer (Boehringer-Mannheim), 300 nM each primer, 1.5 mM MgCl₂, 200 μM each dNTP and 2.6 U of a mixture of Taq and Pwo DNA polymerases. The PCR cycling profile used for the comparison of different template isolation methods was a pre-PCR incubation at 94°C for 2 min, then 10 cycles of 94°C for 15 s, 50°C for 30 s and 68°C for 4 min, which was followed by 15 cycles of 94°C for 15 s, 50°C for 30 s and 68°C for 4 min, with a 20 s extension per cycle and then to 4°C. PCRs were performed in duplicate for both the H₂O₂ and MnTBAP experiments.

External standards and controls for the PCR were performed by amplification of a plasmid construct containing the 4.3 kb mtDNA target clone into pGEMT (Promega, Madison, WI). External standard reactions contained 5 × 10³, 1 × 10⁴, 5 × 10⁴, 1 × 10⁵, 5 × 10⁵ and 7.5 × 10⁵ molecules of the plasmid
construct and were amplified using mtDNA primers L15,322 and H3266. Standard reactions were run with each assay. The results of DPCR were normalized to the external standard and were then transformed to a percentage of control values. Control reactions consisted of treated cells and $5 \times 10^5$ molecules of pGEMT4.3 amplified with vector-specific primers, T7 (5′-TAA TAC GAC TCA CTA TAG GGC GA-3′) and SP6 (5′-ATT TAG GTG ACA CTA TAG AAT AC-3′), which yielded a 4440 bp PCR product. Amplification products were quantitated on a fluorescence plate reader (Cytofluor 4000; Perspective Biosystems, Farmington, MA) using a Pico Green dsDNA Quantitation Kit according to the manufacturer's protocol (Molecular Probes, Eugene, OR).

**RESULTS**

Comparison of direct PCR with other techniques of DNA isolation in the amplification of mitochondrial DNA

DPCR of the 4.3 kb mtDNA target (Fig. 1) was compared to PCR reactions using DNA prepared by phenol/chloroform extraction and the QiaAmp DNeasy DNA Isolation Kit (Fig. 2). Purified mouse lung DNA was used as a reaction control. The band intensities for the DPCR were discrete and comparable to that obtained using 100 ng of purified mouse lung DNA with less smearing. The entire DNA isolate from $2 \times 10^3$ MLFs using the QiaAmp DNeasy DNA Isolation Kit yielded no amplification product. The DNA isolated from $2 \times 10^3$ and DPCR of the same number of cells, the band intensity of DPCR was greater. Analysis using DPCR saved ~3 h needed for DNA isolation by the Qiagen protocol and 1 day required for the protocol we used for phenol/chloroform extraction.

Decrease in amplification efficiency was observed with mtDNA in H$_2$O$_2$-treated cells

DPCR was applied to a modification of a technique developed by Yakes et al. (23) that detects DNA damage by examining the efficiency of *Taq* polymerase to amplify mtDNA. In order to control for variability in PCR efficiency between experiments the results were compared to an external standard that was generated by amplification of pGEMT4.3, a plasmid construct containing the mtDNA target (Fig. 3). MLFs were subjected to oxidative stress by treatment with 200, 400, 600 and 800 µM H$_2$O$_2$ to produce DNA damage (Fig. 4A). Prior to DPCR, monolayers were inspected under an inverted microscope for signs of lysis or debris that would indicate cell death as a result of H$_2$O$_2$ treatment. No observable signs of cytotoxicity were seen in any of the experimental groups. Intensity of PCR products obtained from each treatment were compared as a percentage of the control group, which did not receive H$_2$O$_2$. The 0 h time
point (cells after 60 min of H$_2$O$_2$ treatment) showed a decline in the percentage of amplified mtDNA target for all H$_2$O$_2$ treatments (Fig. 4B). The mtDNA PCR product decreased to 50% of the control value after the 200 µM H$_2$O$_2$ treatment. The amplification efficiency further decreased to 17% of the control value at 400 µM H$_2$O$_2$. The 600 and 800 µM treatments were only 10% of the control value, which could only be detected by the Pico Green fluorescence assay. A similar phenomenon was observed at 24 h, however, the amplification product after the 200 µM H$_2$O$_2$ treatment returned to the control value. The amplification efficiency in the remaining treatments was far less than the control value but recovered slightly from 17.3% at the 0 h to 22.5% at 24 h. There was no detectable effect on pGEMT4.3 amplification in any of the treatment groups (Fig. 4C). Also, amplification of a 4.7 kb nuclear target in the β-globin gene cluster showed no decline in amplification efficiency (data not shown).

**DISCUSSION**

Aged animals, including *Homo sapiens*, are known to accumulate various aberrations of the mitochondrial genome which are thought to be caused by the cumulative effects of oxidative damage. The proximity of mtDNA to the mitochondrial electron transport system that relentlessly leaks oxygen radicals may offer an explanation for these mutations. We attempted to accelerate DNA damage by exposing cultured cells to H$_2$O$_2$. Using DPCR to assay DNA damage, we show that H$_2$O$_2$ selectively reduces the amplification efficiency of a 4.3 kb mtDNA product but does not reduce the amplification efficiency of a 4.7 kb nDNA target in a PCR-based assay used to detect DNA damage. These data are in accord with previous findings that...
show that mtDNA is highly susceptible to oxidative stress induced by H₂O₂, whereas nDNA seems less sensitive to this oxidative stress (10). It may be hypothesized that nDNA is shielded from the deleterious effects of oxidants like H₂O₂ by association with the chromatin complex and other nuclear proteins. However, no reduction in the amplification efficiency of a 4.7 kb nuclear target may not be indicative of the overall state of the nDNA since such a small fraction of the genome is assayed. We estimated the observed fraction of the diploid complement of mouse nDNA to be 8.7 × 10⁻⁷. When this assay is applied to the mtDNA the observed fraction is 0.3, a much broader representation comparatively. Also, the dose to mass ratio between H₂O₂ and nDNA may be too small to create the sort of lesions seen in mtDNA, which result in decreased amplification. We used a plasmid control containing our mtDNA target to assess whether any of the culture conditions employed in these studies may be artificially altering our PCR results. We found that the amplification efficiencies were not affected by conditions that reduced cellular mtDNA amplification. The reduction in mtDNA amplification persists over 24 h. A significant increase in the amplification efficiency of the mtDNA target does seem to occur at a low H₂O₂ dose in our experiments, but decreases at higher doses. This may be partly explained by mtDNA repair. The confluent cells used in these studies did not have the same high energy requirements as do actively dividing cultures. In this quiescent state it is unlikely that the mitochondria would be replicating and replacing damaged mtDNA, but may be repairing DNA already present.

Pretreatment of MLFs with the antioxidant MnTBAP provides protection against a reduction in amplification efficiency in a concentration-dependent manner. The catalytic antioxidant properties of MnTBAP have been previously described as protecting endothelial cells against H₂O₂-mediated injury (14). The planar nature of this class of porphyrins allows intercalation into the double helix, which may provide the DNA with direct protection against attack by H₂O₂ (28). MnTBAP has been shown to protect against DNA strand breaks by lipopoly-saccharide-induced free radical production in macrophages (15). However, Szabo et al. did not examine the effect of MnTBAP on mtDNA specifically. Although MnTBAP is known to protect the mitochondria, whether the protection offered by this compound is localized within the mitochondria or a result of the net effect of the compound within the cell is not known.

DPCR is a sensitive method that was applied to quantitative in vitro detection of target sequences in a discrete cell population using an external standard. The primary advantage of DPCR is that the DNA template has not been subjected to the potentially damaging manipulation involved in DNA purification. Phenol/ chloroform extraction followed by exposure to air and subsequent CsCl centrifugation and UV visualization by ethidium bromide staining can introduce base adducts such as 8-oxodeoxyguanosine and other aberrations into nucleic acids (22). Indeed, the introduction of such modifications during the purification step has caused some controversy in the study of oxidative DNA damage (21). Direct addition of whole cells to the PCR allows rapid processing of samples, as relatively few cells are needed, and no DNA purification is required. Furthermore, the DNA template begins the PCR more closely resembling its natural condition within the cell.

H₂O₂-induced nitroguenous base modifications produce DNA that is a poor template for PCR and results in less efficient amplification of target sequences (19). Using PCR to measure DNA damage is less specific than methods, such as HPLC coupled with electrochemical detection, that assay selected DNA base modifications (18). However, PCR offers a broader measurement of the quality of the DNA template. We found that the results gathered from this assay were consistent within each experiment, but PCR efficiency varied from run to run. Therefore, we stress the use of an external standard to normalize the results and to ensure that the PCR remains within the linear range. DPCR allows the addition of DNA that is protected by cellular antioxidant defenses and excludes extra manipulation of the template that may introduce aberrations. The template is made available to the reaction by lysis of cells upon introduction to a hypotonic environment. The primary heating step further lyzes the cells and inactivates most cellular degradative enzymes. Thus, DPCR may provide a method that more accurately assesses the effects of oxidative stress on the mitochondrial genome when used in conjunction with other techniques.
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