Mitochondrial DNA damage triggers mitochondrial dysfunction and apoptosis in oxidant-challenged lung endothelial cells

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Mitochondrial DNA damage triggers mitochondrial dysfunction and apoptosis in oxidant-challenged lung endothelial cells. Am J Physiol Lung Cell Mol Physiol 288: L530–L535, 2005. First published November 24, 2004; doi:10.1152/ajplung.00255.2004.—Oxidant-induced death and dysfunction of pulmonary vascular cells play important roles in the evolution of acute lung injury. In pulmonary artery endothelial cells (PAECs), oxidant-mediated damage to mitochondrial DNA (mtDNA) seems to be critical in initiating cytotoxicity inasmuch as overexpression of the mitochondrially targeted human DNA repair enzyme, human Ogg1 (hOgg1), prevents both mtDNA damage and cell death (Dobson AW, Grishko V, LeDoux SP, Kelley MR, Wilson GL, and Gillespie MN. Am J Physiol Lung Cell Mol Physiol 283: L205–L210, 2002). The mechanism by which mtDNA damage leads to PAEC death is unknown, and the present study tested the specific hypothesis that enhanced mtDNA repair suppresses PAEC mitochondrial dysfunction and apoptosis evoked by xanthine oxidase (XO). PAECs transfected either with an adenoviral vector encoding hOgg1 linked to a mitochondrial targeting sequence or with empty vector were challenged with ascending doses of XO plus hypoxanthine. Quantitative Southern blot analyses revealed that, as expected, hOgg1 overexpression suppressed XO-induced mtDNA damage. Mitochondrial overexpression of hOgg1 also suppressed the XO-mediated loss of mitochondrial membrane potential. Importantly, hOgg1 overexpression attenuated XO-induced apoptosis as detected by suppression of caspase-3 activation, by reduced DNA fragmentation, and by a blunted appearance of condensed, fragmented nuclei. These observations suggest that mtDNA damage serves as a trigger for mitochondrial dysfunction and apoptosis in XO-treated PAECs.

xanthine oxidase; deoxyribonucleic acid

OXIDANT-INDUCED DEATH AND DYSFUNCTION of pulmonary vascular endothelial cells (ECs) play important roles in the evolution of acute lung injury. A number of molecular targets of reactive oxygen species (ROS) have been identified, including membrane phospholipids, transporters, enzymes, transcription factors, DNA, etc. (16). The mitochondrial genome is far more vulnerable to oxidative stress compared with nuclear DNA (2, 3, 11, 17) because of its open, circular structure and relatively limited repair capacity. Oxidative damage to the mitochondrial genome leads to altered mitochondrial gene expression and an imbalance in the availability of proteins involved in the electron transport chain (2). Respiratory chain dysfunction could lead to increased ROS production and formation of a vicious cycle, thereby killing cells either necrotically, apoptotically, or by some combination thereof (12).

We recently advanced the concept that mitochondrial DNA (mtDNA) in lung vascular ECs serves as a sentinel molecule in which persistent or severe damage promotes oxidant-mediated cell death (5). In support of this idea, we showed, first, that sensitivity to xanthine oxidase (XO)-induced cytotoxicity among pulmonary artery, microvascular, and pulmonary vein EC phenotypes was inversely related to repair of XO-mediated mtDNA damage (10). Second, and more recently, we found that overexpression of a mitochondrially targeted human 8-oxoguanine-DNA glycosylase (human Ogg1; hOgg1) repair enzyme attenuated mtDNA damage and enhanced cell survival in oxidant-challenged pulmonary artery endothelial cells (PAECs) and other cell types (5, 6). However, the mechanism by which oxidant-mediated damage to mtDNA causes cell death remains unknown. In the present study, we tested the specific hypothesis that mtDNA damage is a proximate trigger for mitochondrial dysfunction and linked to activation of an apoptotic death pathway in XO-challenged PAECs.

METHODS

Rat PAEC culture and treatment. Rat PAECs were isolated and cultured as described previously (1). Animal use protocols were approved by the Institutional Animal Care and Use Committee of the University of South Alabama according to National Institutes of Health guidelines. In brief, main pulmonary arteries were isolated from 250- to 300-g Sprague-Dawley rats killed with an overdose of Nembutal. Isolated arteries were opened, and the intimal lining was carefully scraped with a scalpel. The harvested cells were then placed into flasks (Corning, Corning, NY) containing F-12 nutrient mixture and DMEM mixture (1:1) supplemented with 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (GIBCO BRL, Grand Island, NY). Culture medium was changed once per week, and after reaching confluence, the cells were harvested using a 0.05% solution of trypsin (GIBCO BRL). The EC phenotype, confirmed by acetylated LDL uptake, factor VIII-RAg immunostaining, and the lack of immunostaining with smooth muscle cell α-actin antibodies (Sigma, St. Louis, MO) persisted for at least 15 passages.

Enzymatic oxidation of hypoxanthine by XO was used as a source of ROS for cell treatment. Confluent PAECs transfected with adenovirus were challenged for 1 h with the indicated concentrations of XO and 0.5 mM hypoxanthine in HBSS. After treatment, cells were either processed immediately or placed into cell culture media for recovery. Adenoviral vector construct and cell transfection. Recombinant adenoviruses were generated according to established protocols (8) using commercially available plasmids (AdMax; Microbix, Toronto,
PAECs were rinsed three times with PBS and two times with 0.25 M was prepared by the differential centrifugation method. In brief, mitochondrial membrane potential were observed with an Olympus for 30 min at 37°C before cell treatment with XO. Changes in fluorescent JC-1 selectively enters mitochondria and exists as a monomer at low membrane potential. However, at higher potentials, JC-1 in HBSS and immediately lysed with 3 ml of TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 0.5% SDS and 0.3 mg/ml of proteinase K overnight at 37°C. DNA was isolated using extraction with 1 M NaCl for 10 min at room temperature and purified twice with chloroform/isoamyl alcohol, 24:1. After precipitation with ethanol and reconstitution in water, purified DNA was treated with DNase-free RNase and digested with BamHI (Roche Diagnostics, Indianapolis, IN) and 10 U/mg of DNA overnight at 37°C. After restriction, samples were precipitated and dissolved in TE buffer, and precise DNA concentrations were measured on the Hoefer DyNa Quant 200 fluorometer (Pharmacia Biotech, San Francisco, CA) using Hoechst 33258 dye. Samples containing 2 mg of DNA were treated with NaOH (final concentration 0.1 N) during 15 min at 37°C, mixed with loading dye, and resolved in 0.6% agarose alkaline gel at 1.5 V/cm during 16 h. After electrophoresis, gel was washed and DNA was vacuum transferred to a Zeta-Probe GT nylon membrane (Bio-Rad Laboratories, Hercules, CA). Membrane with cross-linked DNA was prehybridized during 30 min and then hybridized with 32P-labeled probe overnight at 55°C. The probe to mtDNA was generated by PCR using rat mtDNA sequence as a template and the following primers: 5'-GCAGGAA-CAGGATGAAAGCT3' for the sense strand and 5'-GTATCCTG-GAAACGATGCAAGGATTAG-3' for the antisense strand. The 725-bp product was hybridized with a 10.8-kb restriction fragment of rat mtDNA digested with BamHI. After hybridization, the membrane was washed and exposed to X-ray film (Kodak XAR-5) to obtain an autoradiographic image or placed on a phosphorimaging screen and scanned with Bio-Rad GS-250 Molecular Imaging System. Changes in the equilibrium lesion density were calculated as negative ln of the quotient of hybridization intensities in treated and control bands (4).

Measurement of mitochondrial membrane potential. The membrane fluorescent dye JC-1 (Molecular Probes, Eugene, OR) was used for the measurement of mitochondrial membrane potential. The green fluorescent JC-1 selectively enters mitochondria and exists as a monomer at low membrane potential. However, at higher potentials, JC-1 forms red fluorescent “J-aggregates” that exhibit a broad excitation spectrum and large shift in emission with a maximum at 590 nm. For dye loading, cells were incubated with 1 μM JC-1 in HBSS for 30 min at 37°C before cell treatment with XO. Changes in mitochondrial membrane potential were observed with an Olympus IX70 fluorescence microscope.

Isolation of mitochondrial fraction. Crude mitochondrial fraction was isolated by the differential centrifugation method. In brief, PAECs were rinsed three times with PBS and two times with 0.25 M sucrose and 10 mM triethanolamine-acetic acid, pH 7.8, at room temperature and harvested in ice-cold 0.25 M sucrose, 1 mM EDTA, and 10 mM triethanolamine-acetic acid, pH 7.8. The following steps were carried out at 0–4°C. The cell suspension was transferred to a Dounce grinder and homogenized with 10 strokes. Cell debris and nuclei were removed by centrifugation at 1,000 g for 10 min. The pellet was then washed with homogenizing buffer twice following centrifugation. Postnuclear supernatants were spun at 15,000 g for 15 min to pellet the mitochondrial fraction. For Western blot analysis, the pellet was resuspended in 2% SDS electrophoresis loading buffer.

Immunoblot analyses of hOgg1 and caspase-3. hOgg1 was detected in crude mitochondrial extract, prepared as described above, and caspase-3 was detected in total cell lysate using Western immunoblot analyses. Control and treated cells were harvested 4 h after XO exposure. For preparation of total cell lysate, cells were washed twice with PBS and lysed in 2% SDS electrophoresis loading buffer, after which 15–25 μg of protein were applied to an SDS/12% polyacrylamide gel. After separation, samples were transferred to nitrocellulose filters (Bio-Rad Laboratories). Membranes blocked in 5% nonfat dry milk in PBS with 0.1% Tween 20 were incubated overnight at 4°C with primary rabbit polyclonal antibodies recognizing either the activated form of caspase-3 (Cell Signaling Technology, Beverly, MA) or hOgg1 (Novus Biologicals, Littleton, CO), both of which were diluted 1:600 in blocking solution. After being washed, the membranes were incubated with 1:7,500 diluted horseradish peroxidase-conjugated goat anti-rabbit IgG (Calbiochem-Novabiochem, San Diego, CA) for 1 h at room temperature and then revealed by chemiluminescence with the ECL detection kit (Amersham).

Immunocytochemical analysis of activated caspase-3. For immunocytochemical analysis of caspase-3, control and XO-treated PAECs were fixed with 2% paraformaldehyde in PBS (1 h at 4°C) 4 h after treatment. Fixed cells were washed three times with 50 mM Tris·HCl (pH 7.4), 150 mM NaCl, and 0.1% Triton X-100 (TBS-T), blocked with 5% normal goat serum in TBS-T, incubated with primary rabbit polyclonal antibodies recognizing either the activated form of caspase-3 (Cell Signaling Technology, and diluted 1:120 in TBS-T with 3% BSA at 4°C overnight. After this, cells were incubated with Texas red-labeled anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:100 in TBS-T with 3% BSA for 1 h at room temperature. Slides were then mounted with fluorescent mounting media (Dako, Carpinteria, CA) and observed with an Olympus IX70 fluorescence microscope. Photomicrographs were taken with a SPOT digital camera using SPOT 2 software (Diagnostic Instruments, Sterling Heights, MI).

Morphological assessment of nuclear condensation. PAECs were prepared for microscopy as described for caspase-3 immunochemistry, except they were stained with Hoechst 33258 dye (Molecular Probes) to demarcate nuclear morphology.

DNA fragmentation assay. Quantitative ELISA detecting mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates was used to determine fragmentation of nuclear DNA during apoptosis caused by XO treatment. Assay was performed with the Cell Death Detection ELISA kit (Roche Diagnostics) according to the supplied protocol. PAECs grown and treated in 24-well culture plates were washed twice with PBS, and 500 μl of lysis buffer per well were added. After incubation for 30 min at room temperature, lysates were transferred to microcentrifuge tubes and centrifuged at 15,000 g for 10 min at 4°C. Supernatant was diluted 1:3 with lysis buffer, and 20 μl were used for ELISA. Reaction product abundance was determined by measuring the absorbance at 405 nm (reference wavelength 490 nm) with a 7520 Microplate Reader (Cambridge Technology, Cambridge, MA).

Statistical analysis. Data are expressed as means ± SE. Depending on the experimental design, differences in mean values were assessed using paired t-tests or one-way ANOVA combined with Newman-Keuls tests. P values <0.05 were taken as evidence of statistical significance.
RESULTS

To test the hypothesis that mtDNA damage is a proximate cause of ROS-induced apoptosis, a construct encompassing the hOgg1 gene fused to MTS from human MnSOD was inserted into an adenoviral vector (Fig. 1, top) and transfected into PAECs. The efficiency of transfection and mitochondrial localization of the recombinant protein was determined by Western blot analysis of mitochondrial fractions. Transient transfection of PAECs with adenoviral vector encoding the hOgg1-MTS construct at 1 and 2 MOI was associated with substantial increases in mitochondrial hOgg1 content (Fig. 1, bottom) compared with cells transfected with empty vector. The increase in mitochondrial hOgg1 was slightly greater at a MOI of 2× relative to 1×.

Initial experiments verified that XO plus hypoxanthine damaged mtDNA and that adenoviral-mediated overexpression of mitochondrially targeted hOgg1 led to a reduction in the XO-induced increase in mtDNA equilibrium lesion density. The representative quantitative Southern blot displayed in Fig. 2 (top) shows that XO, as expected, decreased hybridization intensity of the mtDNA probe in a dose-related manner, thus indicating an increase in equilibrium lesion density. Densitometric analysis of hybridization intensity followed by calculation of the increase in lesions per 10 kb indicated that the 1-h challenge with XO at doses of 2 and 5 mU/ml was accompanied by increases of 0.4 and 1.3 lesions/10 kb, respectively (Fig. 2, bottom). Also, as expected on the basis of previous observations (9, 10), lesions in the nuclear VEGF gene could not be detected at these XO doses using the quantitative Southern blot approach (data not shown). Finally, and also as predicted on the basis of earlier work, mitochondrial overexpression of hOgg1 was accompanied by a dose-related suppression of XO-induced mtDNA damage at both XO concentrations tested (Fig. 2).

Having verified that XO-induced mtDNA damage is suppressed by overexpression of mitochondrially targeted hOgg1, we next addressed the question of whether protection of mtDNA was linked to preservation of mitochondrial function. To address mitochondrial functional activity, we used fluorescence of the mitochondrial membrane potential-dependent dye JC-1. After loading with JC-1, we treated PAECs with XO as described in METHODS and analyzed them 1 h thereafter and again 4 h after treatment. There were no differences in mitochondrial membrane potential compared with control in any samples analyzed at the 1-h time point (data not shown). However, as seen in the photomicrographs and in the pooled data presented in Fig. 3, a marked diminution in JC-1 fluorescence was evident 4 h after XO challenge of vector-transfected PAECs compared with nontreated cells, thus indicating XO-mediated dissipation of mitochondrial membrane potential (Fig. 3, A, C, and E). Whereas transfection with mitochondrially targeted hOgg1 failed to alter JC-1 fluorescence in control cells, hOgg1 overexpression prevented the dissipation in JC-1 fluorescence caused by XO (Fig. 3, B, D, and E).

We next determined whether the observed protective actions of hOgg1 overexpression on mtDNA integrity and mitochondrial function were associated with suppression of XO-induced apoptosis in PAECs. To assess apoptosis, we evaluated the extent of DNA fragmentation, the presence of activated caspase-3, and the appearance of condensed, fragmented, Hoechst-stained nuclei. Initial experiments to delineate the time course of DNA fragmentation indicated the appearance of oligonucleosomes beginning at 3 h with a peak at 4–6 h after the XO treatment (data not shown). All the following apoptosis detection assays were therefore performed 4 h after the cells were challenged with XO for 1 h. The Western blot analyses shown in Fig. 4, top, indicate that whereas XO treatment at doses of 2 and 5 mU/ml increased the extent of active caspase-3, this effect was blunted in hOgg1 overexpressors relative to cells transfected with empty vector. These results were confirmed by immunocytochemistry with antibody specific to activated caspase-3 (Fig. 4, bottom). We wondered whether the residual caspase-3 activation occurring in the...
presence of hOgg1 overexpression at the higher dose of XO (5 mU/ml) was related to oxidant-mediated reduction of mitochondrial hOgg1 or to the possibility that the ROS produced in response to this high XO dose overwhelmed the protection afforded by hOgg1. Mitochondrial hOgg1 was therefore measured by Western blot analyses at the two levels of transfection in control and XO-treated PAECs and was found not to differ between the two treatment groups (data not shown). Thus the residual caspase-3 activation associated with the 5 mU/ml of XO can most likely be attributed to hOgg1 activity that is insufficient to afford complete protection against the high ROS production.

Another index of apoptosis, nuclear condensation, was detected by staining with Hoechst 33258 dye. As seen in the photomicrographs and pooled data presented in Fig. 5, while XO-treated cells displayed numerous condensed, fragmented nuclei (Fig. 5, C and E), overexpression of mitochondrially targeted hOgg1 protein almost completely eliminated this morphological change (Fig. 5, D and E). Finally, the outcome of experiments using the ELISA for DNA fragmentation was consistent with the effects of hOgg1 overexpression on XO-induced caspase-3 activation and formation of apoptotic nuclei. As shown in Fig. 6, PAECs transfected with adenoviral vector encoding the hOgg1-MTS construct exhibited a significant reduction of DNA fragmentation (~40%) after treatment with XO at a dose of 5 mU/ml compared with cells transfected with empty vector. The ELISA assay for oligonucleosome formation failed to detect apoptotic cell death at an XO dose of 2 mU/ml or an effect of vector or hOgg1 transfection alone. Although the disparity between the presence of oligonucleosome formation and caspase-3 activation at the 2-mU/ml XO dose is difficult to explain, it seems reasonable to suspect that it is related to differing time courses of DNA fragmentation and caspase-3 activation and/or different sensitivities of the assays employed.

**DISCUSSION**

Apoptosis in ECs derived from both systemic and pulmonary vascular beds as well as other, non-EC types is an important mechanism of cell death induced by oxidative stress. It is also widely appreciated that mitochondria play a central role in the apoptotic process. Key features within the apoptosis

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**Fig. 3.** Mitochondrial membrane potential, assessed in terms of JC-1 fluorescence, in PAECs 4 h after a 1-h XO treatment. Photomicrographs of vector (A and C)- and hOgg1-MTS (B and D)-transfected PAECs (MOI: 2×) taken 4 h after culture for 1 h in the absence (A and B) or presence of 5 mU/ml of XO (C and D). Cells were loaded with JC-1 (1 μM) during 30 min before XO treatment. Note the mitochondria depolarization in vector-transfected cells after treatment. E: pooled data displaying means ± SE. Shown is JC-1 fluorescence intensity for vector-transfected control cells (Con), cells treated with 5 mU/ml of XO, cells transfected with hOgg1-MTS (Ogg), or cells challenged with XO in the presence of hOgg1 overexpression (XO + Ogg). *Significantly decreased compared with all other groups (n = 4).

**Fig. 4.** Top: representative Western blot analysis of vector (V)- and hOgg1-MTS (Ogg)-transfected cells without treatment or treated with XO (2 or 5 mU/ml) probed with antibody to activated caspase-3. Cells were harvested 4 h after 1-h treatment with XO. Bottom: immunocytochemistry of vector- and hOgg1-MTS (Ogg)-transfected PAECs (MOI: 2×) cultured for 1 h in the absence or presence of 5 mU/ml of XO. Antibodies against the active form of caspase-3 were applied 4 h after XO treatment. Note increased abundance of activated caspase-3 in PAECs 4 h after XO treatment but not in cells overexpressing mitochondrially targeted hOgg1.
report showed that transfection with an identical construct using a similar strategy was accompanied by a prominent increase in Ogg-like activity in the mitochondrial fraction compared with nucleus or cytosol (5). Cells were challenged with ascending doses of XO plus hypoxanthine, and the association between mtDNA damage and oxidatively induced apoptosis was examined.

As expected on the basis of our previous studies (5, 6), hOgg1 overexpression was accompanied by protection of mtDNA against XO-mediated damage. It should be emphasized that at the doses of XO employed, we have never detected oxidative damage to the nuclear genome using either quantitative Southern blot analyses (10) or ligation-mediated PCR methods (9, 10), findings consistent with the high sensitivity of mtDNA to oxidative damage compared with nuclear gene (2, 11, 14, 17). Thus the impact of hOgg1 on mitochondrial function and cell survival can be ascribed to protection of mtDNA and not to an unsuspected effect on oxidant-mediated effects elsewhere in the cell. Protection of mtDNA from XO-mediated damage was associated with preservation of mitochondrial function assessed in terms of mitochondrial membrane potential. This preservation of mitochondrial membrane potential could further enhance the protective actions of hOgg1 overexpression against mtDNA damage since, as pointed out by Santos and coworkers (14), mitochondrial membrane potential is a critical driving force for mitochondrial protein import, including DNA repair enzymes. Most importantly, mitochondrial overexpression of hOgg1 protected against XO-induced apoptosis, as evidenced by blunted activation of caspase-3, accumulation of oligonucleosomes, and formation of condensed, fragmented nuclei. Because of the specificity of hOgg1 as a DNA repair enzyme, its targeting to mitochondria, and the attendant protection of mtDNA from oxidant-mediated damage, these findings support the conclusion that mtDNA damage is a proximate cause of mitochondrial dysfunction and that mtDNA damage is linked specifically to induction of apoptosis in XO-challenged PAECs. These findings, in addition, corroborate observations by Shukla et al. (15) in asbestos-treated, hOgg1 overexpressing HeLa cells.

cascades are dissipation of mitochondrial membrane potential, increased mitochondrial oxidant production, and apoptogenic protein release, caused by opening of the permeability transition pore (13). The processes downstream from the release of proteins from mitochondria, such as apoptosis-inducing factor, cytochrome c, Smac/DIABLO, etc., are also reasonably well established. However, the intramitochondrial events initiated by oxidant challenge that activate the apoptotic cascade are not yet clarified.

The objective of the present study was to test the hypothesis that XO-induced damage to mtDNA was a proximate trigger for mitochondrial dysfunction and apoptosis in cultured PAECs. To test this hypothesis, PAECs were transfected either with empty adenoviral vector or with vector encoding hOgg1 linked to an MTS. 8-Oxoguanine-DNA glycosylase is the principal enzyme for repair of 7,8-dihydro-8-oxoguanine, one of the most important mutagenic lesions in DNA induced by ROS (7). In the present study, mitochondrial targeting of hOgg1 was verified by Western blot analysis. Our previous

![Fig. 5. Nuclear condensation in PAECs after XO treatment. Photomicrographs of vector (A and C) and hOgg1-MTS (B and D)-transfected PAECs (MOI: 2×) cultured for 1 h in the absence (A and B) or presence of 5 μU/ml of XO (C and D) stained with Hoechst 33258. Cells were harvested 4 h after beginning XO treatment. Note the appearance of apoptotic nuclei in vector-transfected cells (arrows). E: pooled data displaying means ± SE of the percentage of condensed, fragmented nuclei for vector-transfected control cells (Con), cells treated with 5 μU/ml of XO, cells transfected with hOgg1-MTS (Ogg), or cells challenged with XO in the presence of hOgg1 overexpression (XO + Ogg). *Significantly increased compared with Con; **significantly increased compared with Con but decreased compared with XO (n = 4–6).]

![Fig. 6. DNA fragmentation in PAECs 4 h after XO treatment. Reduced results of quantitative ELISA assay detecting mono- and oligonucleosomes in the cytoplasmic fractions of vector- and hOgg1-MTS (Ogg)-transfected cells without treatment or treated with XO (2 or 5 μU/ml). Bars reflect means ± SE. *Significantly increased compared with cells not exposed to XO; **significantly reduced compared with vector-transfected cells treated with 5 μU/ml of XO (n = 6).]
Results of this study indicate that the extent of mtDNA damage in response to XO dictates whether PAECs survive or activate an apoptotic death pathway. One of questions associated with this conclusion pertains to the mechanism by which mtDNA damage initiates the apoptotic cascade. Previous reports have suggested that mtDNA damage activates a “feed forward” process wherein mtDNA damage initiates persistent oxidant production that, in cells surviving the oxidant insult, is terminated by mtDNA repair (14). Nonsurviving cells exhibit decreased mitochondrial membrane potential and persistent mtDNA damage. Additional studies will be required to determine whether such a feed forward system, terminated by mtDNA repair, is involved with the PAEC response to oxidant stress.

REFERENCES