Enhanced mtDNA repair capacity protects pulmonary artery endothelial cells from oxidant-mediated death

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Enhanced mtDNA repair capacity protects pulmonary artery endothelial cells from oxidant-mediated death. Am J Physiol Lung Cell Mol Physiol 283: L205–L210, 2002. First published March 1, 2002; 10.1152/ajplung.00443.2001.—In rat cultured pulmonary arterial (PA), microvascular, and venous endothelial cells (ECs), the rate of mitochondrial (mt) DNA repair is predictive of the severity of xanthine oxidase (XO)-induced mtDNA damage and the sensitivity to XO-mediated cell death. To examine the importance of mtDNA damage and repair more directly, we determined the impact of mitochondrial overexpression of the DNA repair enzyme, Ogg1, on XO-induced mtDNA damage and cell death in PAECs. PAECs were transiently transfected with an Ogg1-mitochondrial targeting sequence construct. Mitochondria-selective overexpression of the transgene product was confirmed microscopically by the observation that immunoreactive Ogg1 colocalized with a mitochondria-specific tracer and, with an oligonucleotide cleavage assay, by a selective enhancement of mitochondrial Ogg1 activity. Overexpression of Ogg1 protected against both XO-induced mtDNA damage, determined by quantitative Southern analysis, and cell death as assessed by trypan blue exclusion and MTS assays. These findings show that mtDNA damage is a direct cause of cell death in XO-treated PAECs.

mitochondrial deoxyribonucleic acid; xanthine oxidase; Ogg1; cytotoxicity

THE MITOCHONDRIAL GENOME is ~10–100-fold more sensitive to oxidant damage than nuclear DNA (22). Moreover, mutations and deletions in the mitochondrial genome in cells of the central nervous system and elsewhere have been linked to a variety of disorders (7, 14, 15, 20, 21). Depletion of mitochondrial (mt) DNA with ethidium bromide also is known to suppress ATP synthesis and cause defects in cell function (6). Oxidant damage to mtDNA damage could thus play a causal role in disorders linked to excessive generation of reactive oxygen species.

Pulmonary vascular endothelial cells (ECs) are among the most important targets of reactive oxygen species elaborated in acute lung injury (4, 5, 13). In light of the above considerations pertaining to the sensitivity to, and potential importance of, oxidant-mediated damage to the mitochondrial genome, we recently examined the effects of chemically generated oxidants on mtDNA integrity and cell viability in pulmonary arterial (PA), microvascular (MV), and vein ECs (12). An interesting observation to emerge from this study is that in the case of xanthine oxidase (XO)-generated free radicals, there were EC phenotype-dependent differences in the propensity for mtDNA damage and cell death that were inversely associated with the rate of repair of mtDNA damage. Pulmonary vein ECs, which were most sensitive to XO, were virtually incapable of repairing XO-induced mtDNA damage, whereas PAECs were intermediate and MVECs exhibited very rapid mtDNA repair and a relative insensitivity to XO-induced cell death. One implication of this finding is that damage to mtDNA was a proximate cause of EC death and that pathways repairing mtDNA comprised an important mechanism of EC defense against reactive oxygen species.

The goal of the present experiments was to assess the importance of mtDNA damage as a cause of lung EC death in response to XO. To do so, we took advantage of a recently described strategy to transfect the DNA repair enzyme 8-oxoguanine DNA glycosylase-1 (Ogg1), linked to a mitochondrial targeting sequence to engender a selective increase in DNA repair capacity within the mitochondrial compartment (9). Our studies, conducted in the PAEC phenotype exhibiting an intermediate sensitivity to XO, suggest that oxidant damage to the mtDNA serves as a proximate event initiating cell death.

METHODS

Rat main PAEC cultures. Main PAs and pulmonary veins were isolated from Sprague-Dawley (250–300 g) 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,
restriction fragment subcloned into the EcoRI site of pcDNA3.0neo and sequenced to confirm the presence of an Ogg1-MTS
conjugate. A construct of Ogg1 fused to the MTS from manganese superoxide dismutase (MnSOD) was prepared as described previously (9). In brief, Ogg1 was amplified from a cDNA plasmid using a 5′ primer, GGAATTCATGTTGAGCCGGGCAAGTGTCGACCACGGAGCCTGGGCGCTAGCTTGGGGTGATCGGGCTCACAGGAGTCTGAGCTGAGTATACC
CGAAAGTF, containing the mitochondrial targeting sequence (16), an EcoRI restriction site, and a 3′ primer, CGCGCGTCTCAGGGCTCTTGCCCTTTGGA, containing an Xhol restriction site. The cDNA was amplified with a high-fidelity thermostable DNA polymerase by the PCR procedure under the following conditions: 30-s denaturation (94°C), 1-min annealing (55°C), and 2-min extension (72°C). The PCR fragment so generated consisted of an EcoRI restriction site, the MTS, the Ogg1 coding region, and an Xhol restriction site. The product was digested overnight at 37°C with EcoRI and Xhol, and the restriction fragment subcloned into the EcoRI and Xhol sites of pcDNA3.0neo and sequenced to confirm fidelity. PAECs were transfected with either Ogg1-MTS or empty vector using Fugene 6 reagent according to the manufacturer’s instructions. PAEC cultures were studied 48–72 h after transfection.

Immunocytochemical localization of Ogg1. Cultures of vector- and Ogg1-MTS-transfected PAECs were treated with MitoTracker-Red (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions to stain mitochondria. Cultures were subsequently fixed in paraformaldehyde, washed with phosphate-buffered saline (PBS), and then immersed in ice-cold acetone. After being air-dried and washed with PBS, specimens were blocked with 1% BSA plus 1% normal goat serum in PBS. Rabbit polyclonal antibody to human Ogg1 (Novus Biologicals, Littleton, CO) was then applied overnight, after which specimens were washed in PBS plus 0.1% Triton X-100. FITC-labeled anti-rabbit IgG was employed to localize immunoreactive Ogg1. Finally, specimens were stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Hoechst 33258; Molecular Probes) to localize nuclei. Slides were evaluated using an Olympus IX70 fluorescence microscope (Melville, NY).

Assessment of Ogg1 activity. The general method for determination of Ogg1 activity has been recently described (9). In brief, cellular fractions were prepared from three 75-cm² flasks of Ogg1-MTS- and vector-transfected PAECs, respectively, that were harvested and lysed in an ice-cold digitonin solution. The cell lysate was then applied to a mannositol-sucrose buffer and centrifuged at 800 g for 10 min to pellet nuclei. The supernatant was reserved, the pellet was again resuspended in mannositol-sucrose buffer, and centrifugation was repeated four more times, on each occasion saving the supernatant. The supernatants were then combined and centrifuged a final time to pellet any remaining nuclei, and the resulting supernatant was centrifuged at 10,000 g to pellet mitochondria. Supernatants derived from the initial, lower-speed centrifugations were concentrated (Amicon protein concentrators) to obtain cytosolic fractions.

Ogg1 activity was determined in vector and Ogg1-MTS transfectants by incubating the above-prepared cellular fragments (50 µg mitochondrial, 20 µg nuclear, and 50 µg cytosolic protein, respectively) with a 24-mer, 32P-end-labeled, duplex oligonucleotide containing an 8-oxoguanine at the 10th position (Trevigen, Gaithersburg, MD). An identical oligonucleotide without the 8-oxoguanine was used in parallel reactions. Activity assays contained 0.2 pmol labeled duplex oligonucleotide, 3 µl 10× REC buffer (100 mM HEPES, pH 7.4, 1 M KCl, 100 mM EDTA, and 1 mg/ml BSA), and organelle extracts in a total volume of 30 µl. Organelle extracts contributed >20% of the total reaction volume. Bromophenol blue dye was added, and the reaction contents were resolved on 20% acrylamide, 8 M urea in 1× Tris-borate EDTA.

Detection of mtDNA damage using quantitative Southern analysis. The method used to detect alkali-labile mtDNA damage has been described previously (11, 12). In brief, PAECs cultured on 100-mm petri plates were challenged for 1 h with 10 µM/ml XO plus hypoxanthine (0.5 mM). DNA isolated from ECs was precipitated, resuspended in Tris-EDTA (TE) buffer (pH 8.0), and then treated with DNase-free RNase. Purified DNA was then digested with BamHI, and complete digestion was verified on minigels. After restriction, samples were resolved in a small volume of TE buffer and precisely quantified using a Hoefer TKO microfluorimeter and standards kit. Samples containing 5–10 µg of DNA were heated, then cooled at room temperature, followed by incubation for 15 min with sodium hydroxide (0.1 N) to cleave DNA at sites of oxidative injury to the deoxyribose backbone. Samples were then combined with 5 µl of loading dye, loaded onto a 0.6% alkaline agarose gel, and electrophoresed in an alkaline buffer. After the gel was washed, DNA was transferred by vacuum blotting (Millipore, Bedford, MA) onto a Zeta-Probe GT nylon membrane (Bio-Rad Laboratories, Hercules, CA) and cross-linked to the membrane with a GS Gene Linker (Bio-Rad Laboratories, Hercules, CA). After 10 min of preincubation, the membrane was hybridized with a PCR-generated genomic DNA probe for the mitochondrial genome. The probe used to hybridize to mtDNA was generated via PCR from a mouse mtDNA sequence using the following primers: 5′-CGCGAAACAGGATGAAAGCTGG-3′ from the sense strand and 5′-GTAATCCGAAGACGAGTGTGAG-3′ from the anti-sense strand. The 725-bp product recognized a 10.8-kb restriction fragment when hybridized to rat mtDNA from the sense strand and 5′-GTAATCCGAAGACGAGTGTGAG-3′ from the anti-sense strand. The 725-bp product recognized a 10.8-kb restriction fragment when hybridized to rat mtDNA.

RESULTS

In our initial analysis of Ogg1 immunoreactivity in vector and Ogg1-MTS transfected PAECs, we noted that 10–20% of the cells expressed the transgene product (data not shown). The photomicrographs shown in Fig. 1, representative of four such experiments, com-

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Corning, NY) containing F-12 nutrient mixture and DMEM mixture (1:1) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (GIBCO BRL Products, Grand Island, NY). Cells were harvested using a 0.05% solution of trypsin (GIBCO BRL) and passed up to 15 times. The EC phenotype was confirmed by acetylated low-density lipoprotein uptake, factor VIII-Rag immunostaining, and the lack of immunostaining with smooth muscle cell α-actin antibodies (Sigma, St. Louis, MO).

Transient transfection of PAECs with an Ogg1-MTS construct. A construct of Ogg1 fused to the MTS from manganese superoxide dismutase (MnSOD) was prepared as described previously (9). In brief, Ogg1 was amplified from a cDNA plasmid using a 5′ primer, GGAATTCATGTTGAGCCGGGCAAGTGTCGACCACGGAGCCTGGGCGCTAGCTTGGGGTGATCGGGCTCACAGGAGTCTGAGCTGAGTATACC
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pared the subcellular localization of Ogg1 in clusters of Ogg1-expressing cells to vector-transfected controls. Merged images of Ogg1, mitochondrial, and nuclear-stained vector-transfected PAECs (Fig. 1A) clearly demonstrate blue (DAPI)-stained nuclei and perinuclear, red (MitoTracker)-stained mitochondria. No yellow-staining regions indicative of Ogg1 expression in either of these cellular compartments is evident. However, in Ogg1-MTS transfected PAECs (Fig. 1B), the perinuclear red appearance is replaced by an orangegray color indicative of colocalization of Ogg1 with the mitochondrial stain. Most of the nuclei remained dark blue, suggesting a lack of increased Ogg1 staining, but a yellow color is evident in some and implies a slight increase in nuclear immunoreactive Ogg1 protein.

As an additional means of determining whether the transfection procedure resulted in a mitochondrial-selective increase in Ogg1, we assessed Ogg1-like ac-

tivity in cytoplasmic, nuclear, and mitochondrial fractions, using an oligonucleotide cleavage assay. When aliquots of these fractions from vector-transfected or Ogg1-MTS-transfected cells were applied to a wild-type oligonucleotide devoid of 8-oxoguanine, there was, as expected, no cleavage of substrate oligonucleotide (data not shown). As shown by the representative autoradiogram displayed in Fig. 2, aliquots of cellular fractions derived from vector-transfected PAECs applied to the 8-oxoguanine harboring oligonucleotide were associated with the appearance of a lower-molecular-weight product resulting from Ogg1 cleavage of the 24-mer duplex oligonucleotide at the 8-oxoguanine site. Importantly, the Ogg1-MTS transfectants exhibited a substantial increase in Ogg1 activity in the mitochondrial fraction, as shown by an increase in the lower-molecular-weight product, with a less impressive increase also evident in the cytosolic fraction. Similar results were obtained in three experiments, and the increase in Ogg1-like product noted in Ogg1-MTS-transfected cells over vector-transfected PAECs was estimated by densitometry at 30–40%.

To determine whether Ogg1-MTS transfectants were protected from mtDNA damage, we challenged vector-transfected and Ogg1-overexpressing PAECs for 1 h with 10 mU/ml XO plus 0.5 mM hypoxanthine, and quantitative Southern analysis was used to detect alkali-labile damage to the mitochondrial genome. The representative Southern analysis shown in Fig. 3, top, displays two interesting features. First, relative to vector-transfected control cells, hybridization intensity in the controls transfected with the Ogg1-MTS construct is notably enhanced, thus indicating that overexpression of the DNA repair enzyme decreased the equilibrium density of constitutive mtDNA lesions. More importantly, XO-induced decreases in hybridization intensity, indicative of mtDNA damage, appear to be more pronounced in vector-transfected PAECs than in those overexpressing the Ogg1-MTS construct. Hybridization intensities from four or five such experiments were pooled and used to calculate changes in equilibrium lesion density from control for vector and Ogg1-MTS PAEC populations. As shown in Fig. 3,
Ogg1-Transfection with (1), can limit availability of key components of the gene expression, known to accompany mtDNA damage pathway. In this regard, a reduction in mitochondrial age could generate signals that activate the cell death mitochondrial apoptotic machinery where mtDNA damage resides in close proximity to the mitochondrial DNA. It could be central to the death-initiating processes. Mitochondria, which are the sites of mtDNA damage, trigger cytotoxicity is unknown, but for several reasons it is tempting to speculate that mtDNA damage directly triggers PAEC death.

As a first step towards resolving this issue, we capitalized on a recently reported approach to stably transfact HeLa cells with a construct encoding human Ogg1 linked to an MTS (9). The advantage of this strategy is that, to the extent that the transgene product is confined to the mitochondrial compartment, only the mtDNA repair rate should be affected; other elements associated with oxidant-mediated toxicity or signaling should proceed unabated. Nevertheless, the potential obfuscating effect of nuclear localization of Ogg1 needs to be considered inasmuch as the human Ogg1 gene used in the present study, as well as in the report by Dobson et al. (9), encodes a nuclear localization signal. We hoped to minimize the possibility of nuclear localization by incorporating a strong MTS into the construct in a position that would place this targeting sequence closer to the protein’s NH2 terminus than the nuclear targeting sequence. The rationale for this configuration stems from the observation that, when multiple targeting sequences are present on a protein, the one most proximate to the NH2 terminus predominates (17). Additionally, a mitochondrial targeting sequence from MnSOD was employed because electron transport chain, which, in turn, could reroute the flow of electrons and thereby augment mitochondrial oxidant production (3). Mitochondrially derived oxidants have been demonstrated to serve as proximate activators of the apoptotic pathway (8, 10). Diminished availability of electron transport chain components also could reduce ATP synthesis, which, in turn, could have an impact on a range of cellular functions necessary for survival. In light of these considerations and the recognition that the mitochondrial genome is considerably more sensitive to oxidative damage than nuclear DNA (1, 12, 22), the present studies tested the hypothesis that mtDNA serves as a sentinel molecule in which oxidant damage directly triggers PAEC death.

Fig. 3. Top: representative quantitative Southern analysis of alkali-detectable mitochondrial (mt) DNA damage in vector (V)- and Ogg1-MTS (Ogg1)-transfected cells cultured under control (C) conditions or immediately after 60-min exposure to 10 mU xanthine oxidase (XO). Note increased hybridization intensity in control Ogg1-MTS-transfected cells relative to vector-transfected controls and more prominent decrease in hybridization intensity evoked by XO in vector transfectants relative to PAECs overexpressing Ogg1-MTS. Bottom: results are expressed as the mean increase in calculated equilibrium lesion density ± SE for 4–5 experiments. Transfection with Ogg1 suppressed (*P < 0.05) mtDNA damage caused by XO relative to damage in vector-transfected cells.

bottom, XO increased the equilibrium density of alkali-detectable lesions in vector-control PAECs by nearly 4 lesions/10 kb, whereas in Ogg1-MTS transfectants the XO-mediated increase in lesion density was reduced by ~40%.

A final experiment was conducted to determine whether mitochondrial-selective overexpression of Ogg1 conferred protection against XO-induced cytotoxicity. Vector and Ogg1-MTS transfected PAECs were treated for 1 h with XO in concentrations ranging from 5 to 50 mU/ml, and cell viability was assessed 24 h thereafter. Data presented in Fig. 4 show that XO caused dose-related cytotoxicity that, importantly, was attenuated at all XO doses tested in the Ogg1 overexpressers.

DISCUSSION

There are numerous molecular targets of oxygen radicals within PAECs, including membrane proteins and lipids, transmembrane transporters, cytosolic regulatory proteins, and the nuclear and mitochondrial genomes (19). Which of these is most critical in terms of triggering cytotoxicity is unknown, but for several reasons it is tempting to speculate that mtDNA damage could be central to the death-initiating processes. Mitochondrial DNA resides in close proximity to the mitochondrial apoptotic machinery where mtDNA damage could generate signals that activate the cell death pathway. In this regard, a reduction in mitochondrial gene expression, known to accompany mtDNA damage (1), can limit availability of key components of the
this is a strong signal that effectively directs a variety of proteins to localize in mitochondria (18).

The results of our microscopic analysis of Ogg1 localization show that most of the transgene product appeared to be within mitochondria, although some PAECs exhibited a modest degree of nuclear immunoactivity as well. On the other hand, when Ogg1-like activity was determined, there was little if any increase in the nuclear fraction. The cytosolic fraction increased somewhat after transfection with the Ogg1-MTS construct, but the mitochondrial fraction exhibited a marked elevation in Ogg1 activity. The increase in cytoplasmic Ogg1 activity is not surprising, because this is the location of its synthesis. More importantly, the increase in cytosolic Ogg1 activity would not seem to obfuscate data interpretation, since, in this cellular compartment, there is no DNA target of oxidants, and free radical reactions with cytoplasmic constituents would not be affected by a DNA repair enzyme. Although the conclusion that transfection with an Ogg1-MTS construct causes a prominent elevation in mitochondrial Ogg1 activity seems adequately justified, the equivocal results in terms of nuclear Ogg1 could confound data interpretation. However, it is widely appreciated that mtDNA is far more sensitive to oxidant damage than nuclear DNA (22). Indeed, at the XO doses used in the present study, 5–50 mU/ml, we have been unable to detect any evidence of nuclear DNA damage, even with the highly sensitive ligation-mediated PCR technique, while integrity of the mitochondrial genome is severely impaired (12). Because mtDNA appears to be the exclusive target of XO at the doses employed, it seems most reasonable to suspect that any effect of the Ogg1-MTS transfection on cell viability is associated with an increase in mtDNA repair capacity and not with the minor elevation in nuclear Ogg1. That mitochondrial overexpression of Ogg1 was effective at reducing the equilibrium density of mtDNA lesions is suggested by two observations. First, in control PAECs transfected with the Ogg1-MTS construct but not challenged with XO, there was increased hybridization of the mitochondrial DNA probe, indicating a reduction in the constitutive level of damage. More importantly, however, the increase in equilibrium lesion density evoked by 10 mU/ml XO was reduced by nearly 40% in the Ogg1-MTS transfectants. We think that the most significant finding in the present study is that protection of mtDNA afforded by Ogg1-MTS overexpression was associated with attenuation of PAEC cytotoxicity evoked by XO in doses ranging from 5 to 50 mU/ml. Inasmuch as the only known function of Ogg1 involves DNA repair, and since the most likely site at which this activity is exerted in the Ogg1-MTS transfectants is within mitochondria, these findings support the hypothesis that mtDNA serves as a sentinel molecule in PAECs in which excessive or persistent damage triggers cell death pathways. The concept that mtDNA damage is a proximate cause of cell death has implications for future areas of investigation. First, the specific mechanisms linking mtDNA damage to necrotic or apoptotic death could warrant additional study. For the reasons outlined above, mtDNA damage could be lethal to cells by triggering either or both of these pathways. Second, our finding that enhancement of mtDNA repair capacity suppresses XO-mediated PAEC death suggests that overexpression of mtDNA repair enzymes may be of potential use in gene therapy of oxidant-mediated lung injury.

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