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Mitochondria-targeted Ogg1 and Aconitase-2 Prevent Oxidant-induced Mitochondrial DNA Damage in Alveolar Epithelial Cells*

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Background: Mitochondrial Ogg1 prevents oxidant (H_2O_2 and asbestos)-induced Aco-2 degradation and apoptosis.
Results: Oxidant stress caused preferential AEC mtDNA > nuclear DNA damage, mt-p53 translocation, and apoptosis; effects were blocked by mt-hOgg1 or Aco-2.
Conclusion: mt-hOgg1 and Aco-2 preserve AEC mtDNA, preventing oxidant-induced p53 activation and apoptosis.
Significance: mt-hOgg1/Aco-2 effects on mtDNA may be an innovative target for preventing degenerative diseases.

Mitochondria-targeted human 8-oxoguanine DNA glycosylase (mt-hOgg1) and aconitase-2 (Aco-2) each reduce oxidant-induced alveolar epithelial cell (AEC) apoptosis, but it is unclear whether protection occurs by preventing AEC mitochondrial DNA (mtDNA) damage. Using quantitative PCR-based measurements of mitochondrial and nuclear DNA damage, mtDNA damage was preferentially noted in AEC after exposure to oxidative stress (*e.g.* amosite asbestos (5–25 μg/cm^2) or H_2O_2 (100–250 μM)) for 24 h. Overexpression of wild-type mt-hOgg1 or mt-long α/β 317–323 hOgg1 mutant incapable of DNA repair (mt-hOgg1-Mut) each blocked A549 cell oxidant-induced mtDNA damage, mitochondrial p53 translocation, and intrinsic apoptosis as assessed by DNA fragmentation and cleaved caspase-9. In contrast, compared with controls, knockdown of Ogg1 (using Ogg1 shRNA in A549 cells or primary alveolar type 2 cells from *ogg1*$^{-/-}$ mice) augmented mtDNA lesions and intrinsic apoptosis at base line, and these effects were increased further after exposure to oxidative stress. Notably, overexpression of Aco-2 reduced oxidant-induced mtDNA lesions, mitochondrial p53 translocation, and apoptosis, whereas siRNA for Aco-2 (siAco-2) enhanced mtDNA damage, mitochondrial p53 translocation, and apoptosis. Finally, siAco-2 attenuated the protective effects of mt-hOgg1-Mut but not wild-type mt-hOgg1 against oxidant-induced mtDNA damage and apoptosis. Collectively, these data demonstrate a novel role for mt-hOgg1 and Aco-2 in preserving AEC mtDNA integrity, thereby preventing oxidant-induced mitochondrial dysfunction, p53 mitochondrial translocation, and intrinsic apoptosis. Furthermore, mt-hOgg1 chaperoning of Aco-2 in preventing oxidant-mediated mtDNA damage and apoptosis may afford an innovative target for the molecular events underlying oxidant-induced toxicity.

Reactive oxygen species (ROS)[2] generated under physiologic conditions from the mitochondrial electron transport chain and other sources activate cellular signaling important for survival; however, higher levels of ROS cause oxidative DNA damage and apoptosis that promote aging, tumorigenesis, and degenerative diseases (1–3). Mitochondrial DNA (mtDNA) damage is more abundant and longer lasting than nuclear DNA damage after exposure to oxidative stress because of the proximity of mtDNA to the electron transport chain and because mtDNA lacks histones that serve as a barrier against ROS (2–4). 8-Oxo-7,8-dihydroxyguanine (8-oxoG), which is one of the most abundant DNA adducts caused by oxidative stress, is causally associated with several cancers and neurodegenerative diseases because it induces mutations by preferentially mispairing with adenosine during replication (1, 2). 8-Oxoguanine DNA glycosylase (Ogg1), a key enzyme in base excision repair, excises and repairs 8-oxoG in the mitochondria and nucleus (1–6). Mitochondrial base excision repair enzymes, which are all nuclear encoded and imported into the mitochondria, are primarily responsible for removing abnormal DNA adducts, including 8-oxoG from mtDNA (1, 6). Accumulating evidence implicates a direct association between mtDNA damage and mitochondria-regulated (intrinsic) apoptosis; most notably, overexpression of mitochondria-targeted Ogg1 (mt-Ogg1) prevents mtDNA damage and intrinsic apoptosis in response to a variety of oxidative stresses in various cell types (7–12). How-

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[2] The abbreviations used are: ROS, reactive oxygen species; 8-oxoG, 8-oxo-7,8-dihydroxyguanine, Ogg1, 8-oxoguanine DNA glycosylase; hOgg1, human Ogg1; AEC, alveolar epithelial cell(s); AT2, alveolar type II; Aco-2, aconitase-2; Q-PCR, quantitative PCR.

ever, whether alternative mechanisms modulate mtDNA damage that affect intrinsic apoptosis are not fully established.

Ineffective repair of damaged alveolar epithelial cells (AEC) and AEC apoptosis are implicated in mediating pulmonary fibrosis in humans with idiopathic pulmonary fibrosis and in animal models of fibrotic lung disease, including asbestosis (13–16). Notably, targeted injury of alveolar type II (AT2) cells appears necessary for triggering pulmonary fibrosis (17). We previously reported that oxidative stress caused by exposure to asbestos fibers (exogenous) or H_2O_2 (endogenous) induces AEC mitochondrial dysfunction, mitochondrial ROS production, p53 activation, and intrinsic apoptosis (11, 16, 18, 19). Furthermore, we showed that overexpression of wild-type mt-hOgg1 (mt-hOgg1-WT) and a mt-long α/β 317–323 hOgg1 mutant incapable of DNA repair (mt-hOgg1-Mut) each prevent intrinsic AEC apoptosis despite high levels of mitochondrial ROS stress in part because a novel function of hOgg1 chaperoning mitochondrial aconitase (Aco-2) from oxidative degradation (11). Aco-2, one of the enzymes participating in the tricarboxylic acid cycle (TCA), acts as a biosensor for oxidative stress and preserves mtDNA in yeast independent of Aco-2 activity (20, 21). However, the role of mtDNA damage in triggering AEC apoptosis as well as whether Aco-2 modulates mtDNA damage in eukaryotic cells (including AEC) are unknown.

In this study we reasoned that preservation of mtDNA by mitochondria-targeted hOgg1 and/or Aco-2 is necessary for preventing oxidant (amosite asbestos or H_2O_2)-induced AEC mitochondrial dysfunction, p53 translocation to the mitochondria, and intrinsic apoptosis. Using a quantitative-PCR (Q-PCR)-based mitochondrial and nuclear DNA damage assay, we show that oxidative stress induces preferential mtDNA damage in AEC. Furthermore, using hOgg1 and Aco-2 over- and underexpression conditions, we establish that both hOgg1 and Aco-2 are important in limiting oxidant-induced mtDNA damage and subsequent p53 mitochondrial translocation and intrinsic apoptosis. Intriguingly, primary AT2 cells from *ogg1*$^{-/-}$ mice have reduced Aco-2 expression and increased levels of mtDNA lesions and intrinsic apoptosis under untreated conditions as compared with wild-type AT2 cells, and these deleterious effects are further enhanced after exposure to oxidative stress. Finally, we show that siRNA against Aco-2 alters the protective effects of mt-hOgg1-Mut but not mt-hOgg1-WT against oxidant-induced mtDNA damage and apoptosis. Collectively, these findings establish a novel role for mt-hOgg1 and Aco-2 in preserving AEC mtDNA integrity that prevents downstream mitochondrial dysfunction, p53 activation, and intrinsic apoptosis.

EXPERIMENTAL PROCEDURES

Reagents—Amosite asbestos fibers utilized herein were Union International Centere le Cancer reference standard samples kindly supplied by Drs. V. Timbrell and Andy Ghio (Environmental Protection Agency) as characterized and handled as described previously (18). All other reagents were purchased from Sigma unless otherwise stated.

Cell Culture—The A549 human lung adenocarcinoma cell line, MLE-12 mouse lung epithelial cell line, and RLE-6TN rat AT2 cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Primary isolated AT2 cells were isolated from the lungs of wild-type C57/BL6 and *ogg*$^{-/-}$ mice using techniques previously described (22) and approved by the Animal Care and Use Committee. A549 cells, primary mice AT2 cells, and MLE-12 cells were maintained in DMEM (Invitrogen) with 2 mM L-glutamine supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. RLE-6TN cells were maintained at 37 °C in Ham-F12 (Invitrogen) with 2 mM L-glutamine supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated in 6-well plates or 100-mm dishes and grown to confluence before adding asbestos or H_2O_2 for 24 h as described (11).

hOgg1 and Aco-2 Overexpression—Aco-2 (Invitrogen), wild-type, and mutant α-Ogg1 plasmid constructs were previously described (11). Exogenous expression of hOgg1-WT and hOgg1-Mut was identified by immunostaining using c-Myc antibody (Santa Cruz Biotechnology, Dallas, TX). The plasmids were transiently transfected into A549 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommendations. The transfection efficiency is >95%. After transfection for 48 h, the cells were tested for Western blot and DNA fragmentation as previously described (11, 18, 19) as well as mtDNA damage (see below).

Gene Silencing—hOgg1-specific shRNA with the target sequences of 5′- UCCAAGGUGUGCGACUGCUGCGACA-3′ (Integrated DNA Technologies, Coralville, IA), RNA interference for Aco-2 (Invitrogen), and scrambled shRNA controls (Invitrogen) were transiently transfected into A549 cells using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer's recommendations. After transfection for 48 h, the cells were tested for Western blot and DNA fragmentation as previously described (11) as well as mtDNA damage (see below).

mtDNA Damage Assay—Genomic DNA, including both nuclear and mtDNA, was assessed by Q-PCR as described elsewhere (23). Briefly, genomic DNA was extracted using the Qiagen Genomic-tip 20/G and Qiagen DNA Buffer Set (Qiagen, Gaithersburg, MD) per the manufacturer's protocol. The cells were incubated in a lysis buffer containing RNase A (Invitrogen) and proteinase K (Qiagen) for 2 h. Total DNA contained in the extracts is bound to Qiagen Genomic-tip 20/G columns, washed, and eluted. Eluted DNA was incubated with isopropyl alcohol at −80 °C overnight and centrifuged 12,000 × *g* for 60 min. To wash DNA, we evenly added 0.7 ml (70%) of ethanol to each aliquot and centrifuged samples at 9500 − *g* for 30 min. After removing the supernatant, Tris-EDTA buffer (pH 7.5) was added the each tube, and the DNA concentration was measured. PCR was performed using Ex-taq (Clontech, Mountain View, CA) with specific primers (Table 1) to amplify a fragment of the mitochondrial genome, both a short and long fragment and nuclear DNA (β-globulin) as described (23). Each DNA was quantified by Pico-green (Invitrogen) using the FL600 Microplate Fluorescence Reader parameters excitation and emission wavelengths 485 and 530 nm. Then the data were obtained from the small fragment were subsequently used to normalize the results of the mitochondrial long fragment (23).

TABLE 1
The sequences of primer pairs to amplify human, mouse, and rat target genes for Q-PCR-based DNA damage assay

Human		
β-Globin gene (nucleus,13.5 kb)	5′-TTG AGA CGC ATG AGA CGT GCA G-3′	Sense
	5′-GCA CTG GCT TAG GAG TTG GAC T-3′	Antisense
Mitochondria long fragment (8.9 kb)	5′- TCT AAG CCT CCT TAT TCG AGC CGA-3′	Sense
	5′-TTT CAT CAT GCG GAG ATG TTG GAT GG-3′	Antisense
Mitochondria short fragment (222 bp)	5′-CCC CAC AAA CCC CAT TAC TAA ACC CA-3′	Sense
	5′-TTT CAT CAT GCG GAG ATG TTG GAT GG-3′	
Mouse		
β-Globin gene (nucleus,8.7 kb)	5′-TTG AGA CTG TGA TTG GCA ATG CCT-3′	Sense
	5′-CCT TTA ATG CCC ATC CCG GAC T-3′	Antisense
Mitochondria long fragment (10 kb)	5′-GCC AGC CTG ACC CAT AGC CAT AAT AT-3′	Sense
	5′-GAG AGA TTT TAT GGG TGT AAT GCG G-3′	Antisense
Mitochondria short fragment (117 bp)	5′-CCC AGC TAC TAC CAT CAT TCA AGT-3′	Sense
	5′-GAT GGT TTG GGA GAT TGG TTG ATG T-3′	
Rat		
TRPM-2 (nucleus,12.5 kb)	5′-AGA CGG GTG AGA CAG CTG CAC CTT TTC-3′	Sense
	5′-CGA GAG CAT CAA GTG CAG GCA TTA GAG-3′	Antisense
Mitochondria long fragment (13.4 kb)	5′-AAA ATC CCC GCA AAC AAT GAC CAC CC-3′	Sense
	5′-GGC AAT TAA GAG TGG GAT GGA GCC AA-3′	Antisense
Mitochondria short fragment (235 bp)	5′-CCT CCC ATT CAT TAT CGC CGC CCT TGC-3′	Sense
	5′-GTC TGG GTC TCC TAG TAG GTC TGG GAA-3′	

The number of mitochondrial lesions was calculated by the equation, $D = (1–2^{-(\Delta long - \Delta short)}) \times 10{,}000$ (bp)/size of the long fragment (bp).

Apoptosis Assays—DNA fragmentation for apoptosis was assessed using a histone-associated DNA fragmentation (mono and oligonucleosomes) Cell Death detection kit (Cell Signaling Technology, Beverly, MA) as previously described (11, 18, 19).

Apoptosis was also determined by flow cytometric analysis of Annexin V staining using an APC Annexin V kit (BD Pharmingen) according to the manufacturer's recommendations. Briefly, the cells were washed twice using cold PBS and then resuspended in 1× binding buffer (10 mM Hepes (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$) at a concentration of 1×10^6 cells/ml. 1×10^5 cells were transferred to a new test tube, and 5 μl of APC Annexin V was added for 15 min at room temperature in the dark. 400 μl of 1× binding buffer with 3 μM 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI, Invitrogen) were added to each tube. At the end of the incubation, the cells were analyzed by a FACSAria 4-Laser (BD Pharmingen). The values were determined with H_2O_2-induced A549 cells and are described under "Results" ($n = 3$).

Western Blot—Cell lysates were collected, and immunoblotting was performed as described (11, 19). For p53 localization studies, we separated the total cellular protein into the mitochondrial and the cytosolic fractions using a Mitochondria Isolation kit (Thermo Fisher Scientific Inc., Rockford, IL) according to the manufacturer's recommendations as previously described (19). Protein concentration was quantified by BCA protein assay kit (Thermo Fisher Scientific). Proteins were resolved in 4∼20% acrylamide gel (Bio-Rad), transferred onto a nitrocellulose membrane, and incubated with specific antibodies. Membranes were developed with an ECL chemiluminescence detection kit (GE Healthcare Bio-Sciences, Pittsburgh, PA). The antibodies for Western blotting included polyclonal antibodies directed against hOgg1 (Novus Biological, Cambridge, UK), mitochondrial aconitase (Abcam, Cambridge, UK), cleaved caspase-9 (Cell Signaling Technology), and cytochrome oxidase IV (Cell Signaling Technology). Anti-GAPDH, c-Myc, and p53 were purchased from Santa Cruz Biotechnologies. The protein bands were visualized by enhanced chemiluminescence reaction (GE Healthcare Bio-Sciences) and quantified by densitometry using Eagle Eye software (Stratagene, La Jolla, CA).

Statistical Analysis—The results of each experimental condition were determined from the mean of duplicate-triplicate trials. Data was expressed as the means ± S.E. ($n = 3$ unless otherwise stated). A two-tailed Student's t test was used to assess the significance of differences between two groups. Analysis of variance was used when comparing more than two groups; differences between two groups within the set were analyzed by a Fisher's protected least significant differences test as well as Tukey tests. Probability values <0.05 were considered significant.

RESULTS

Oxidative Stress Induces mtDNA Damage in Several Types of AEC—To investigate the effect of oxidative stress (asbestos or H_2O_2) on AEC DNA damage, we used a Q-PCR-based measurement of mitochondrial and nuclear DNA damage. We found that mtDNA lesions in A549 cells are increased in a dose-dependent manner after exposure to asbestos (5–25 $\mu g/cm^2$) or H_2O_2 (100–250 μM) over 24 h (Fig. 1*A*). Oxidative stress also induced mtDNA damage in primary isolated murine AT2, MLE-12, and RLE-6TN rat AT2 cells, although the levels of mtDNA lesions were less than that noted in A549 cells (Fig. 1*A*). Increased mtDNA lesions after exposure to asbestos or H_2O_2 corresponded with slightly reduced mtDNA copy number in all four AEC (Fig. 1*B*). Notably, negligible levels of nuclear DNA damage to β-globulin (human and mice) or clusterin (rat) were detected in these AEC after asbestos or H_2O_2 exposure over 24 h (Fig. 1). These data show that oxidative stress from either asbestos or H_2O_2 induces greater levels of mtDNA as compared with nuclear DNA damage in various types of AEC.

Mitochondrial-targeted hOgg1 Blocks Asbestos-induced mtDNA Damage and Apoptosis—Previous studies have established that mitochondrial-targeted mt-hOgg1-WT prevents oxidant-induced apoptosis in part by augmenting repair of mtDNA damage, but a similar role in AEC is unclear (8–10, 13,

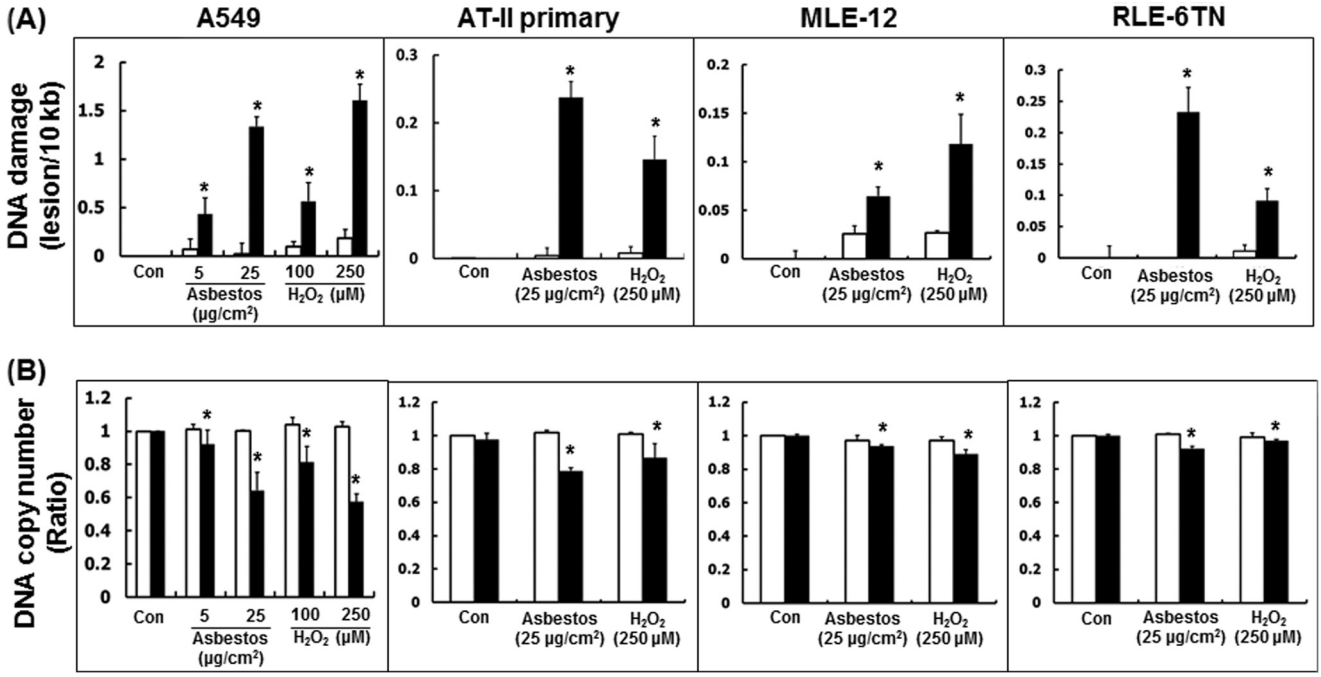

FIGURE 1. **Damaged mtDNA was increased in several types of AEC exposed to asbestos or H_2O_2.** A549 cells were exposed to asbestos (5–25 μg/cm^2) or H_2O_2 (100–250 μM) for 24 h in a dose-dependent manner. Primary ATII cells, MLE-12, and RLE-16TN were exposed to asbestos (25 μg/cm^2; ASB) or H_2O_2 (250 μM) for 24 h. Nuclear DNA and mtDNA damage assay was performed by a Q-PCR-based measurement using isolated whole genomic DNA from each condition. Each pico-green-stained value was calculated to lesions per 10 kb of the nucleic and mitochondrial genome (*A*) and their copy number (*B*). The damage and copy number of nuclear DNA was converted after assay by a Q-PCR-based measurement using β-globin primer (human and mouse) and clusterin (rat). The *open bar* is nuclear DNA, and the *closed bar* is mtDNA. *, $p < 0.05$ *versus* control (*Con*); $n = 3$).

24). We reported that mt-hOgg1-WT and mt-hOgg1-Mut lacking DNA binding and incapable of 8-oxo-G DNA glycosylase activity were both fully capable of preventing oxidant (asbestos and H_2O_2)-induced A549 cell mitochondrial dysfunction and intrinsic apoptosis by chaperoning Aco-2 from oxidative degradation (11). Because Aco-2 has an alternative function outside the TCA cycle in preserving mtDNA integrity in yeast (21), we determined whether the protective effects of mt-hOgg1-WT and mt-hOgg1-Mut against oxidant-induced apoptosis occur by attenuating oxidant-induced mtDNA damage. As expected, mt-hOgg1-WT blocked oxidant-induced mtDNA lesion formation and apoptosis as assessed by DNA fragmentation and cleaved caspase-9 formation (Fig. 2). Notably, mt-Ogg1-Mut, which is incapable of DNA repair yet able to block intrinsic AEC apoptosis (11, 25), also prevented oxidant-induced AEC mtDNA damage and intrinsic apoptosis similar to mt-hOgg1-WT (Fig. 2). Furthermore, H_2O_2-induced empty vector A549 cell apoptosis (11.4 $\pm$ 4.5%) was reduced by either mt-hOgg1-WT (5.2 $\pm$ 2.2%) or mt-Ogg1-Mut (5.3 $\pm$ 2.5%) as assessed by Annexin V staining.

Oxidative stress-induced DNA damage triggers intrinsic apoptosis in part by activating both nuclear p53 that transcriptionally activates pro-apoptotic target genes as well as cytosolic/mitochondrial p53 expression that can interact with Bcl-2 family members to promote Bax and Bak oligomerization (15, 26, 27). We previously showed that p53 mediates asbestos-induced AEC mitochondrial dysfunction and intrinsic apoptosis by both pathways (19). Herein we examined whether mtDNA damage activates mitochondrial translocation of p53 in asbestos-exposed A549 cells transfected with mt-Ogg1-WT or mutant. As shown Fig. 2*C*, compared with empty vector controls, asbestos increased mitochondrial p53 expression in a manner similar to our previous study (19). Notably, asbestos-induced mitochondrial p53 expression was decreased by either mt-Ogg1-WT or mt-hOgg1-Mut, both of which reduced mtDNA damage (Fig. 2). Taken together, our data demonstrate that overexpression of mt-Ogg1-WT or mt-hOgg1-Mut incapable of DNA repair each prevents asbestos-induced AEC mtDNA damage and subsequent mitochondrial p53 localization and intrinsic apoptosis.

Loss of Ogg1 Leads to Enhanced mtDNA Damage and Intrinsic Apoptosis—To investigate whether silencing hOgg1 in AEC augments mtDNA lesions and intrinsic apoptosis, we transfected hOgg1 shRNA in A549 cells and then assessed oxidant-induced mtDNA damage and intrinsic apoptosis. As compared with scramble shRNA controls, down-regulation of hOgg1 enhanced mtDNA damage, mitochondrial p53 localization, and apoptosis (cleaved-caspase 9 activation and DNA fragmentation) under control conditions, and these deleterious effects were enhanced further after exposure to asbestos or H_2O_2 (Fig. 3). Additionally, silencing of hOgg1 augments H_2O_2-induced A549 cells apoptosis activity as compared with scramble transfected control (27.4 $\pm$ 7.4 *versus* 18.1 $\pm$ 3.3%, respectively) as detected by Annexin V staining. Our findings that mtDNA damage induced by hOgg1 depletion results in p53 mitochondrial localization and apoptosis in A549 cells are in accord with a study showing that Ogg1 attenuates oxidative stress-induced apoptosis in fibroblasts through a p53-mediated signaling pathway (28).

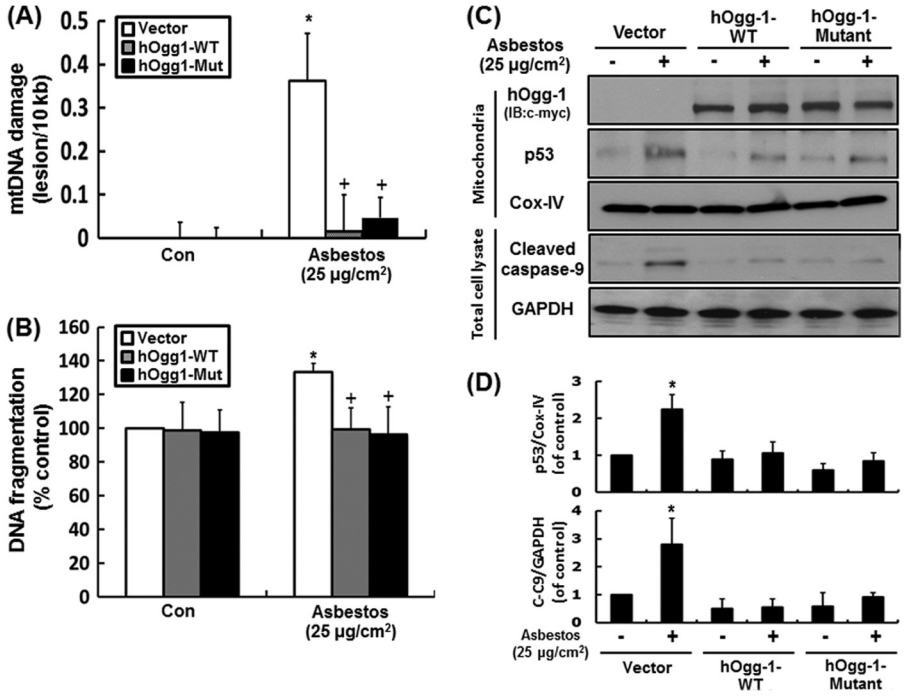

FIGURE 2. **Overexpression of mitochondrial hOgg1 or hOgg1 mutant that is incapable of DNA repair each prevents oxidant-induced A549 cell mtDNA damage and apoptosis.** Mt-Ogg1 WT or mutant was transiently overexpressed by plasmids containing the α isoform of the hOgg1 gene with a mitochondrial targeting sequence and hOgg1 mutant plasmid constructs lacking 8-oxoguanine DNA repair capacity (317–323 long α/β) done by Lipofectamine in A549 cells as described under "Experimental Procedures." A549 cells were exposed to asbestos (25 μg/cm^2; ASB) for 24 h. mt-Ogg1-WT or mutant prevented mtDNA damage (*A*) and DNA fragmentation (*B*) in asbestos-induced A549 cells. *C* and *D*, mitochondria and total cellular proteins were extracted from asbestos-exposed A549 cells transfected with mt-Ogg1-WT or mutant. Cleaved caspase-9 in total cell lysate and p53 levels in mitochondria protein were assessed by Western blotting (*IB*) showing representative figures (*C*). *Bars* represent densitometric quantifications of the p53/Cox-IV and cleaved caspase-9/GAPDH bands normalized to control group with no treatment (*D*). The values are represented as the mean ± S.E. (*, $p < 0.05$ *versus* control, +, $p < 0.05$ *versus* asbestos; $n = 5$).

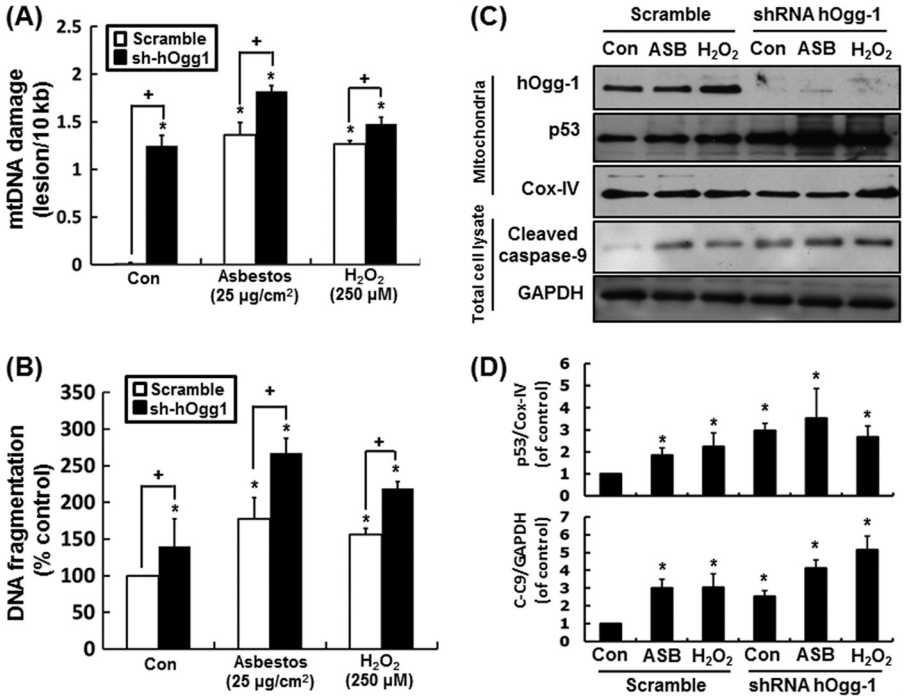

FIGURE 3. **Silencing hOgg1 by shRNA enhances mtDNA damage and apoptosis in asbestos or H_2O_2-exposed A549 cells.** shRNA targeted to hOgg1 was transiently transfected in A549 cells. Cells were exposed to asbestos (25 μg/cm^2; *ASB*) or H_2O_2 (250 μM) for 24 h. mtDNA damage (*A*) and DNA fragmentation (*B*) were increased in asbestos or H_2O_2-induced A549 cells with shRNA for hOgg1. *C* and *D*, mitochondria and total cellular proteins were extracted from asbestos (*ASB*)- or H_2O_2-exposed A549 cells transfected with shRNA for hOgg1. Cleaved caspase-9 in total cell lysate and p53 levels in mitochondria protein were assessed by Western blotting showing representative figures (*C*). *Bars* represent densitometric quantifications of the p53/Cox-IV and cleaved caspase-9/GAPDH bands normalized to control group with no treatment (*D*). The values are represented as the mean ± S.E. (*, $p < 0.05$ *versus* control. +, $p < 0.05$ *versus* Scramble; $n = 3$).

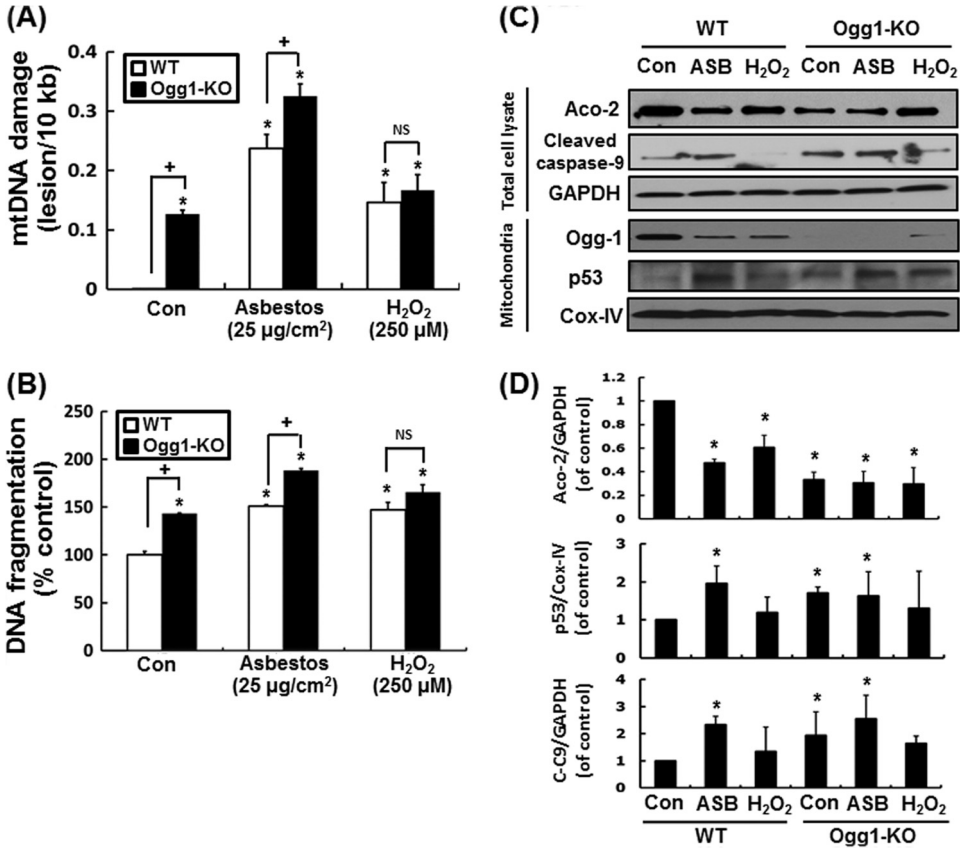

FIGURE 4. **ATII cells from Ogg1$^{-/-}$ increased mtDNA lesions and intrinsic apoptosis after oxidative stress compared with ATII cells from wild-type mice.** ATII cells from wild-type and Ogg1$^{-/-}$ mice were placed on 6-well plate and exposed to asbestos (25 μg/cm^2) or H_2O_2 (250 μM) for 24 h. mtDNA damage (*A*) and DNA fragmentation (*B*) detected more increase in asbestos or H_2O_2-indueced AT-II cells from Ogg1$^{-/-}$ mice than wild-type mice. *NS*, not significant. *C* and *D*, mitochondria and total cellular proteins were extracted from asbestos- or H_2O_2-induced AT-II cells. Aco-2 and cleaved caspase-9 in total cell lysate and p53 level in mitochondria protein were assessed by Western blotting showing representative figures (*C*). *D* shows averaged data for -fold change in Aco-2, p53, and cleaved caspase-9 level as -fold change over basal. The values are represented as the mean ± S.E. (*, $p < 0.05$ *versus* control; $^+$, $p < 0.05$ *versus* WT; $n = 3$).

Transgenic Ogg1 overexpressing and knock-out mice studies suggest an important role for Ogg1 in maintaining mtDNA and mitochondrial function in cardiac muscle, skeletal muscle, and neural stem cells (24, 29, 30). We next investigated whether primary isolated AT2 cells from *ogg1*$^{-/-}$ mice have enhanced mtDNA lesions and intrinsic apoptosis as compared with AT2 cells from Ogg1 wild-type mice. As shown in Fig. 4, compared with wild-type AT2 cells, *ogg1*$^{-/-}$ AT2 cells have increased mtDNA lesions (Fig. 4*A*), reduced Aco-2 expression (Fig. 4, *C* and *D*), p53 mitochondrial expression (Fig. 4, *C* and *D*), and apoptosis (cleaved-caspase 9 activation and DNA fragmentation) under control untreated conditions (Fig. 4, *B* and *C*). These deleterious effects were augmented after exposure to oxidative stress in WT AEC cells (Fig. 4, *A* and *B*). Together, these data show that Ogg1 depletion increases mtDNA damage that leads to the accumulation of mitochondrial p53 and intrinsic apoptosis in AEC and that oxidative stress further promotes these changes.

Aco-2 Prevents Oxidant-induced mtDNA Damage, p53 Activation, and Intrinsic Apoptosis in AEC—Previous studies in yeast have established that Aco-2 has a dual role as a mitochondrial TCA cycle enzyme as well as in preserving mtDNA independent of Aco-2 catalytic activity (21, 31). We showed that Aco-2 attenuates oxidant-induced AEC intrinsic apoptosis (11). To determine whether the protective effects of Aco-2 in AEC are mediated by attenuating mtDNA damage in the setting of oxidative stress, we overexpressed Aco-2 in A549 cells and assessed oxidant-induced mtDNA damage. As shown in Fig. 5*A*, overexpression of Aco-2 reduced mtDNA lesions after exposure to both asbestos or H_2O_2 as compared with empty vector-transfected controls. In accord with our earlier study, overexpression of Aco-2 reduced oxidant-induced AEC intrinsic apoptosis as assessed by cleaved-caspase 9 activation and DNA fragmentation (Fig. 5, *B* and *C*). Furthermore, overexpression of Aco-2 reduced H_2O_2-induced A549 cell apoptosis as compared with empty vector controls (5.2 ± 2.7 *versus* 11.8 ± 5.9%, respectively) as assessed by Annexin V staining. Notably, compared with empty vector controls, Aco-2 overexpression also reduced mitochondrial translocation of p53 caused by oxidative stress (Fig. 5*C*). These data show that Aco-2 overexpression attenuates A549 cell mtDNA damage, p53 mitochondrial expression, and intrinsic apoptosis after exposure to asbestos or H_2O_2.

To investigate whether silencing of Aco-2 augments oxidant-induced AEC mtDNA damage and intrinsic apoptosis, A549 cells were transiently transfected with Aco-2 siRNA. Compared with scramble siRNA-treated control cells, we observed that blocking Aco-2 protein expression augmented mtDNA lesions under control untreated conditions and that these effects were increased further after exposure to asbestos or H_2O_2 (Fig. 6). As

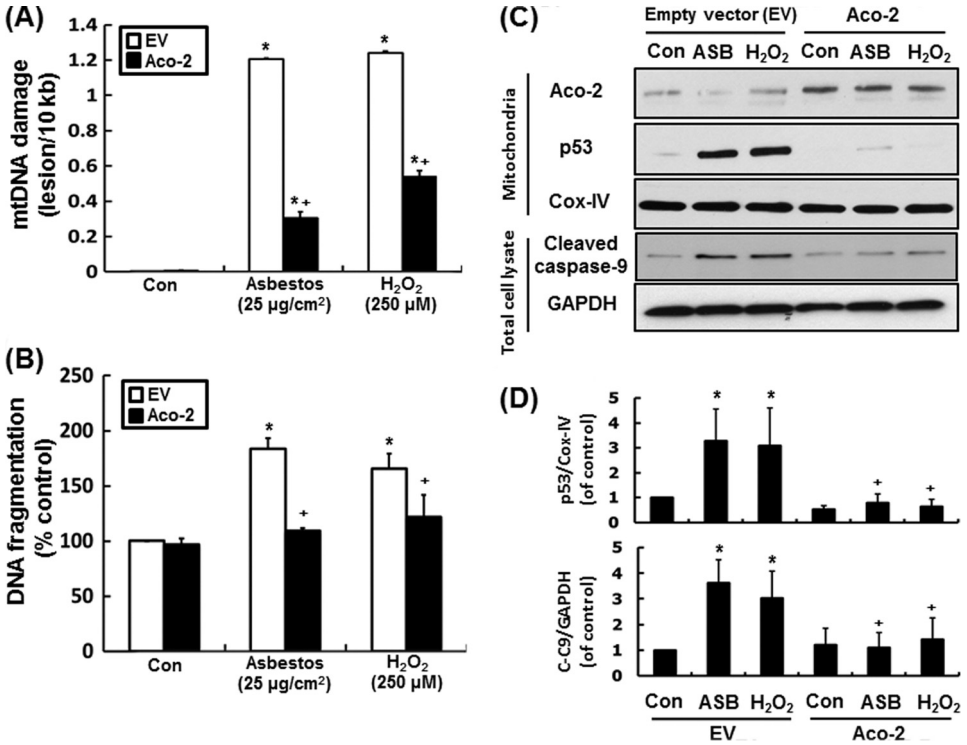

FIGURE 5. **Mitochondria-targeted aconitase overexpression prevents A549 cell mtDNA damage and intrinsic apoptosis exposure to asbestos or H_2O_2.** Mitochondria-targeted aconitase overexpression blocks oxidative stress-induced mtDNA damage (*A*) and DNA fragmentation (*B*) caused by H_2O_2 (250 μM) or asbestos (25 μg/cm^2) for 24 h in A549 cells. *C* and *D*, mitochondria and total cellular proteins were extracted from asbestos-exposed A549 cells transfected with Aco-2 plasmid. Cleaved caspase-9 in total cell lysate and p53 levels in mitochondria protein were assessed by Western blotting showing representative figures (*C*). *ASB*, asbestos. *Bars* represent densitometric quantifications of the p53/Cox-IV and cleaved caspase-9/GAPDH bands normalized to control group with no treatment (*D*). The values are represented as the mean ± S.E. *, $p < 0.05$ *versus* control. +, $p < 0.05$ *versus* empty vector (*EV*); $n = 3$.

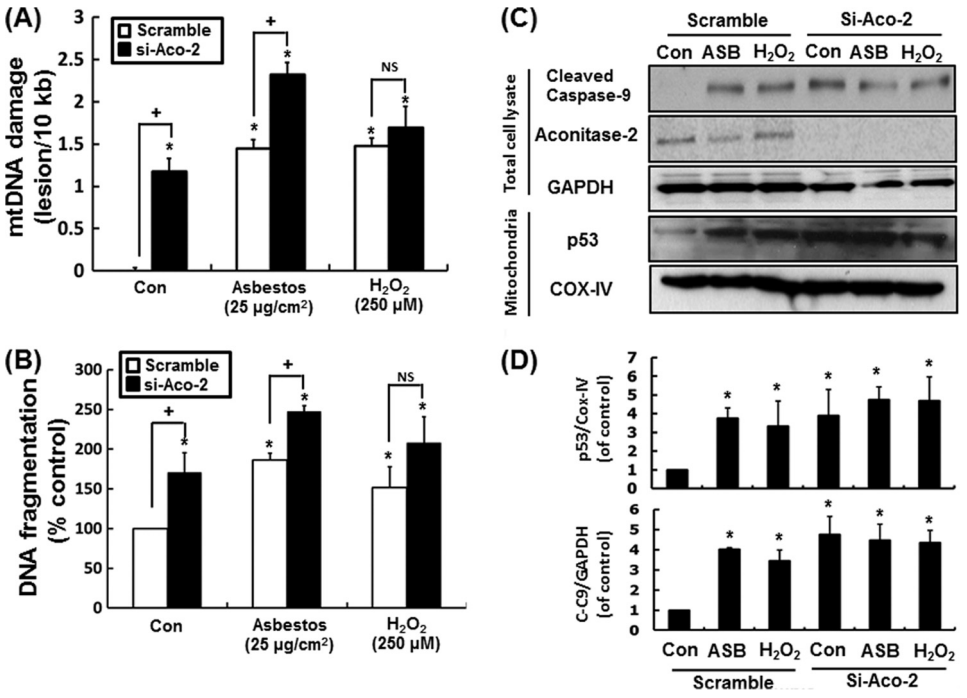

FIGURE 6. **Silencing Aco-2 by siRNA increases mtDNA damage and apoptosis in A549 cells after exposure to oxidative stress.** Cells were transfected with control siRNA or Aco-2 siRNA for 48 h and exposed to asbestos (25 μg/cm^2) or H_2O_2 (250 μM) for 24 h. mtDNA damage (*A*) and DNA fragmentation (*B*) were increased in asbestos or H_2O_2-indueced A549 cells with siRNA for Aco-2. *NS*, not significant. *C* and *D*, mitochondria and total cellular proteins were extracted from asbestos (*ASB*)- or H_2O_2-exposed A549 cells transfected with siRNA for Aco-2. Cleaved caspase-9 in total cell lysate and p53 levels in mitochondria protein were assessed by Western blotting showing representative figures (*C*). *Bars* represent densitometric quantifications of the p53/Cox-IV and cleaved caspase-9/GAPDH bands normalized to control group with no treatment (*D*). The values are represented as the mean ± S.E. *, $p < 0.05$ *versus* control. +, $p < 0.05$ *versus* scramble; $n = 3$.

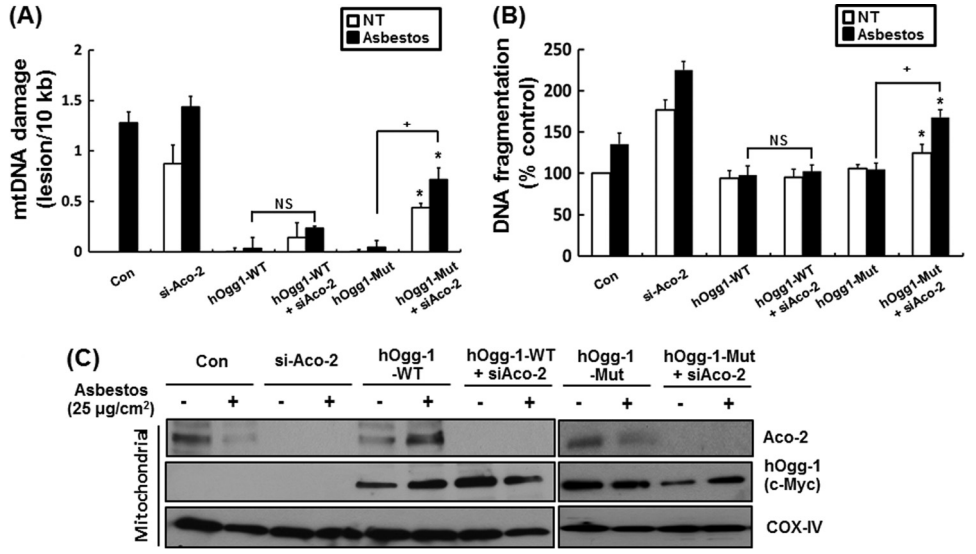

FIGURE 7. **Aco-2 is necessary for the protective effect of mitochondrial hOgg1 mutant, but not mt-hOgg1-WT, overexpression against oxidant-induced mtDNA damage and apoptosis.** Cells were transfected with control siRNA or Aco-2 siRNA. After 6 h, mt-Ogg1 WT or mutant was transiently overexpressed by plasmids in Aco-2 siRNA-transfected cells for 48 h, and then cells were exposed to asbestos (25 μg/cm^2) or H_2O_2 (250 μM) for 24 h showing mtDNA damage (*A*) and DNA fragmentation (*B*). *NS*, not significant; *NT*, nontreatment. *C*, mitochondria proteins were extracted from each cell, and the expression of Aco-2 and mtOgg1 was assessed by Western blotting. *, $p < 0.05$ *versus* Con-NT; +, $p < 0.05$ *versus* NT; #, $p < 0.05$ *versus* con-asbestos; $n = 3$.

compared with controls, Aco-2 siRNA augmented basal levels of apoptosis by nearly 50% as assessed by DNA fragmentation as well as increased cleavage of caspase-9 and mitochondrial p53 translocation (Fig. 6, *B* and *C*). Additionally, compared with scramble controls, Annexin V staining confirmed that Aco-2 siRNA augmented H_2O_2-induced A549 cell apoptosis (18.1 ± 3.3 *versus* 29.5 ± 8.7%, respectively). Asbestos and, to a lesser degree H_2O_2, each modestly induced additional DNA fragmentation, cleavage of caspase-3/9, and p53 mitochondrial expression (Fig. 6, *B* and *C*). Taken together, our findings demonstrate that Aco-2 protects AEC mtDNA from oxidative damage, which subsequently prevents mitochondrial p53 translocation and intrinsic apoptosis.

Aco-2 Is Necessary for the Protective Effects of mt-hOgg1-Mut, but Not mt-hOgg1-WT, in AEC mtDNA Exposed to Oxidative Stress—We previously identified a novel function of mt-hOgg1-WT and mt-hOgg1-Mut in chaperoning Aco-2 from oxidative degradation, thereby preventing intrinsic AEC apoptosis (11). We reasoned that Aco-2 levels would be reduced in AT2 cells from *ogg1*$^{-/-}$ mice and that Aco-2 serves an alternative function in preserving eukaryotic mtDNA integrity in a manner similar to yeast. Compared with AT2 cells from wild-type *ogg1*$^{+/+}$ mice, AT2 cells from *ogg1*$^{-/-}$ mice had reduced Aco-2 levels as expected (Fig. 4*C*). Furthermore, oxidative stress caused additional reductions in AT2 cell Aco-2 levels (Fig. 4*D*). This suggests that mitochondrial Ogg1 modulates Aco-2 expression in AT2 cells. To test whether Aco-2 is necessary for the protective effect of mitochondrial Ogg1 in AEC, we co-transfected Aco-2 siRNA and either mt-hOgg1-WT or mt-hOgg1-Mut in A549 cells. As shown in Fig. 7, compared with scrambled controls, Aco-2 siRNA decreased Aco-2 expression and increased mtDNA damage and DNA fragmentation under basal conditions (nontreatment), and these effects were further enhanced after exposure to asbestos. These changes were similar to what was noted in Fig. 6. Likewise, overexpression of either mt-hOgg1-WT or mt-hOgg1-Mut attenuated mtDNA damage and apoptosis in a manner similar to what was noted earlier (Fig. 2). Notably, co-transfection with Aco-2 siRNA and mt-hOgg1-WT did not block the protective effects of mt-hOgg1-WT despite negligible levels of Aco-2 detected. This suggests that mt-hOgg1-WT overexpression is sufficient for preserving mtDNA integrity and reducing oxidant-induced apoptosis. In contrast with mt-hOgg1-WT, overexpression of mt-hOgg1-Mut did not protect the asbestos-induced mtDNA damage and DNA fragmentation in A549 cells transfected by Aco-2 siRNA (Fig. 7). These data indicate that Aco-2 is necessary for the protective effect of mt-hOgg1-Mut, but not mt-Ogg1-WT, overexpression against oxidant-induced mtDNA damage and apoptosis.

DISCUSSION

Mitochondrial DNA damage by oxidative stress is implicated in contributing to the pathophysiology of diverse conditions, such as heart failure, atherosclerosis, aging, mechanically ventilated injured human diaphragms, and cancer (29–33). Beyond the well described mitochondrial DNA repair pathways, there is a paucity of information about the molecular mechanisms modulating mtDNA damage (1–3, 6). In this study we report that oxidative stress from amosite asbestos or H_2O_2 induces preferential mtDNA damage in a variety of AEC. Furthermore, overexpression of mt-hOgg1-WT or a mt-hOgg1-Mut that is incapable of DNA repair but chaperones Aco-2, each, blocked AEC oxidant-induced mtDNA damage as well as subsequent mitochondrial p53 translocation and intrinsic apoptosis. Compared with controls, Ogg1 knockdown augmented mtDNA lesions and intrinsic apoptosis at base line, and these effects were enhanced further after exposure to oxidative stress. Notably, overexpression of Aco-2 reduced oxidant-induced mtDNA lesions, mitochondrial p53 translocation, and apoptosis, whereas siRNA for Aco-2 (siAco-2) enhanced mtDNA damage,

mitochondrial p53 translocation, and apoptosis. Finally, siAco-2 attenuated the protective effects of mt-hOgg1-Mut but not mt-hOgg1-WT against oxidant-induced mtDNA damage and apoptosis. Collectively, these data demonstrate a novel role for mt-hOgg1 and Aco-2 in preserving AEC mtDNA integrity, thereby blocking oxidant-induced mitochondrial dysfunction, p53 mitochondrial translocation, and intrinsic apoptosis. Importantly, mt-hOgg1 chaperoning of Aco-2 in blocking oxidant-mediated mtDNA damage and apoptosis may be an innovative target for the molecular events underlying oxidant-induced toxicity as seen in a wide variety of settings.

In this study we show that oxidative stress induces preferential mtDNA damage in a variety of AEC (Fig. 1) and that mt-hOgg1-WT overexpression is protective (Fig. 2*A*). The preferential effects of oxidative stress on mtDNA noted herein concur with others (4, 5). Furthermore, the protective effects of mt-hOgg1-WT we show are in accordance with studies from several groups as well as our own (7–12). Although unclear with AEC, a causal link between mtDNA damage and apoptosis has been established in various cell types (7–10, 12). Compared with nuclear DNA, the mutation and damage rate of mtDNA by oxidative stress is severalfold higher in part because of the close proximity of mtDNA to the electron transport chain-generating ROS, the lack of histone-containing proteins that shield mtDNA, and the limited mtDNA repair systems (1–6). A novel finding of this study is that mt-hOgg1-Mut, which lacks the crucial amino acids necessary for 8-oxoG DNA repair (25), prevents oxidant-induced AEC mtDNA damage in a manner similar to mt-hOgg1-WT (Fig. 2). Consistent with our prior study (11), mt-hOgg1-Mut and mt-hOgg1-WT each blocked oxidant-induced intrinsic apoptosis in AEC. Herein, we extend these observations by showing that mt-hOgg1-Mut and mt-hOgg1-WT each attenuate mitochondrial p53 expression, suggesting a reduced DNA damage response (Fig. 2). An important role of mitochondrial ROS in mediating mtDNA damage in our model is supported by several lines of evidence that include the following. 1) p0-A549 cells lacking mtDNA and incapable of mitochondrial ROS production are protected against asbestos-induced p53 activation and apoptosis (18, 19, 37). 2) Using highly sensitive reduction-oxidation-sensitive green fluorescent proteins (RoGFP) targeted to the mitochondria or cytoplasm to detect ROS production, we showed that the mitochondria are the primary source of ROS production (11). 3) The mitochondria-regulated death pathway is the principal pathway mediating AEC apoptosis *in vitro* (18, 34). 4) Antioxidants, including those targeted to the mitochondria, attenuate oxidative stress-induced endoplasmic reticulum stress and intrinsic apoptosis in AEC as well as other cell types (35–37). Thus, the levels of oxidative stress-induced AEC mtDNA damage are crucial in triggering a DNA damage response (*e.g.* p53) and intrinsic apoptosis.

An important observation in this study is that both mt-hOgg1-Mut and Aco-2 protect mtDNA from oxidative damage in a manner similar to mt-hOgg1-WT. Notably, we showed that knockdown of Ogg1 by using either Ogg1 shRNA in A549 cells (Fig. 3) or primary AT2 cells from *ogg1*$^{-/-}$ mice (Fig. 4), each, augments mtDNA damage, p53 mitochondrial expression, and intrinsic apoptosis at base line. We previously reported that Ogg1 shRNA reduces Aco-2 levels in A549 cells and augments apoptosis (11). Herein we report that AT2 cells from *ogg1*$^{-/-}$ mice have decreased Aco-2 levels and that oxidative stress causes a further decline (Fig. 4). Moreover, oxidative stress further enhanced mtDNA damage, mitochondrial p53 translocation, and apoptosis (Figs. 3 and 4). Also, Aco-2 overexpression attenuated oxidant-induced mtDNA lesions as well as mitochondrial p53 expression and intrinsic apoptosis (Fig. 5). In contrast, siRNA for Aco-2 enhanced mtDNA damage, mitochondrial p53 translocation, and apoptosis (Fig. 6). Collectively, these findings establish a novel role for Aco-2 in protecting mtDNA in a eukaryotic cell (AEC) from oxidative damage. To determine whether Aco-2 is necessary for the protective effect of mt-hOgg1 in our model, we co-transfected Aco-2 siRNA and either mt-hOgg1-WT or mt-hOgg1-Mut in A549 cells. We found that depleting Aco-2 attenuated the protective effects of mt-hOgg1-Mut but not mt-hOgg1-WT against oxidant-induced mtDNA damage and intrinsic apoptosis (Fig. 7). The above findings with oxidative stress-induced mtDNA damage are in accord with our earlier study showing that mt-hOgg1-WT, mt-hOgg1-Mut, or Aco-2 overexpression, each, prevent oxidant-induced AEC apoptosis despite high levels of mitochondrial ROS production, an effect that was attributed to a newly described chaperone function of mt-hOgg1-WT or mt-hOgg1-Mut in preventing oxidative degradation of Aco-2 (11). We reason that Aco-2 is necessary for the protective effects of mt-hOgg1-Mut but not mt-hOgg1-WT because the former is incapable of repairing mtDNA damage and Aco-2, and its mtDNA chaperone function is depleted by siACO-2 (11, 25). Our findings concur with investigations showing that Ogg1 depletion promotes oxidant-induced apoptosis and extends these studies by demonstrating that Ogg1 and Aco-2 function coordinately to preserve mtDNA in the setting of oxidative stress (28, 38). Aco-2 is a bifunctional mitochondrial protein that is vital for energy metabolism in the TCA cycle converting citrate to isocitrate as well as important for mtDNA maintenance in yeast. Aco-2 is an iron-sulfur-containing protein that is vulnerable to oxidative inactivation and is implicated as a mitochondrial redox-sensor (21, 39). Ogg1 may be acting similar to frataxin, which is an iron chaperone protein that prevents aconitase oxidative inactivation by Lon protease after oxidative modification and/or augments aconitase reactivation controlling (40, 41). In this regard, Aco-2 binds and provides metabolically coupled protection to mitochondrial DNA independent of Aco-2 catalytic activity (21, 39). Thus, these data demonstrate a novel role for mt-hOgg1 and Aco-2 in preserving AEC mtDNA integrity, thereby preventing oxidant-induced mitochondrial dysfunction, p53 mitochondrial translocation, and intrinsic apoptosis.

The precise molecular mechanisms by which mtDNA damage leads to apoptosis are not fully understood. Our findings of a direct relationship between oxidative stress-induced mtDNA damage and mitochondrial p53 expression are consistent with activation of a DNA damage response. As reviewed in detail elsewhere (5, 15, 26, 27, 42), the mechanisms by which p53, a redox-sensitive transcriptional factor, modulates apoptosis by transcriptional activation of pro-apoptotic molecules and/or transcriptional independent mechanisms acting at the mito-

chondria are complex and not fully established. Our results herein showing p53 mitochondrial expression in the setting of mtDNA damage concur with our earlier study showing that p53 mediates asbestos-induced AEC p53 mitochondrial translocation and intrinsic apoptosis (19). In accord with our findings, p53 activation is required for H_2O_2-induced apoptosis in hOgg1-deficient human fibroblast cells (28), and mitochondrial matrix p53 sensitizes HepG2 cells to oxidative stress by reducing mtDNA (43). Several lines of evidence demonstrate a potentially key association between p53, Ogg1, and Aco-2 including; 1) p53 regulates the transcription of the *OGG1* gene in colon and renal epithelial cells (28), 2) p53-deficient cells have reduced Ogg1 protein expression and activity (28), and 3) p53 can reduce Aco-2 gene expression as well as Aco-2 activity (44, 45). However, p53 has diverse functions in the nucleus and the mitochondria that can promote cell survival or cell death depending upon the experimental conditions. For example, recent studies have shown that under certain experimental conditions mitochondrial p53 can promote mtDNA repair, induce an anti-apoptotic response, or activate necrotic cell death (46–48). Alternative mechanisms may also account for cell death after mtDNA damage. In the pulmonary artery endothelial cells, oxidative stress-induced mtDNA damage and subsequent apoptosis have been attributed to impaired ATP synthesis and reduced mitochondrial gene expression (8, 49). Although the precise molecular mechanisms by which mitochondrial p53 and Aco-2 modulate AT2 cell mtDNA and apoptosis as well as their broader function in other cell types await further study, our results suggest an important role for Aco-2 in maintaining AEC mtDNA integrity and thereby reducing mitochondrial p53 expression and apoptosis.

The *in vivo* relevance of our *in vitro* findings implicating that mt-hOgg1 and Aco-2 preserve mtDNA against oxidative stress are unclear. As reviewed elsewhere, AEC apoptosis and p53 activation are prominently implicated in the pathophysiology underlying idiopathic pulmonary fibrosis and animal models of pulmonary fibrosis, including that due to asbestos exposure (13–16). The potential role of prompt DNA damage repair by Ogg1 has been prominently implicated in several diverse cellular events/diseases including early embryonic development, cardiac fibrosis, diabetes, lung cancer, and neurodegenerative diseases (2, 24, 50–53). OGG1-deficient mice have been shown to accumulate 8-oxoG particularly in mtDNA but are otherwise normal under normal breeding conditions (54–56). Loss of Ogg1 protein function predisposes mice to lung tumorigenesis (57). As compared with wild-type neuronal stem cells, loss of plasticity toward a terminally differentiated astrocytic lineage occurs in Ogg1-deficient neuronal stem cells that accumulate mtDNA damage (58). Ogg1 also has an emerging important role in linking DNA repair to Rac1 and Ras cellular signaling pathways (59, 60). These findings support the notion that defects in Ogg1 and 8-oxoG DNA repair play an important role in enhancing mtDNA damage. Future studies exploring the *in vivo* role of Ogg1 in maintaining AT2 cell Aco-2 levels and mtDNA integrity and in modulating AEC apoptosis using transgenic murine models as well as human lungs in health and disease will be of considerable interest.

In summary, we show that oxidative stress (asbestos and H_2O_2) causes mtDNA lesions that activate downstream p53 mitochondrial expression and mitochondria-regulated AEC apoptosis. Our findings implicate a novel role for mitochondrial Ogg1 and Aco-2 in preserving AEC mtDNA integrity in the setting of oxidative stress that significantly reduces activating the p53 DNA damage response and intrinsic apoptosis. We reason that prevention of oxidant-mediated mtDNA damage and apoptosis by mt-hOgg1 chaperoning of Aco-2 may afford an innovative target for the molecular events underlying oxidant-induced toxicity as occurs in the setting of pulmonary fibrosis and possibly other degenerative diseases.

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