# Modulation of Mitochondrial Gene Expression in Pulmonary Epithelial Cells Exposed to Oxidants

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Oxidants are important in the regulation of signal transduction and gene expression. Multiple classes of genes are transcriptionally activated by oxidants and are implicated in different phenotypic responses. In the present study, we performed differential mRNA display to elucidate genes that are induced or repressed after exposure of rat lung epithelial (RLE) cells to $H_2O_2$ or crocidolite asbestos, a pathogenic mineral that generates oxidants. After 8 or 24 hr of exposure, RNA was extracted, reverse transcribed, and amplified by polymerase chain reaction with degenerate primers to visualize alterations in gene expression. The seven clones obtained were sequenced and encoded the mitochondrial genes, NADH dehydrogenase subunits ND5 and ND6, and 16S ribosomal RNA. Evaluation of their expression by Northern blot analysis revealed increased expression of 16S rRNA after 1 or 2 hr of exposure to $H_2O_2$. At later time periods (4 and 24 hr), mRNA levels of 16S rRNA and NADH dehydrogenase were decreased in $H_2O_2$-treated RLE cells when compared to sham controls. Crocidolite asbestos caused increases in 16S rRNA levels after 8 hr of exposure, whereas after 24 hr of exposure to asbestos, 16S rRNA levels were decreased in comparison to sham controls. In addition to these oxidants, the nitric oxide generator spermine NONOate caused similar decreases in NADH dehydrogenase mRNA levels after 4 hr of exposure. The present data and previous studies demonstrated that all oxidants examined resulted in apoptosis in RLE cells during the time frame where alterations of mitochondrial gene expression were observed. As the mitochondrion is a major organelle that controls apoptosis, alterations in expression of mitochondrial genes may be involved in the regulation of apoptosis. — *Environ Health Perspect* 106(Suppl 5):1191–1195 (1998). *http://ehpnet1.niehs.nih.gov/docs/1998/Suppl-5/1191-1195janssen/abstract.html*

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Alveolar epithelium is a major target for oxidant stress as a result of inhalation of oxidant gases or pathogenic minerals, including asbestos, which is implicated in the development of bronchogenic carcinoma arising in the tracheobronchial epithelium of asbestos workers (*1*). Furthermore, the elicitation of an inflammatory response that occurs after inhalation of these toxicants also generates several reactive oxygen or nitrogen species (ROS/RNS) with potent reactivities. The response of the pulmonary epithelial cell to oxidative stress may depend on the type and concentration of oxidant encountered. For instance, low levels of ROS/RNS can evoke adaptive responses that involve the induction of antioxidant defenses or DNA repair proteins that enable cells to become resistant to conditions of extensive oxidative stress (*2*). Known genes induced by oxidants include antioxidant enzymes, metallothionein, hemeoxygenase, etc. In contrast, exposure of alveolar epithelial cells to high levels of ROS/RNS can result in apoptosis or necrosis (*2,3*).

It is unclear whether the epithelial cells respond to $H_2O_2$, nitric oxide (•NO), or asbestos with expression of the same classes of genes. Different sources of oxidants may evoke unique genetic responses that may lead to distinct functional end points. For instance, in bacteria, different genetic regulons coordinate the expression of distinct classes of genes in response to different oxidants (*4,5*). These OxyR and SoxR/S regulons activate expression of genes induced in response to peroxide or superoxide anion radical ($O_2^{\cdot -}$)-generating agents, respectively. Many of the activated genes encode protein important in antioxidant defense or DNA repair (*4,5*).

Conceivably, mammalian cells may also activate distinct genes in response to different classes of oxidants. In order to examine this, we performed differential mRNA display, a polymerase chain reaction (PCR)-based approach that allows the investigation of all expressed RNA species (*6,7*). With the use of degenerate primers and four classes of 3′ oligo DT primers, multiple classes of genes can be visualized on a sequencing gel and provide a representation of expressed genes. The technique has an advantage over conventional subtractive hybridization approaches, in that it allows the simultaneous evaluation of mRNAs that are induced or repressed. Use of this approach in different models has resulted in identification of numerous genes important in different physiologic processes as well as disease (*8–11*).

Here, we report the modulation of two mitochondrial genes, NADH dehydrogenase, subunits ND5 and 6, and 16S rRNA in rat lung epithelial (RLE) cells exposed to either $H_2O_2$, asbestos or the •NO generator spermine NONOate. After initial exposure to $H_2O_2$, levels of 16S rRNA are increased but decrease at later time periods that coincide with the development of apoptosis. mRNA levels of NADH dehydrogenase are decreased in comparison to sham controls after 1 to 24 hr of exposure to spermine

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Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; NBS, newborn bovine serum; •NO, nitric oxide; $O_2^{\cdot -}$, superoxide; PCR, polymerase chain reaction; RLE cells, rat lung epithelial cells; RPM cells, rat pleural mesothelial cells; RNS, reactive nitrogen species; ROS, reactive oxygen species.

NONOate or $H_2O_2$. Transient increases in levels of 16S rRNA occur in response to asbestos and are followed by decreases compared to controls after 24 hr of exposure. The alterations in expression of these mitochondrial genes coincide with the development of apoptosis, suggesting a link between alterations in mitochondrial transcription and apoptosis.

## Methods

### Cell Culture and Exposure to Test Agents

The rat alveolar epithelial cell line RLE-6TN was propagated in Dulbecco's modified Eagle's medium/F12 containing penicillin and streptomycin and 10% newborn bovine serum (NBS). RLE-6TN expresses characteristics of alveolar type II cells (*12*). Twenty-four hours before the addition of test agents, cells were switched to 1% NBS. Cells were exposed to 200 µM $H_2O_2$ or crocidolite asbestos for 8 hr from the National Institute of Environmental Health Sciences and total RNA was extracted for reverse transcription and PCR.

### RNA Extraction and Synthesis of cDNA

Total RNA was extracted in guanidinium isothiocyanate according to standard procedures (*13*). Contaminating DNA was removed by incubation with RNAse-free RNAse (Promega, Madison, WI) for 1 hr at 37°C. RNA was re-extracted with phenol and precipitated. Two micrograms of RNA were used in differential display reactions as described by the manufacturer's instructions (Genhunter, Nashville, TN). Briefly, RNA was reverse transcribed with Moloney murine leukemia virus reverse transcriptase using an oligo dT primer anchored to the beginning of the poly (A)tail for 1 hr at 37°C. The reaction was terminated by heat inactivation and cDNAs were stored at –20°C until analysis.

### PCR, Reamplification, and Cloning of Differentially Expressed Genes

cDNAs were PCR amplified in the presence of $^{33}$P-deoxyadenosine triphosphate on a Perkin Elmer thermocycler 9600 (Perkin Elmer, Branchburg, NJ) using cycling conditions as follows: denaturing 95°C, 30 sec; annealing 40°C, 2 min; extension at 72°C, 30 sec for 40 cycles, followed by a final extension at 72°C for 5 min. PCR products were resolved on a 6% denaturing polyacrylamide sequencing gel in Tris–borate–EDTA–buffer, dried, and exposed to Kodak-X-omat film. Differentially expressed bands were cut out of the sequencing gel, and reamplified using the same PCR conditions and primer sets. To verify differential expression by Northern blotting, we performed reamplification in the presence of 25 µCi $^{32}$P-deoxycytidine triphosphate (dCTP). cDNAs were next cloned into a Bluescript SK+ vector (Stratagene, La Jolla, CA) and sequenced using standard procedures (*14*). Sequences were checked using the Genbank database.

### Northern Blot Analysis

Ten micrograms of RNA was electrophoresed on formaldehyde 3-(*N*-morpholino)propanesulfonic acid agarose gels, blotted onto nitrocellulose using a semidry turboblotter system (Schleicher and Schuell, Keene, NH). Blots were prehybridized overnight in 50% formamide, 4X standard sodium citrate (SSC), Denhards, and 10 µg/ml salmon testis DNA at 50°C. NADH dehydrogenase or 16S rRNA probes, generated by PCR, were added in hybridization fluid for 16 hr, and blots were washed in a series of SSC and sodium dodecyl sulfate washes as described before. Hybridized signals were visualized by autoradiography and quantitated by phosphoimage analysis. Selected blots were hybridized with glyceraldehyde-3-phosphate dehydrogenase cDNA to verify the RNA content between groups and revealed approximately 10% variability of hybridization signals as has been published elsewhere (*15*) (data not shown).

### Evaluation of Apoptosis

The development of apoptosis in RLE cells exposed to $H_2O_2$ or spermine NONOate was evaluated by flow cytometry using propidium iodide to measure DNA content. The percentage of cells undergoing apoptosis characterized by their hypolipoid DNA content was quantitated. The use of this technique has been described elsewhere (*16*) and confirmed by other techniques (*3*).

### Statistical Analysis

Data were analyzed by ANOVA with the use of Duncan's procedure to adjust for multiple comparisons.

## Results

The use of different combinations of primers in differential display reactions led to unique patterns of mRNA species, observed after electrophoresis on a sequencing gel (Figure 1). Multiple bands are either increased or decreased after exposure to $H_2O_2$ or asbestos in comparison to sham controls (Figure 1). These bands were excised from the sequencing gel, reamplified in the presence of $^{32}$P-dCTP and used in Northern blot analysis. Performing these Northern blots revealed the presence of about 80% false-positive bands that could either not be detected or were not affected when evaluated by Northern blot analysis (data not shown).

We next cloned seven differentially expressed species into an SK$^+$ superscript vector and sequenced the clones. Sequence analysis revealed that five clones encoded the 16S rRNA species, whereas the other mRNA was identified as NADH dehydrogenase, subunits ND5 and ND6. Interestingly, both genes are encoded in the mitochondria.

To determine the time frame of alterations in mRNA levels of NADH dehydrogenase or 16S rRNA, we performed a time-course study with $H_2O_2$. Exposure to 300 µM $H_2O_2$ caused decreases in NADH dehydrogenase mRNA levels after time periods of exposure that range from 1 to 24 hr (Figure 2, A). In contrast, as demonstrated in Figure 2B, early increases

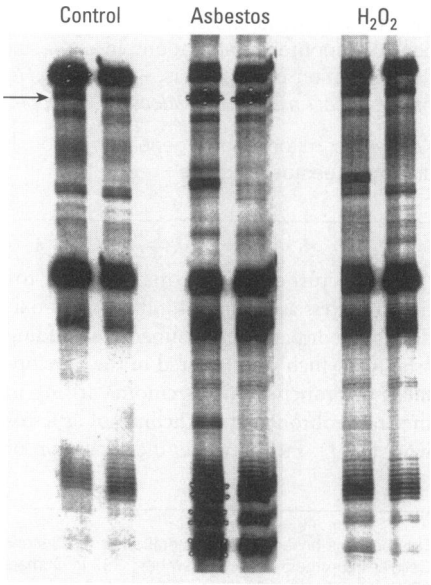


**Figure 1.** Example of a differential display sequencing gel demonstrating modulation of expressed RNAs after exposure to 200 µM $H_2O_2$ or 5 µg/cm$^2$ of crocidolite asbestos. Cells were treated with agents for 8 hr and RNA extracted, reverse transcribed, and PCR amplified in the presence of $^{33}$P and primer combinations T12MG-AP-1 (Genhunter). The arrow represents 16S rRNA. Duplicate lanes are shown for each group. NADH dehydrogenase was identified using additional differential display reactions (not shown here).

in mRNA expression of 16S rRNA occur after 1-hr exposure to $H_2O_2$, whereas after 24-hr exposure to $H_2O_2$, the expression of 16S rRNA is decreased. The differences in hybridization signal in the sham control groups apparent at different time points evaluated are due to hybridization of different blots. Thus, fluctuations in steady-state levels of 16S rRNA or NADH dehydrogenase mRNA in control cultures over time cannot be evaluated here.

Because asbestos caused an increase in the band representing 16S rRNA on the sequencing gel, we evaluated its expression by Northern blot analysis. As shown in Figure 3, asbestos caused increases in 16S rRNA after 8 hr of exposure, whereas 24 hr postexposure, 16S rRNA was decreased compared to sham controls. Interestingly, these alterations were not restricted to RLE cells. Exposure of rat pleural mesothelial (RPM) cells to asbestos for 24 hr also led to decreased expression of 16S rRNA without affecting expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*17*).

We next examined whether the •NO donor, spermine NONOate, caused similar alterations in mRNA expression. As shown in Figure 4, dosage-dependent decreases in mRNA levels of NADH dehydrogenase are observed following 4 hr of exposure to spermine NONOate. Relative decreases in NADH dehydrogenase mRNA induced by 300 μM and 1 mM spermine NONOate, respectively, compared to sham controls are 29 and 13% after 2 hr, and 45 and 60% after 4 hr. At these time points, no alterations in the expression of 16S rRNA were observed in response to spermine NONOate exposure.

Exposure of RLE cells to asbestos or ROS/RNS at concentrations examined

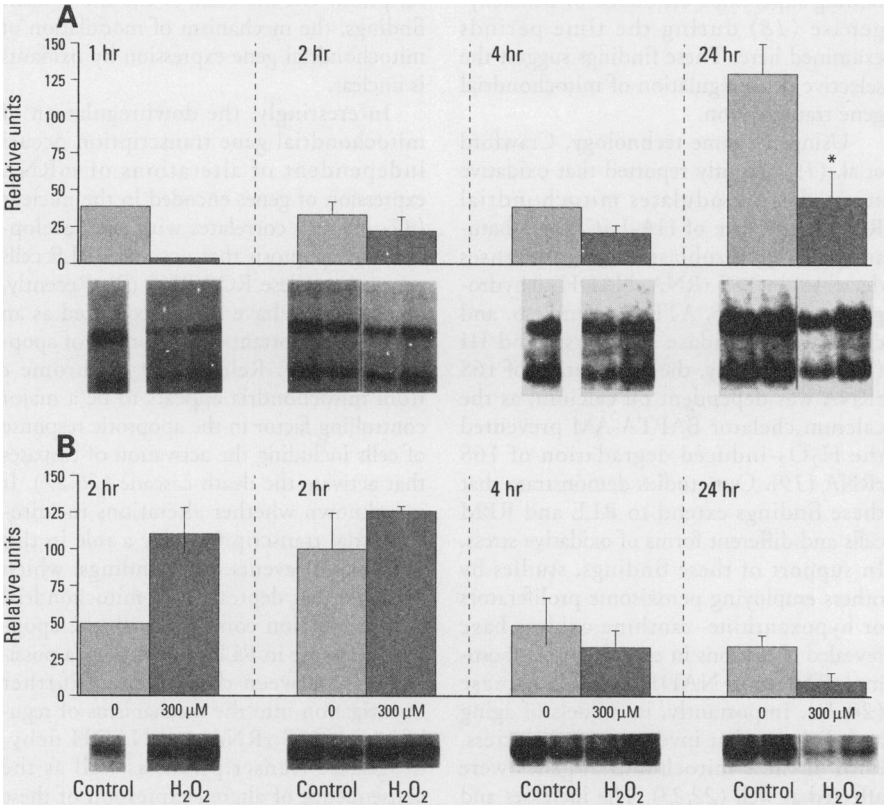


**Figure 2.** Northern blot analysis of (*A*) NADH dehydrogenase and (*B*) 16S rRNA in RLE cells exposed to 300 μM $H_2O_2$ for 1, 2, 4, or 24 hr. RNA was extracted, blotted, and incubated with NADH dehydrogenase and 16S rRNA probes generated by PCR. Blots were quantitated by phosphoimage analysis and expressed as relative units (mean ± SEM) on the *Y*-axis. Control = sham control, $H_2O_2$ = 300 μM ($n$ = 1–2/group, indicated under each bar graph). Note that the differences in hybridization signals in the control groups at different time points is due to different hybridization conditions of separate blots. Percent changes in NADH dehydrogenase mRNA in $H_2O_2$-treated cells compared to controls at different time points are 1 hr, −90%; 2 hr, −33%; 4 hr, −46%; and 24 hr, −65%. Percent changes in 16S rRNA in $H_2O_2$-treated cells compared to controls at different time points are 1 hr, +130%; 2 hr, +30%; 4 hr, −35%; and 24 hr, −70%. * $p<0.05$, ANOVA.

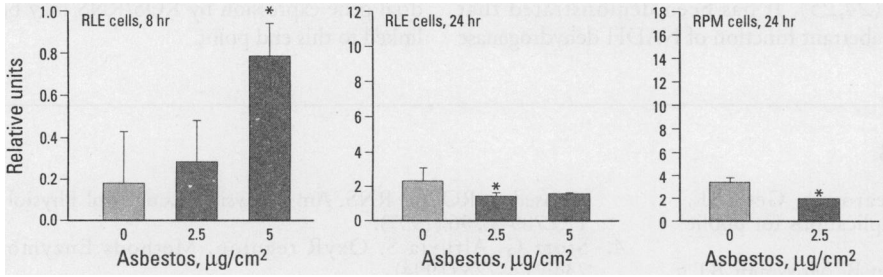


**Figure 3.** Quantitation of a Northern blot analysis evaluating 16S rRNA in RLE or RPM cells exposed to crocidolite asbestos for 8 or 24 hr; $n$=2/group. The GAPDH hybridization signal of the identical blot is published elsewhere (*17*) and does not vary. Blots were quantitated by phosphoimage analysis and expressed as mean ± SEM, and relative units are indicated on the *Y*-axis. *$p<0.05$, ANOVA.

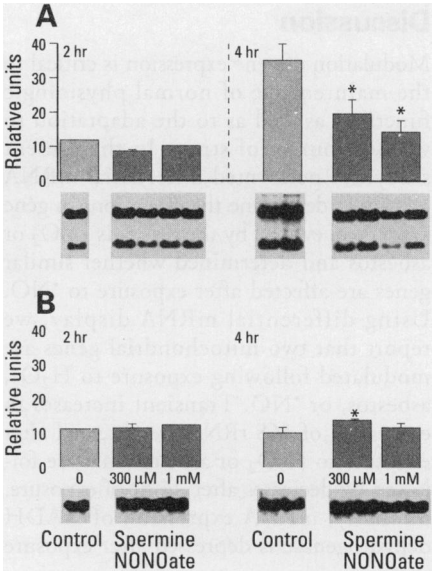


**Figure 4.** Northern blot analysis of (*A*) NADH dehydrogenase or (*B*) 16S rRNA in RLE cells exposed to the •NO donor, spermine NONOate for 2 or 4 hr. Cells were exposed to 300 μM or 1 mM spermine NONOate, which releases •NO over a time frame of 2–4 hr, and RNA was extracted for evaluation by Northern blot analysis. Blots were quantitated by phosphoimage analysis ($n$=2/group, mean ± SEM) and relative units are expressed on the *Y*-axis. Note that the differences in hybridization signals in the control groups at different time points are due to different hybridization conditions of separate blots. * $p<0.05$, ANOVA.

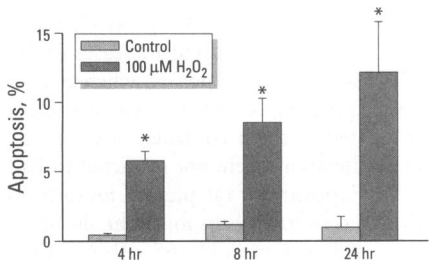


**Figure 5.** Quantitation of apoptosis in RLE cells after exposure to $H_2O_2$. RLE cells were exposed to 100 μM of $H_2O_2$ and analyzed for DNA content via propidium iodide staining and flow cytometry after 4, 8, and 24 hr of exposure. * $p<0.05$, ANOVA.

here causes apoptosis (*3*). To determine whether apoptosis is observed during the time frame of alterations in RNA expression, we performed flow cytometry and analyzed cells for DNA content. As demonstrated in Figure 5, exposure of RLE cells to $H_2O_2$ causes increases in the percentage of cells undergoing apoptosis characterized by a hypodiploid DNA content as early as 4 hr postexposure. These results demonstrate that alterations in mitochondrial gene expression correlate with an apoptotic response in RLE cells.

## Discussion

Modulation of gene expression is critical to the maintenance of normal physiologic function, as well as to the adaptation to various sources of stress. In the present study, we performed differential mRNA display to determine the alterations in gene expression evoked by the oxidants $H_2O_2$ or asbestos and determined whether similar genes are affected after exposure to $^{\bullet}NO$. Using differential mRNA display, we report that two mitochondrial genes are modulated following exposure to $H_2O_2$, asbestos, or $^{\bullet}NO$. Transient increases in expression of 16S rRNA are observed after exposure to $H_2O_2$ or asbestos that are followed by decreases after 24 hr of exposure. Similarly, mRNA expression of NADH dehydrogenase is depressed after exposure to these ROS/RNS. These changes in mitochondrial gene expression were observed independent of alterations in expression of GAPDH used as a housekeeping gene. Furthermore, these ROS/RNS led to increases in expression of protooncogenes (*3*) or the oxidative stress-responsive genes, manganese containing superoxide dismutase or hemeoxygenase (*18*) during the time periods examined here. These findings suggest the selective downregulation of mitochondrial gene transcription.

Using the same technology, Crawford et al. (*19*) recently reported that oxidative stress down modulates mitochondrial RNAs. Exposure of HA-1 (Chinese hamster HA-1) fibroblasts to $H_2O_2$ causes decreases in 16S rRNA, NADH dehydrogenase subunit 6, ATPase subunit 6, and cytochrome oxidase subunits I and III (*19*). Importantly, the degradation of 16S rRNA was dependent on calcium, as the calcium chelator BAPTA-AM prevented the $H_2O_2$-induced degradation of 16S rRNA (*19*). Our studies demonstrate that these findings extend to RLE and RPM cells and different forms of oxidative stress. In support of these findings, studies by others employing peroxisome proliferators or hypoxanthine–xanthine oxidase have revealed alterations in expression of ribosomal RNAs or NADH dehydrogenase (*20,21*). Importantly, in models of aging and diabetes that involve oxidative stress, both of these mitochondrial genes were affected as well (*22,23*). The increases and decreases in RNAs observed in these models may reflect the type or duration of oxidative stress encountered (*20,21*).

It is unclear to date whether these alterations in mitochondrial gene expression are associated with decreases in protein levels or mitochondrial function. Importantly, NADH dehydrogenase is located in the electron transport chain at a site critical in the formation of $O_2^{\bullet -}$ (*24,25*). It has been demonstrated that aberrant function of NADH dehydrogenase or excess levels of the substrate NADH can promote the formation of $O_2^{\bullet -}$ in mitochondria. Conceivably, oxidant-induced downmodulation of mitochondrial gene transcription may prevent the further formation of oxidants. Alternatively, the increased exposure to oxidants may damage mitochondrial function and repress transcription. On the basis of the the present findings, the mechanism of modulation of mitochondrial gene expression by oxidants is unclear.

Interestingly, the downregulation of mitochondrial gene transcription occurs independent of alterations of mRNA expression of genes encoded in the nucleus (*17–19*) and correlates with the development of apoptosis that occurs in RLE cells exposed to these ROS/RNS (*3*). Recently, mitochondria have been recognized as an organelle important in the control of apoptosis (*26,27*). Release of cytochrome *c* from mitochondria appears to be a major controlling factor in the apoptotic response of cells including the activation of caspases that activate the death cascade (*26,27*). It is unknown whether alterations in mitochondrial transcription play a role in this sequence of events. Our findings, which illustrate that depression of mitochondrial gene expression coincides with the apoptotic response in RLE cells, suggest a possible link between these events. Further investigation into the mechanisms of regulation of 16S rRNA and NADH dehydrogenase transcription, as well as the consequence of altered expression of these genes, is needed to address this question.

In summary, our study has utilized the differential display technique and reports the modulation of expression of 16S rRNA and NADH dehydrogenase, two mitochondrial genes in response to $H_2O_2$ or an $^{\bullet}NO$ donor, and the modulation of 16S rRNA in response to asbestos. In view of the importance of the mitochondria in the regulation of apoptosis, the modulation of mitochondrial gene expression by ROS/RNS may be linked to this end point.

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