Mitochondrial oxidant production by a pollutant dust and NO-mediated apoptosis in human alveolar macrophage

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Huang, Yuh-Chin T., Joleen Soukup, Shirley Harder, and Susanne Becker. Mitochondrial oxidant production by a pollutant dust and NO-mediated apoptosis in human alveolar macrophage. Am J Physiol Cell Physiol 284: C24–C32, 2003. First published September 11, 2002; 10.1152/ajpcell.00139.2002.—Residual oil fly ash (ROFA) is a pollutant dust that stimulates production of reactive oxygen species (ROS) from mitochondria and apoptosis in alveolar macrophages (AM), but the relationship between these two processes is unclear. In this study, human AM were incubated with ROFA or vanadyl sulfate (VOSO₄), the major metal constituent in ROFA, with or without nitro-l-arginine methyl ester (L-NAME), diphenyleneiodonium (DPI), and mitochondrial electron transport inhibitors. Interactions among production of ROS, nitric oxide (NO), and apoptosis of AM were determined. ROFA-stimulated ROS production was attenuated by DPI, rotenone, antimycin, and NaN₃, but not by L-NAME, a pattern mimicked by VOSO₄. ROFA-induced apoptosis was inhibited by L-NAME and a caspase-3-like protease inhibitor, but not by mitochondrial inhibitors. ROFA enhanced NO-mediated increase in caspase-3-like activity. VOSO₄ had minor effects on apoptosis. Thus ROFA-stimulated production of ROS from mitochondria was independent of apoptosis of AM, which was mediated by activation of caspase-3-like proteases and NO. The pro-oxidant effect but not the proapoptotic effect of ROFA was mediated by vanadium.

The alveolar macrophage (AM) is one of the cell types in the lungs constantly exposed to the ambient environment. Upon contact with certain environmental particulate pollutants, AM are activated and produce a large quantity of reactive oxygen species (ROS) in the form of chemiluminescence burst. Examples of pollutant particles capable of enhancing the production of ROS by AM include oil fly ash and residual oil fly ash (ROFA) (3, 4, 30, 39) and the Utah Valley dust (48). The exact sources for ROS produced by pollutant-activated AM vary depending on the particles used. The membrane NADPH oxidase appears to be an important source in AM stimulated with quartz dusts, metal-containing dusts, or silica particles coated with a single metal oxide (17). AM may also produce ROS from mitochondria when exposed to combustion-derived particles, such as ROFA (3). ROS produced by AM may then serve as signaling molecules for downstream events including inflammation, cell growth, and cell death, although previous studies have shown that ROFA-induced ROS production does not predict activation of NF-κB or induction of IL-8 (4, 39).

Environmental pollutants may also cause apoptotic cell death. Human AM exposed to ROFA and urban particles showed morphological features and DNA changes consistent with apoptosis (26). The human fibroblast cell line MRC-5 undergoes apoptosis when exposed to extracts of automobile exhaust (53). Diesel exhaust particles induce apoptosis in AM and RAW264.7 cells (25). The programmed cell death is considered an integral part of the host mechanisms by which the homeostasis of the microenvironment is maintained.

In the present study, we determined how ROFA-induced mitochondrial production of ROS is related to apoptosis in human AM. Because vanadium is the major metal constituent in ROFA and is also capable of inducing chemiluminescence (23, 45), we also determined the role of vanadium in mediating the pro-oxidant and proapoptotic effects of ROFA. In addition, because nitric oxide (NO) is a known stimulant for apoptosis of macrophages (1) and its levels can be affected by superoxide, we also determined how ROFA-

EXPOSURE TO PARTICULATE MATTER is consistently associated with increased morbidity and mortality attributable in part to respiratory illnesses (13, 43). Patients with chronic lung conditions, such as asthma or chronic obstructive pulmonary disease (COPD), are among the most susceptible (20, 51). For asthmatics, an increase of 10 μg/m³ in PM₁₀ was associated with a 3–6% increase in outpatient visits (21), a 3–4% increase in emergency room visits (46), and a 2–3% increase in hospital admissions (13, 47). For patients with COPD, an increase of 10 μg/m³ in PM₁₀ was linked to a 1–3% increase in emergency room visits (49) and a 1–2% increase in hospital admissions (9). These adverse pulmonary effects are thought to be related to pulmonary inflammation as a result of activation of resident lung cells.

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Table 1. ROFA produces a dose-dependent increase in AM apoptosis and necrosis

<table>
<thead>
<tr>
<th>ROFA, μg/ml</th>
<th>Hoechst (+), %</th>
<th>PI (+), %</th>
<th>Hoechst (-)/PI (-), %</th>
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<tbody>
<tr>
<td>0</td>
<td>1.4(0.7)</td>
<td>5.9(1.0)</td>
<td>92.8(1.0)</td>
</tr>
<tr>
<td>10</td>
<td>3.9(0.8)</td>
<td>4.7(0.5)</td>
<td>91.4(1.3)</td>
</tr>
<tr>
<td>50</td>
<td>4.8(0.8)</td>
<td>15.8(1.7)</td>
<td>79.5(0.9)</td>
</tr>
<tr>
<td>100</td>
<td>6.7(0.6)</td>
<td>14.7(0.8)</td>
<td>78.6(0.7)</td>
</tr>
<tr>
<td>200</td>
<td>8.8(0.9)</td>
<td>25.7(1.4)</td>
<td>65.5(2.2)</td>
</tr>
</tbody>
</table>

Residual oil fly ash (ROFA) produces a dose-dependent increase in the proportion of alveolar macrophages (AM) with positive Hoechst stain (apoptosis) and positive propidium iodide (PI) uptake (necrosis). AM were incubated with different concentrations of ROFA for 24 h and stained with Hoechst 33342 dye for 15 min at 37°C. At least 200 cells were counted. Cells that were positive for both Hoechst and PI were counted as Hoechst positive. Each value represents the average (SE) from 3 independent experiments.

induced ROS production modulated NO production and NO-mediated apoptosis.

MATERIALS AND METHODS

Reagents and Chemicals

Vanadyl sulfate (VOSO$_4$) was obtained from Johnson Matthey (Ward Hill, MA). DetANOate was obtained from Cayman Chemical (Ann Arbor, MI). 4,5-Diaminofluorescein diacetate (DAF-2DA) was obtained from Calbiochem-Novabiochem (San Diego, CA). DEVD was obtained from R&D Systems (Minneapolis, MN). All other chemicals, unless otherwise specified, were obtained from Sigma Chemical (St. Louis, MO). Residual oil fly ash (ROFA) was acquired from Southern Research Institute (Birmingham, AL). The sample was collected downstream from the cyclone of a power plant in Florida that was burning a low-sulfur no. 6 residual oil (collection temperature of 250–300°C). The metal contents in ROFA (in μg/mg) are 41.7 V, 37.5 Ni, 23.3 Fe, 1.0 Zn, and 0.2 Cu (11).

Isolation of Human AM

Human AM were obtained by bronchoalveolar lavage (BAL) from a total of 67 bronchoscopies in normal individuals according to procedures described previously (18). Subjects were informed of the procedures and potential risks, and each signed an informed consent. The protocol was approved by University of North Carolina School of Medicine Committee on Protection of the Rights of Human Subjects. Briefly, the fiberoptic bronchoscope was wedged into a segmental bronchus of the lingual lobe. Six aliquots of sterile saline were instilled and immediately aspirated. The first was 20 ml and was not used for AM isolation. The remaining five aliquots were 50 ml each. The procedure was repeated on the right middle lobe. BAL samples were put on ice immediately and centrifuged at 300 g for 10 min at 4°C. The lavaged cells were washed once with ice-cold RPMI 1640 medium with 20 mg/ml gentamicin (Life Technologies, Rockville, MD). Cell counts were performed using a hemocytometer. Cytocentrifuge slides were prepared and stained with Diff Quick (Leukostat solution; Fisher Scientific, Atlanta, GA) to check for AM purity. The cell preparation consisted of 85–95% AM. The viability of AM was determined by trypan blue exclusion. Viability exceeded 85% in all samples.

Production of ROS

Production of ROS by human AM was quantified by measuring the chemiluminescence (4). The assay was performed on Berthold’s LB953 autolumat using luminol (ExOxEmis, Little Rock, AR). Human AM treated with ROFA and other interventions were aliquoted into twelve 75-mm polypipette tubes. These cells (10$^6$ cells in 100 μl of RPMI) and 600 μl of luminol reagent were automatically injected simultaneously into the tubes, and resultant chemiluminescence (cpm) was measured over a 30-min period.

Measurements of GSH and Ascorbate

Glutathione was measured by using the enzymatic recycling method of Anderson (2). Ascorbate was analyzed by high-performance liquid chromatography electrochemical detection using the method of Kutnik et al. (33).

Measurements of NO Production in AM

NO production by human AM was measured by oxidation of DAF-2DA using a flow cytometer. DAF-2DA is a cell-permeable fluorescence dye that is oxidized by NO to form an

Fig. 1. Production of reactive oxygen species (ROS) by human alveolar macrophages (AM) treated with residual oil fly ash (ROFA). A: chemiluminescence was measured after AM were incubated with ROFA for 24 h with or without diphenyleneiodonium (DPI; 4 μM), a flavoprotein inhibitor, or nitro-l-arginine methyl ester (L-NAME; 100 μM), a nitric oxide synthase (NOS) inhibitor. B: chemiluminescence was measured after AM were incubated with ROFA for 24 h, followed by incubation for 45 min with 3 mitochondrial electron transport inhibitors: rotenone (Rot; 2.5 μM), antimycin (Ant; 4 μM), and sodium azide (NaN$_3$; 0.3%), which inhibit complexes I, III, and IV, respectively. R, ROFA. *P < 0.05 vs. control. #P < 0.05 vs. ROFA alone. The results in each group were obtained from experiments on AM from 5–10 different individuals.
irrelevant antibodies of the same isotype as the receptor antibodies were used as controls to establish background fluorescence and nonspecific antibody binding.

**Caspase-3-like activity.** Human AM (1 × 10⁶ cells) in RPMI 1640 medium supplemented with 2% fetal bovine serum (FBS) were incubated with test agents for 4 h. The cells were then split into two Eppendorf tubes and centrifuged. The pellets and the supernatant were separated. Cell pellets were lysed with 25 μl of caspase-3 kit lysis buffer on ice for 10 min and then stored at −20°C until further analysis. Caspase-3-like activity was measured by using EnzChek caspase-3 assay kit no. 1 with fluorescence-labeled Z-DEVD-7-aminomethylcoumarin (AMC) as the substrate (Molecular Probes). The assay was performed according to the manufacturer’s recommended procedures, and the fluorescence was measured with a fluorescence plate reader (Perkin-Elmer) using an excitation wavelength of 350 nm and an emission wavelength of 450 nm. The reference standard was AMC. The caspase-3-like activity was expressed as the amount of AMC released in the reaction (in nmol·min⁻¹·mg protein⁻¹).

**Statistical Analysis**

All data are expressed as means ± SE and were compared using a paired t-test adjusted for multiple comparisons. The statistical analysis was performed with StatView (version 4.0; SAS, Cary, NC). A P value <0.05 was considered statistically significant.

**RESULTS**

**ROFA and Cytotoxicity**

We first determined how ROFA affected cell death. Treatment with ROFA for 24 h produced a dose-dependent increase in cells with apoptotic or necrotic features (Table 1). On the basis of this dose-response result, a 100 μg/ml dose was chosen for all subsequent experiments because it produced more apoptotic cell death than a 50 μg/ml dose but less necrotic cell death than a 200 μg/ml dose. The dose response of ROFA in AM with these endpoints was similar to that of oil fly ash with the use of trypan blue uptake as reported by our group (4).

**Effects of ROFA on ROS Production**

ROFA drastically increased ROS production measured by luminol-dependent chemiluminescence burst. The increase could be inhibited by Cu²⁺SOD (100 U/ml) by >50% and deferoxamine by ~70%, indicating that the

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**Table 2. Effects of ROFA and mitochondrial inhibitors on NO**

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<thead>
<tr>
<th>Inhibitors</th>
<th>Control, %</th>
<th>ROFA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100(0)</td>
<td>145.5(38.1)#</td>
</tr>
<tr>
<td>+ Rotenone</td>
<td>83.5(6.9)</td>
<td>127.2(26.0)</td>
</tr>
<tr>
<td>+ Antimycin</td>
<td>73.9(9.5)</td>
<td>99.7(12.0)#</td>
</tr>
<tr>
<td>+ NaN₃</td>
<td>88.0(8.0)</td>
<td>108.4(24.2)</td>
</tr>
</tbody>
</table>

Effects of ROFA and 3 mitochondrial inhibitors (rotenone, antimycin, and NaN₃) on intracellular NO concentration measured by oxidation of DAF-2DA. The values are normalized to the control without inhibitors and expressed as mean percentage of the control value (SE). *P < 0.05 vs. no inhibitors. #P = 0.063 vs. control. The results in each group were obtained from experiments on AM from 5 different individuals.

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primary signals of chemiluminescence were from superoxide and its derived ROS. The ROFA-induced chemiluminescence burst was inhibited by 40% by diphenyleneiodonium (DPI; 4 μM), a flavoprotein inhibitor, but not by nitro-L-arginine methyl ester (L-NAME; 100 μM), a nitric oxide synthase (NOS) inhibitor (Fig. 1A). AM were also treated with rotenone (2.5 μM), antimycin (4 μM), and sodium azide (0.3%), which block mitochondrial electron transport at complex I (NADH-Q reductase), complex III (cytochrome reductase), and complex IV (cytochrome oxidase), respectively, for 45 min after 24-h incubation with ROFA. All three mitochondrial electron transport inhibitors attenuated ROFA-induced ROS production (Fig. 1B).

Effects of ROFA on Antioxidants

ROFA decreased GSH levels ~30% by 60 min, consistent with increased production of peroxides. The
inhibition was restored by rotenone, sodium azide, and DPI but not by antimycin (Fig. 2). ROFA also inhibited ascorbate level by 90%, and this was not reversed by any of the inhibitors used (Fig. 2).

Effects of Mitochondrial Inhibitors on NO Production

Because superoxide may react rapidly with NO, we determined how the excessive electron leaks from the mitochondrial electron transport chain induced by ROFA may affect intracellular NO concentration by measuring oxidation of a cell-permeable fluorescent dye, DAF-2DA. Control AM showed oxidation of DAF-2DA over 45 min of incubation, which was completely inhibited by 100 μM L-NAME (data not shown). ROFA tended to increase DAF-2DA oxidation (P = 0.063). The increase was inhibited by antimycin but not by rotenone or sodium azide. A similar pattern of inhibition was also seen in control AM not stimulated by ROFA (Table 2).

ROFA and Apoptosis

Approximately 10% of ROFA-treated AM expressed annexin V (early apoptosis), 12% showed increased PI uptake (necrosis), and 9% showed both increased annexin V expression and increased PI uptake (late apoptosis) (Fig. 3A). ROFA also increased histone release more than twofold (Fig. 3B). The caspase-3-like activity of AM was also assessed. Control AM showed a level of caspase-3-like activity of 31.0 ± 7.7 nmol·min⁻¹·mg protein⁻¹. ROFA treatment for 4 h increased the activity approximately twofold (Fig. 4A). The caspase-3-like activity in control and ROFA-treated cells was completely inhibited by DEVD, a caspase-3-like protease inhibitor (Fig. 4A). AM treated with ROFA demonstrated nuclear morphology consistent with apoptosis (Fig. 4B), which was also inhibited by DEVD.

Effects of Mitochondrial ROS on Apoptosis

To determine how mitochondrial ROS production regulates apoptosis, we measured the effects of mitochondrial electron transport inhibitors on the activity of caspase-3-like proteases in control and ROFA-treated AM. Sodium azide but not rotenone or antimycin increased caspase-3-like activity in both control and ROFA-treated cells (Fig. 5).

Effects of NO on Apoptosis

Because NO is known to induce apoptosis in macrophages and ROFA increased NO production, we further determined the role of NO in ROFA-induced apoptosis. L-NAME inhibited the increase in caspase-3-like activity in ROFA-treated AM (Fig. 6A) as well as nuclear apoptotic morphology, whereas L-arginine (3 mM) and a NO donor, detrNONOate (3 mM), but not L-ornithine (3 mM), the amino acid metabolite of L-arginine by arginase, increased caspase-3-like activity approximately twofold in control cells (Fig. 6B). ROFA further increased caspase-3-like activity in AM treated with L-arginine and detrNONOate (Fig. 6B). A similar pattern of inhibition by L-NAME was also observed for annexin V expression (Fig. 7). Note that cell necrosis as stained by PI (necrosis) was not affected by L-NAME.

Effects of Vanadium on ROS Production

Because vanadium is the major soluble metal in ROFA, we determined the contribution of vanadium to
ROFA-induced ROS production. Human AM were incubated with 50 \( \mu \)M of VOSO\(_4\) for 24 h with or without inhibitors. This concentration of VOSO\(_4\) was equivalent to the vanadium content in 100 \( \mu \)g/ml ROFA.

VOSO\(_4\) increased the chemiluminescence burst, and, like that for ROFA, the chemiluminescence burst was partially inhibited by DPI but not by L-NAME (Fig. 8A). All three mitochondrial inhibitors attenuated the vanadium-induced chemiluminescence burst, a pattern similar to that seen with ROFA (Fig. 8B). Mitochondrial inhibitors did not alter intracellular NO concentration in VOSO\(_4\)-treated AM (Table 3).

**Fig. 8.** Production of ROS by AM treated with VOSO\(_4\) (50 \( \mu \)M). A: chemiluminescence was measured after AM were incubated with VOSO\(_4\) for 24 h with or without DPI (4 \( \mu \)M), a flavoprotein inhibitor, or L-NAME (100 \( \mu \)M), a NOS inhibitor. B: chemiluminescence was measured after AM were incubated with VOSO\(_4\) for 24 h, followed by incubation for 45 min with 3 mitochondrial electron transport inhibitors: rotenone (2.5 \( \mu \)M), antimycin (4 \( \mu \)M), and sodium azide (0.3%), which inhibit complexes I, III, and IV, respectively. * \( P < 0.05 \) vs. control. # \( P < 0.05 \) vs. VOSO\(_4\) alone. The results in each group were obtained from experiments on AM from 5–10 different individuals.

### Table 3. Effects of VOSO\(_4\) and mitochondrial inhibitors on NO

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Control, %</th>
<th>VOSO(_4), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100(0)</td>
<td>122.6(17.2)</td>
</tr>
<tr>
<td>+ Rotenone</td>
<td>83.5(6.9)</td>
<td>134.7(23.4)</td>
</tr>
<tr>
<td>+ Antimycin</td>
<td>73.9(9.5)</td>
<td>101.9(12.4)</td>
</tr>
<tr>
<td>+ NaN(_3)</td>
<td>88.0(8.0)</td>
<td>101.4(8.1)</td>
</tr>
</tbody>
</table>

Effects of vanadyl sulfate (VOSO\(_4\)) and 3 mitochondrial inhibitors (rotenone, antimycin, and NaN\(_3\)) on intracellular NO concentration measured by oxidation of DAF-2DA. The values are normalized to the control without inhibitors and expressed as mean percentage of the control value (SE). The results in each group were obtained from experiments on AM from 5 different individuals.

### Effects of Vanadium on Apoptosis

VOSO\(_4\) had a small effect on caspase-3-like activity compared with ROFA. The increase by VOSO\(_4\) was \(-30\%\), which was not statistically different from that in control AM (Fig. 9A). Unlike ROFA, VOSO\(_4\) did not enhance the increase in caspase-3-like activity induced by detaNONOate or L-arginine (Fig. 9B). VOSO\(_4\) also did not affect annexin V expression. Approximately 3.8% of cells expressed annexin V in VOSO\(_4\)-treated AM, which was not statistically different from 2.6% in control AM.

### DISCUSSION

Human alveolar macrophages, when stimulated with a combustion pollutant dust, ROFA, produced a large quantity of ROS detected as chemiluminescence burst. A significant portion of these oxidants was produced as a result of electron leaks from the mitochondrial electron transport chain. Membrane NADPH oxidase but not NOS may also contribute to ROFA-induced ROS production.
production, because DPI but not L-NAME attenuated the chemiluminescence burst.

The mitochondrial electron transport chain is an important source for ROS in other cells stimulated by proinflammatory stimulants. In human umbilical vein endothelial cells, TNF-α stimulates the production of ROS at the ubiquinone site (10). In keratinocytes, ultraviolet irradiation stimulates ROS produced at complex III (19). In rat alveolar macrophages, 12-o-tetradecanoyl phorbol-13-acetate stimulates ROS from mitochondrial sources, mainly at complexes I and III (42). In A549 cells, vanadate stimulates the production of ROS (mainly hydrogen peroxide) from mitochondria, which produces growth arrest (52). The present study and a previous study from this laboratory (3) have further shown in human AM that ROFA stimulates ROS production from mitochondria. Because all three mitochondrial inhibitors attenuate ROFA-induced chemiluminescence burst, it is difficult to pinpoint the exact sites of electron leaks from the mitochondrial electron transport chain.

The main species of mitochondrial ROS produced by ROFA-stimulated AM should include hydrogen peroxide, given the presence of Mn SOD and the decrease in GSH level that could be reversed by rotenone and sodium azide. Also, ROFA increased oxidation of dihydrorhodamine 123, a reaction product between dihydrorhodamine 123 and hydrogen peroxide in the presence of peroxidase, cytochrome c, or ferrous iron (data not shown). Notably, ROFA decreased intracellular ascorbate levels, and this was not reversible by mitochondrial inhibitors or DPI. This finding may indicate consumption of reduced ascorbate by oxidants and/or disruption of ascorbate uptake and recycling processes (37), which would render the cells more susceptible to oxidative stress (7).

ROFA contains a significant amount of redox active metals, especially vanadium (14) (32). Many proinflammatory effects produced by ROFA are mediated by vanadium, including alveolar neutrophil influx and in vitro activation of rat AM (32), pulmonary vasoconstriction (29), airway epithelial injury and gene expression of MIP-2 and IL-6 (15), and protein tyrosine phosphorylation (44). Here we have shown that vanadium also is likely to mediate ROFA-induced production of ROS from mitochondria, because VOSO₄ mimics ROFA in the stimulation of chemiluminescence burst and in patterns of inhibition by various inhibitors of ROS-producing enzymes. The ability of ROFA to stimulate production of ROS from mitochondria indicates that the particles or its components may reach these subcellular organelles. Vanadium, the most abundant metal contained in ROFA, is known to permeate cell membrane avidly. Once inside the cells, vanadium chelates to many intracellular ligands, especially phosphates, e.g., ATP and creatine phosphate, which may partially explain its long elimination half-life (~12 days) (40, 41). Because mitochondria are rich in phosphate compounds, it is reasonable to anticipate a significant portion of intracellular vanadium in mitochondria. When vanadium loads exceed the capacity of the chelators, free vanadyl ions may interact with redox-active ligands and induce electron leaks (24, 35). Alternatively, vanadium may initiate oxidative stress by enhancing tyrosine phosphorylation of membrane receptors such as epidermal growth factors (23). The exact enzymatic sites of electron leaks and the mechanisms by which vanadium promotes electron leaks from these mitochondrial sources require further study.

We also have shown that an increased number of ROFA-treated AM undergo apoptosis, as shown by condensation and fragmentation of nuclei, increased histone release, annexin V expression, and caspase-3-like activity. These findings are consistent with a previous study using cell death ELISA and DNA laddering to demonstrate the proapoptotic property of ROFA (26). The ROFA-induced increase in caspase-3-like activity was not inhibited by mitochondrial inhibitors. In fact, the caspase-3-like activity was increased by so-
dioimid azide. Furthermore, VOSO₄, which also stimu-
lated production of ROS from mitochondria, has rela-
tively weak effects on apoptosis. These results indicate 
that ROS produced from mitochondria in response to 
ROFA do not induce, and might even inhibit, apopto-
sis of AM. ROS produced from cytosolic sources have been 
shown to inhibit NO-induced apoptosis in RAW264.7 
cells (5, 8).

ROFA-induced apoptosis was inhibited by L-NAME 
and DEVD, suggesting the dependency on NO and 
activation of caspase-3-like proteases, respectively. 
ROFA further enhanced caspase-3-like activity in-
duced by an exogenous NO donor, detaNONOate, and 
ROFA further enhanced caspase-3-like activity in-
toactivation of caspase-3-like proteases, respectively. 
and DEVD, suggesting the dependency on NO and 
ROS produced from cytosolic substrate of NOS, L-arginine. The in-
crease in caspase-3-like activity, however, cannot be 
the direct effect of NO because NO is known to inacti-
vate caspsases by S-nitrosylation or oxidation (12, 31, 
34, 38). Most likely, NO initiates apoptosis by activat-
ing upstream events, including p53 accumulation (6), 
permeation transition (28), cytochrome c release (27), 
and ATF6/CHOP-related endoplasmic reticulum stress 
pathways (22).

ROFA tended to increase NO production measured 
by oxidation of DAF-2DA dye, which would support the 
contention that ROFA-induced apoptosis is mediated 
by NO. Antimycin and sodium azide, however, de-
creased NO concentration. This finding indicates that 
there is little interaction between mitochondrial ROS 
and NO. In activated RAW264.7 cells, rotenone and 
antimycin enhanced cellular NO production, but a 
chemical uncoupler decreased NO production (50). The 
reason for the discrepancy is unclear but could be 
related to the cell types (transformed macrophage cell 
lines vs. primary human AM) or the stimuli used to 
activate macrophages.

Unlike its role in mediating ROFA-induced produc-
tion of ROS, vanadium plays a relatively minor role in 
mediating the proapoptotic effects of ROFA. VOSO₄ at 
a concentration equivalent to vanadium content in 100 
µg/ml ROFA had a relatively small effect on caspase-
3-like activity and annexin V expression. VOSO₄ did 
not increase NO production and did not enhance the 
increase in caspase-3-like activity induced by deta-
NONOate or L-arginine. It remains a possibility that 
vanadium in combination with other transition metals 
contained in ROFA, e.g., Ni or Fe, may have more 
pronounced proapoptotic effects. Alternatively, the 
timing for the apoptotic events induced by soluble van-
adium may be different from that induced by ROFA.

Extrapolation of our results to human exposure to 
ambient particulate matter should be made with cau-
tion. ROFA is a complex combustion-related particle 
that is collected from a variety of emission sources, e.g., 
power plants and boilers. Compared with ambient par-
ticular matter, ROFA contains higher concentrations 
of soluble metals and sulfate and thus does not repre-
sent typical “real-world” pollutant particles. The doses 
of ROFA to which AM were exposed were much higher 
than the one-time exposure in most ambient settings in 
humans. The contribution of apoptosis of AM to the 
health effects of particulate matter remains unclear, 
although in vivo exposure to environmental oxidative 
stimuli such as ozone and hyperoxia are known to 
cause apoptosis (16, 36).

In summary, human AM can produce a significant 
quantity of ROS from mitochondria as chemilumines-
cence burst when stimulated with the pollutant dust 
ROFA. Inhibition of oxidant production generated from 
mitochondrial sources did not inhibit apoptosis of AM, 
indicating that the prooxidant effects and the proapop-
totic effects of ROFA are likely mediated by different 
mechanisms. The ability to regulate independently 
these two important host defense mechanisms under-
scores the role of alveolar macrophages in maintaining 
the homeostasis of alveolar microenvironment that is 
exposed constantly to ambient pollutant particles.

We thank Dr. Andy Ghio, Maryann Bassett, Debbie Levin, and 
Sue Darrenbacher in the Medical Station for assisting with 
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and measuring ascorbate and GSH.

The research described in this article has been reviewed by the 
Health Effects and Environmental Research Laboratory, United 
States Environmental Protection Agency and has been approved for 
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reflect the views and policies of the Agency, nor does mention of the 
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