Pollutant particles enhanced H$_2$O$_2$ production from NAD(P)H oxidase and mitochondria in human pulmonary artery endothelial cells

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Over the past decade, a growing body of epidemiological and clinical evidence has raised the possibility of the potentially deleterious effects of ambient particulate matter (PM) on cardiovascular health. Exposure to PM has been associated consistently with increased hospitalization and mortality due to cardiovascular diseases (39). It has been estimated that for each 10-$\mu$g/m$^3$ increase in PM$_{10}$ (PM <10 $\mu$m in aerodynamic diameter), the daily cardiopulmonary mortality increased by 0.3% (16). The risk is especially high in patients with congestive heart failure, frequent arrhythmias, or both (19, 33), and the catastrophic cardiac events may occur as early as hours after PM exposure (37). The mechanisms for the acute increase in cardiovascular events are not entirely clear. Several hypotheses have been proposed, including imbalance in autonomic systems, increases in pro-coagulant activities and systemic release of inflammatory mediators (10, 18).

Recent in vivo and in vitro evidence indicates that PM may also cause endothelial dysfunction and vasoconstriction. Exposure to concentrated ambient particles (CAPs) (median, 182.75 $\mu$g/m$^3$) for 5 h/day for 3 days decreased the lumen/wall area ratio of small pulmonary arteries (PA) in rats indicating increased pulmonary vascular resistance (4). PM from motorcycle exhaust enhanced constriction of rat aortic rings induced by phenylephrine (45). Exposure to PM for 4 wk increased atherosclerotic plaque formation in rabbits (42). Co-exposure to CAPs (~150 $\mu$g/m$^3$) and ozone (120 ppb) for 2 h causes acute constriction of brachial artery in healthy human adults (9). An air pollution episode in Germany was associated with increases in systemic blood pressure by as much as 8 mmHg (26). Various vasoconstrictor mechanisms are affected by PM, including the release of endothelins (8, 44), activation of the epithelial growth factor receptor (25), and inhibition of nitric oxide (NO) production (2, 8, 25, 27). More recently, we demonstrated that PM caused pulmonary vasoconstriction and activated mitogen-activated protein kinase (MAPK) via activation of angiotensin II receptor subtype 1 (ATIR) (32).

PM also is known to produce oxidative stress in lung cells (17). Chapel Hill particles increased the production of reactive oxygen species (ROS) in human alveolar macrophages and bronchial epithelial cells (6). Diesel particles induced oxidative stress in macrophages (23). PM-induced secretion of amphiregulin was blocked by the antioxidant N-acetylcysteine (7). Ottawa ambient particles (EHC-93) induced blood oxidative stress markers in rats (46). The oxidative stress in these lung cells have been linked mostly to PM-induced pulmonary inflammation. Because the cardiovascular adverse effects induced by PM may occur without lung inflammation, we reasoned that PM may induce oxidative stress directly in vascular endothelial cells. The induction of endothelial oxidative stress is an important mechanism linking many known cardiovascular risk factors, including hyperglycemia, dyslipidemia, hypertension, and cigarette smoking (11), with the development of...
vascular diseases. To test this hypothesis, we exposed PA endothelial cells to PM and measured extracellular H₂O₂ production. Our goals were to determine the enzymatic sources of ROS production in vascular endothelial cells and to investigate the effects of ROS from different sources on MAPK activation and vasoconstriction, two cellular events known to occur with PM exposure (32). The study was performed in human PA endothelial cells (HPAECs), isolated PA ring, and isolated buffer-perfused lungs.

MATERIALS AND METHODS

Reagents and chemicals. HPAECs were obtained from Cell Applications (San Diego, CA). Endothelial growth medium (EGM-2) and supplements were purchased from Clonetics (BioWhittaker, Walkersville, MD). Diphenylethionemodinum (DPI), apocynin, indomethacin, cinetidine, rotenone, allopurinol, antimycin, sodium azide and N³-methyl-l-arginine (L-NAME) were obtained from Sigma (St. Louis, MO). Monoclonal antibodies against phospho-p38, total p38, phospho-ERK1/2, and total ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Molecular mass standards, polyclonal antibodies, and buffers were from Bio-Rad (Richmond, CA). The ECL Western blot analysis detection reagents were purchased from Amersham Biosciences (Piscataway, NJ).

Source of particles. Urban particles (SRM 1648) were purchased from the National Institute of Standards and Technology (Gaithersburg, MD). They were prepared from urban PM collected in the St. Louis, MO, area in a baghouse over a period of 12 mo. The material was removed from the filter bags, combined in a single lot, screened through a fine mesh sieve to remove extraneous materials and thoroughly blended in a v-blender. The material was then packaged into sequentially numbered bottles. The major constituent elements are (mass fraction in %) the following: 3.4% Al, 3.9% Fe, 1.1% K and 2.7% Zn. There are also metal constituents (in ng/mg), including 115 As, 76 Cd, 403 Cr, 609 Cu, 786 Mn, 82 Ni, 27 Se, 5.5 U, and 127 V. UP was suspended in distilled deionized water for the use in experiments.

Isolated rat PA ring model. Segments of the right and left main PAs were obtained from Sprague-Dawley rats (250–350 g) for measurements requiring ~2–3 mm were removed and placed in the Krebs-Henseleit buffer, as described previously (31). The artery segments were then suspended in distilled deionized water for the use in experiments. The blotted was blocked with 5% milk in PBS with 0.05% Tween 20 for 1 h at room temperature, washed briefly, and then probed with goat antibodies against human phospho-p38 or phospho-ERK1/2 overnight at 4°C. This was followed by incubation with HRP-conjugated secondary antibodies. Bands were detected with the use of ECL and films. The blot was then stripped and reprobed with monoclonal antibodies against human p38 or ERK1/2 and appropriate HRP-conjugated secondary antibodies.

Measurements of H₂O₂. Cells were cultured in 24-well plates. Production of extracellular H₂O₂ was measured by the Amplex red reagent (10-acetyl-3,7-dihydroxynaphoxazine; Molecular Probes-Invitrogen, Carlsbad, CA). Amplex red reagent reacts with H₂O₂ to produce highly fluorescent resorufin in the presence of HRP. The fluorescent signals were measured over 120 min with a Bioassay HTS7000 plate reader (Perkin Elmer, Wellesley, MA) with HTSoft version 1.0 software (PE Applied Biosystems, Weiterstadt, Germany). The excitation and emission wavelengths were 530 and 590 nm, respectively. Generation of H₂O₂ was calculated by subtracting the baseline signal at 0 min (baseline) from that at 120 min. Positive controls using H₂O₂ (0.5–2 µM) were included for all Amplex red assays. All inhibitors were added 20 min before Amplex red reaction.

Transfection of HPAEC with p47phox siRNA. Cells were grown to ~60–70% confluency in 6-well plates. They were then transfected with GeneSilencer transfecting agent plus (Gene Therapy System, San Diego, CA) with p47phox siRNA (100 nM; DHarmacco, Chicago, IL) in serum-free EGM-2 medium for 3 h according to the manufacturer’s recommendation. Control cells were incubated with Gene Silencer without p47phox siRNA. Fresh EGM-2 medium with 2% fetal bovine serum was then added, and cells were cultured for an additional 48 h. Cell lysates from some wells were collected for p47phox protein expression and phospho-ERK1/2 and phospho-p38 MAPKs by Western blot analysis. Other wells were used for measuring H₂O₂ production.

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Statistical analysis. Data shown in text and figures are means ± SE. Data from the artery ring experiments and perfused rabbit lungs were analyzed by the repeated-measures ANOVA. Data from the cell experiments were analyzed by ANOVA, followed by Scheffé’s test for post hoc comparisons. The statistical analysis was performed using commercially available software (Statview version 5.0.1, SAS Institute, Cary, NC). A P value of <0.05 was taken as statistically significant.

RESULTS

Effects of UP on H$_2$O$_2$ release. We first measured extracellular release of H$_2$O$_2$ using the Amplex red reagent. UP at up to 100 µg/ml did not produce cytotoxicity in 20 min as evidenced by the lack of lactate dehydrogenase release. UP increased H$_2$O$_2$ production in a time- and dose-dependent manner (Fig. 1). The increase was statistically significant as early as 5 min (the first time point) after incubation. H$_2$O$_2$ released over 120 min by cells treated with 100 µg/ml of UP was 0.80 ± 0.02 µM or 230% that by the control cells. The enhanced Amplex red signal induced by UP was completely abolished by the addition of catalase to the medium (changes in the Amplex red signal = 7,319 ± 210).

Enzymatic sources of H$_2$O$_2$ stimulated by UP. To determine the enzymatic sources of H$_2$O$_2$, we measured UP-induced
H$_2$O$_2$ release in the presence of inhibitors for different H$_2$O$_2$-producing enzymes. DPI and apocynin, two structurally distinct NAD(P)H oxidase inhibitors, and NaN$_3$, a mitochondrial complex IV (cytochrome $c$ oxidase) inhibitor, attenuated UP-induced H$_2$O$_2$ release (Fig. 2). The inhibitory effects of DPI and apocynin occurred within 10 min while the inhibition by NaN$_3$ was not statistically significant until 20 min after UP exposure. The UP-induced H$_2$O$_2$ release was not affected by CuSO$_4$ (50 µM) for 2 h. Amplex red (50 µM) was given 20 min before CuSO$_4$, and the fluorescence signals were read with a fluorescent plate reader. The CuSO$_4$-induced H$_2$O$_2$ production was inhibited by 4 µM DPI (C), 10 µg/ml APO (D), and 0.3% NaN$_3$ (E). *P < 0.05 vs. time 0; n = 6–10 independent experiments performed in triplicate.

Fig. 3. Effects of Cu on H$_2$O$_2$ production. A: the cells were incubated with various concentrations of CuSO$_4$ for 2 h. B: cells were incubated with CuSO$_4$ (50 µM) for 2 h. Amplex red (50 µM) was given 20 min before CuSO$_4$, and the fluorescence signals were read with a fluorescent plate reader. The CuSO$_4$-induced H$_2$O$_2$ production was inhibited by 4 µM DPI (C), 10 µg/ml APO (D), and 0.3% NaN$_3$ (E). *P < 0.05 vs. time 0; n = 6–10 independent experiments performed in triplicate.

Fig. 4. Effects of V on H$_2$O$_2$ production. A: cells were incubated with various concentrations of vanadyl sulfate (VOSO$_4$) for 2 h. B: cells were incubated with 50 µM VOSO$_4$ for 2 h. Amplex red (50 µM) was given 20 min before VOSO$_4$, and the fluorescence signals were read with a fluorescent plate reader. The VOSO$_4$-induced H$_2$O$_2$ production was not inhibited by 4 µM DPI (C) or 10 µg/ml APO (D) but was inhibited by 0.3% NaN$_3$ (E). *P < 0.05 vs. time 0; n = 6–10 independent experiments performed in triplicate.
inhibitors of other oxidant-producing enzyme systems, including cimetidine (a cytochrome P-450 inhibitor, 5 mM), L-NAME (an NO synthase inhibitor, 100 μM), allopurinol (a xanthine oxidase inhibitor, 100 μM), indomethacin (a cyclooxygenase inhibitor, 50 μM), rotenone (a mitochondrial complex I inhibitor, 2.5 μM) and antimycin (a mitochondrial complex III inhibitor, 4 μM). These results indicate that vascular NAD(P)H oxidase and the mitochondria are major sources for the increased H₂O₂ release following UP exposure.

Fig. 5. Effects of 4 μM DPI (A) and 0.3% NaN₃ (B) on UP-induced pulmonary vasoconstriction. The pulmonary arterial ring was pretreated with DPI or NaN₃ for 15 min before UP administration (1–100 μg/ml). The constriction was measured by ring tension and expressed as % maximum tension induced by 1 μM phenylephrine. *P < 0.05 vs. UP; n = 6 rings from different animals. C: changes in pulmonary artery pressure (PAP) induced by glucose/glucose oxidase, which generates H₂O₂. d-Glucose (1 mM) and glucose oxidase (10 U/ml) were given as a bolus into the perfusate of isolated lungs at time 0. PAP was then monitored for 25 min. *P < 0.05 vs. time 0; n = 6.

Fig. 6. Effects of DPI on UP-induced phosphorylation of ERK1/2 (A) and p38 (B). Quiescent cells were pretreated with DPI (4 μM) for 30 min and incubated with 10 μg/ml UP for 10 min. Phosphorylation of ERK1/2 and p38 in cell lysates was measured by Western blot analysis. The blots were then stripped and probed for total ERK1/2 and p38. A representative Western blot and the densitometry results for ERK1/2 and p38 are shown. Data are means ± SE; n = 4 independent experiments.
Effects of UP-associated metals on H2O2 release. The water-soluble fraction of UP was sufficient to induce H2O2 release. The increase in amplex red signals over 120 min was ~60% that by the whole particles. Because Cu and V are two abundant redox-active metals in the water-soluble fraction of UP, we further tested the effects of these two metals on H2O2 release. As shown in Fig. 3, A and B, copper sulfate (CuSO4) increased H2O2 release in a time- and dose-dependent manner. The Cu-stimulated release of H2O2 was inhibited by DPI, apocynin and NaN3 (Fig. 3, C–E). Vanadyl sulfate (VOSO4) also increased H2O2 release in a time- and dose-dependent manner (Fig. 4, A and B). The V-stimulated release of H2O2 was inhibited by NaN3 but not by DPI or apocynin (Fig. 4, C–E).

Effects of UP-induced H2O2 on PA constriction. To determine whether H2O2 production mediated UP-induced vasocon-
striction, we determined the effects of DPI and NaN₃ on the constriction of isolated rat PA rings. UP-induced pulmonary vasoconstriction was inhibited by DPI but not NaN₃ (Fig. 5, A and B). To determine whether or not extracellular H₂O₂ has vasoactive effects, we administered low concentration of glucose oxidase (10 U/ml or 0.05 mg/ml) into the perfusate of isolated lungs. UP suspension was not used because the insoluble residues of the particles would plug the capillaries and damage the lung. Glucose oxidase can oxidize α-glucose and produce H₂O₂ (12, 14). The system has been used to investigate the signaling events and lung injury induced by extracellular H₂O₂ (41). Figure 5C shows that addition of glucose/glucose oxidase to the pulmonary circulation increased PA pressure. Because the lung was perfused by a constant flow, increases in PA pressure indicated pulmonary vasoconstriction.

Effects of UP-induced H₂O₂ on MAPK activation. In a manner consistent with our previous study (32), UP increased phosphorylation of ERK1/2 and p38 MAPKs within 20 min. No increases in phosphorylation of c-Jun NH₂-terminal kinase (JNK) were detected within this short time frame (data not shown); however, we cannot exclude the possibility that JNK may be activated at later time points. Figures 6 and 7 show that UP-induced increases in ERK1/2 and p38 phosphorylation were also inhibited by DPI and apocynin, indicating that H₂O₂ produced by NAD(P)H oxidase likely mediated UP-induced activation of ERK1/2 and p38 MAPKs. In contrast, NaN₃ did not inhibit UP-induced ERK1/2 and p38 phosphorylation. In fact, phosphorylation of ERK1/2 and p38 was increased by NaN₃ (Fig. 8). Inhibitors of other H₂O₂-producing enzymes had no consistent effects on ERK1/2 and p38 MAPK activation (data not shown).

Effects of p47phox siRNA. To confirm the involvement of NAD(P)H oxidase, we performed additional experiments using p47phox siRNA to knock down p47phox gene expression. p47 siRNA significantly suppressed the protein expression of p47phox, phosphorylation of ERK, and partially attenuated the UP-induced production of H₂O₂ (Fig. 9).

DISCUSSION

Oxidative stress in endothelial cells is an important molecular mechanism for endothelial dysfunction that encompasses altered anticoagulant and anti-inflammatory properties of the endothelium, impaired modulation of vascular growth, dysregulation of vascular remodeling, and impaired endothelium-dependent vasorelaxation (11). Several common risk factors for cardiovascular diseases, including hyperglycemia, dyslipidemia, hypertension, and cigarette smoking, are known to cause oxidative stress in vascular endothelial cells. In this study, we showed that PM can stimulate PA endothelial cells to release H₂O₂. The induction of oxidative stress in endothelial cells by PM may be a mechanism by which PM exposure exacerbates atherosclerotic plaque formation in hypercholesterolemic mice (13) and increases cardiovascular morbidity and mortality in patients with diabetes (5, 29, 35, 36).
H$_2$O$_2$ released by the UP-stimulated endothelial cells comes from two major enzymatic sources, the NAD(P)H oxidase and the mitochondrial electron transport chain, because the release of H$_2$O$_2$ was inhibited by two structurally distinct NAD(P)H oxidase inhibitors (DPI and apocynin), p47$^{phox}$ siRNA and a mitochondrial complex IV inhibitor (NaN$_3$). Other enzyme systems that are potential sources for H$_2$O$_2$, including xanthine oxidase, cytochrome P-450, NO synthase, and cyclooxygenase-1, were less likely sources because their respective inhibitors, (allupurinol, cimetidine, L-NAME, and indomethacin) did not inhibit UP-induced H$_2$O$_2$ release. The NAD(P)H oxidase activity in vascular endothelial cells can be stimulated by many plasma membrane-mediated events, including cytokines, hormones, growth factors, G protein receptor agonists, and shear forces, and activation of NAD(P)H oxidase is known to be involved in the pathogenesis of vascular disease (15, 20, 21, 24, 34). Our results show that UP is a common stimulus for both systems because inhibitors for each system almost completely inhibit the UP-stimulated production of H$_2$O$_2$. It is not possible, however, to assess the relative contribution of each system in part because of the nonspecific effects of these inhibitors. Because DPI and apocynin inhibit UP-induced H$_2$O$_2$ production earlier than NaN$_3$, UP may activate NAD(P)H oxidase first. This hypothesis would be consistent with the cell membrane localization of NAD(P)H oxidase that makes it more accessible to extracellularly administered UP.

The exact components of UP responsible for activating NAD(P)H oxidase and the mitochondrial electron transport chain are not clear. The water-soluble fraction of UP was sufficient to stimulate the release of H$_2$O$_2$. Its transition metals, Cu and V, also enhanced H$_2$O$_2$ release. The patterns of inhibition by DPI, apocynin, and NaN$_3$ on Cu- and V-induced H$_2$O$_2$ production were similar to those on UP-induced H$_2$O$_2$ production. These results indicate that some components in the water-soluble fraction are responsible for changes induced by UP. Whether Cu and V are among the active components will require further studies in the future.

H$_2$O$_2$ produced by NAD(P)H oxidase contributed to PM-induced vasoconstriction because DPI inhibited UP-induced constriction of PA ring and intravascular administration of extracellular H$_2$O$_2$ produced by glucose and glucose oxidase produced acute vasoconstriction in perfused lungs. We and others have previously identified several signaling pathways that mediate PM-induced vasoconstriction, including epidermal growth factor receptor, angiogenin II receptor subtype 1, endothelins, and NO (8, 25, 27, 31, 32, 44). Our results indicate that H$_2$O$_2$ produced by the membrane NAD(P)H oxidase should also be added to the list. The cell membrane localization of NAD(P)H oxidase would allow H$_2$O$_2$ produced by this enzyme to be released directly into the extracellular space and exert its effects on the adjacent smooth muscle cells. The mitochondrial source of H$_2$O$_2$, on the other hand, appears not involved in regulating vascular reactivity because inhibition of H$_2$O$_2$ production by NaN$_3$ did not affect UP-induced vasoconstriction despite increases in ERK1/2 and p38 MAPK phosphorylation. We speculate that a subfamily of mitochondrial MAPKs may be affected by NaN$_3$ (1, 3). These mitochondrial MAPKs show a different response to ROS than the cytosolic MAPKs because H$_2$O$_2$ inhibits rather than increases phosphorylation of mitochondrial ERK1/2 (1). The primary function of the mitochondrial MAPKs appears different from that of the cytosolic MAPKs. Activation of the mitochondrial p38 MAPK mediates ceramide-induced apoptosis (30). Activation of mitochondrial ERK MAPK induces phosphorylation of a pro-apoptotic protein Bad preventing cell death in murine heart (3). Activation of the mitochondrial MAPKs subfamily and the specific role of mitochondrial MAPKs in cell death signaling may explain why NaN$_3$ did not affect UP-induced vasoconstriction despite an increase in phosphorylated ERK1/2 and p38 MAPKs. More studies in the future will be needed to test this hypothesis.

Extrapolation of our in vitro results to human exposure to ambient PM should be made with caution. The doses of UP to which endothelial cells were exposed were much higher than the one-time exposure in most ambient settings. With repeated exposures during severe pollution episodes (38, 40) and under certain occupational conditions (22, 47), the amount of PM inhaled into the lung may increase several hundred fold. Although the whole inhaled particles are unlikely in direct contact with vascular endothelial cells, water-soluble components contained in ambient PM can more readily permeate the alveolar-capillary barrier and gain access to the endothelial cells. Our study shows that PM can directly induce the production of H$_2$O$_2$ from NAD(P)H oxidase and the mitochondrial electron transport chain in vascular endothelial cells and that H$_2$O$_2$ produced from NAD(P)H oxidase mediates the PM-induced vasoconstriction. These results provided further evidence that PM may be an important risk factor for the development of cardiovascular diseases.

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DISCLOSURES

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 publication.

REFERENCES


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