Pollutant Particles Produce Vasoconstriction and Enhance MAPK Signaling via Angiotensin Type I Receptor

Zhuowei Li,1 Jacqueline D. Carter,2 Lisa A. Dailey,2 and Yuh-Chin T. Huang2

1Center for Environmental Medicine, Asthma and Lung Biology, University of North Carolina, Chapel Hill, North Carolina, USA; 2National Health and Environmental Effects Research Laboratory, Office of Research and Development, Environmental Protection Agency, Research Triangle Park, North Carolina, USA

Exposure to particulate matter (PM) is associated with acute cardiovascular mortality and morbidity, but the mechanisms are not entirely clear. In this study, we hypothesized that PM may activate the angiotensin type 1 receptor (AT1R), a G protein-coupled receptor that regulates inflammation and vascular function. We investigated the acute effects of St. Louis, Missouri, urban particles (UPs; Standard Reference Material 1648) on the constriction of isolated rat pulmonary artery rings and the activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and p38 mitogen-activated protein kinases (MAPKs) in human pulmonary artery endothelial cells with or without losartan, an antagonist of AT1R. Up's at 1–100 μg/ml induced acute vasoconstriction in pulmonary artery. UPs also produced a time- and dose-dependent increase in phosphorylation of ERK1/2 and p38 MAPK. Losartan pretreatment inhibited both the vasoconstriction and the activation of ERK1/2 and p38. The water-soluble fraction of UPs was sufficient for inducing ERK1/2 and p38 phosphorylation, which was also losartan inhibitable. Copper and vanadium, two soluble transition metals contained in UPs, induced pulmonary vasoconstriction and phosphorylation of ERK1/2 and p38, but only the phosphorylation of p38 was inhibited by losartan. The UP-induced activation of ERK1/2 and p38 was attenuated by captopril, an angiotensin-converting enzyme inhibitor. These results indicate that activation of the local renin–angiotensin system may play an important role in cardiovascular effects induced by PM. Key words: air pollutant, angiotensin II, angiotensin-converting enzyme, copper, ERK, p38, vanadium. Environ Health Perspect 113:1009–1014 (2005). doi:10.1289/ehp.7736 available via http://dx.doi.org/ [Online 15 April 2005]

Over the last decade, a growing body of epidemiologic and clinical evidence has raised the possibility of the potentially deleterious effects of ambient pollutant particles on cardiovascular health. Exposure to particulate matter (PM) has consistently been associated with increased hospitalization and mortality due to cardiovascular diseases (Pope et al. 2004). It has been estimated that for each 10-μg/m3 increase in PM10 (PM < 10 μm in aerodynamic diameter), the daily cardiopulmonary mortality increased by as much as 8 mm Hg (Ibald-Mulli et al. 2000). An air pollution episode in Germany was associated with increases in systemic blood pressure by as much as 8 mm Hg (Ibald-Mulli et al. 2001). Various vasoconstrictor mechanisms have been demonstrated, including the release of endothelins (Bouthillier et al. 1998; Thomson et al. 2004), activation of the epithelial growth factor receptor (EGFR; Huang et al. 2002), and inhibition of nitric oxide production (Bai et al. 2001; Bouthillier et al. 1998; Huang et al. 2002; Ikeda et al. 1995). These mechanisms, however, could not completely explain the epidemiologic findings of the acute effects of PM on cardiovascular events, which have a lag time of hours. The endothelins are potent vasoconstrictors, but the increased release occurs 24 hr after PM exposure. Vasoconstriction caused by the activation of EGFR is relatively weak, and the inhibition of NO production results in a loss of vasodilator activity.

The circulating and local renin–angiotensin systems have been known to play a key role in the pathogenesis of cardiovascular diseases (Dzau 1988). The end product of this pathway, angiotensin II, is one of the most potent vasoconstrictors, and its effects are mediated primarily by the G protein-coupled angiotensin type 1 receptor (AT1R; Daugherty and Cassis 2004). Agonist binding of AT1R activates mitogen-activated protein kinases (MAPKs; Touyz and Schiffrin 2000), a common early signaling event induced by PM exposure (Roberts et al. 2003; Silbajoris et al. 2000). In preliminary experiments, we found that pulmonary vasoconstriction induced by St. Louis, Missouri, urban particles [UPs; Standard Reference Material (SRM) 1648] could be inhibited by losartan, an AT1R antagonist. In the present study, we characterized the role of AT1R in UP-induced vasoconstriction and MAPK activation. The study was performed in the isolated pulmonary artery ring system and human pulmonary artery endothelial cells (HPAECs).

Materials and Methods

Reagents and chemicals. We obtained HPAECs from Cell Applications, Inc. (San Diego, CA). We purchased endothelial growth medium (EGM-2) and supplements from Clonetics (Bio Whittaker Inc., Walkersville, MD), and vanadyl sulfate (VOSO4) and copper sulfate (CuSO4) from Johnson Matthey Co. (Warishill, MA). We obtained Captopril from Sigma Chemical Co. (St. Louis, MO); SB203580, a p38 MAPK inhibitor, and PD98059 an ERK1/2 MAPK inhibitor, from Calbiochem-Novabiochem Corp. (San Diego, CA); and

Address correspondence to Y.-C.T. Huang, CB 7315, 104 Mason Farm Rd., Chapel Hill, NC 27599 USA. Telephone: (919) 843-9504. Fax: (919) 966-6271. E-mail: huang.tony@epa.gov

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losartan potassium from Merck & Co., Inc. (West Point, PA). Monoclonal antibodies against phospho-p38, total extracellular signal-regulated kinases 1 and 2 (ERK1/2), total p38, and phospho-ERK1/2 were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Horse heart peroxidase (HRP)-conjugated goat anti-rabbit and goat anti-mouse IgG were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). We purchased molecular weight standards, polyacrylamide, and buffers from Santa Cruz Biotechnology (Santa Cruz, CA). We purchased goat anti-rabbit and goat anti-mouse IgG were purchased from Amersham Biosciences Corp. (Piscataway, NJ).

Urban particles. We purchased St. Louis UPs (SRM 1648) from the National Institute of Standards and Technology (Gaithersburg, MD). They were prepared from urban PM collected in the St. Louis, Missouri, area in a baghouse over a period of >12 months. The material was removed from the filter bags, combined in a single lot, screened through a baghouse over a period of >12 months. The major constituent elements are (mass fraction) aluminum, 3.4%; iron, 3.9%; and potassium, 1.1%; the minor constituent elements are sodium, 0.43%; lead, 0.66%; and zinc, 0.48%. There are also trace constituents (nanograms per milligram), including arsenic (115), cadmium (75), chromium (403), copper (609), manganese (786), nickel (82), selenium (27), uranium (5.5), and vanadium (127). UPs were suspended in distilled deionized water for use in experiments.

Preparation of the water-soluble and -insoluble fraction of UP. The water suspension of UPs was centrifuged for 10 min at 14,000 rpm; we then collected the supernatant and centrifuged it again. We repeated this procedure several times until no sediments could be seen in the supernatant. This final supernatant was designated as the water-soluble fraction of UP. The pellets from the first centrifuge were washed using deionized distilled water for three times and then resuspended in appropriate volume of water.

Isolated perfused rat pulmonary artery ring model. We removed segments of the right and left main pulmonary arteries of Sprague-Dawley rats (250–350 g) measuring approximately 2–3 mm and placed them in the Krebs-Henseleit (KH) buffer as described previously (Li et al. 2004). The artery segments were then suspended in the Radnoti four-unit tissue organ bath system (Glass Technology Inc., Monrovia, CA). The reservoirs held...
20 mL KH buffer and were bubbled constantly with 21% O2/5% CO2 gas. After a 10–15 min stabilization period, the baseline tension of the rings was adjusted to 1 g before all experiments. We exposed the artery rings to increasing doses of particles from 1 to 100 µg/mL, and the rings were washed with buffer between the two doses of particles. We recorded the maximum tension within 5 min after each dose of particles.

**Cultured HPAECs.** HPAECs were grown in endothelial growth medium (EGM-2) supplemented with 2% fetal bovine serum, 0.04% hydrocortisone, 0.4% human fibroblast growth factor-B, 0.1% vascular endothelial growth factor, 0.1% R3-insulin growth factor-1, 0.1% ascorbic acid, 0.1% human epithelial growth factor, 0.1% GA-1000, and 0.1% heparin. We used cells at passages 4–9 grown to 80% confluence in six-well plates for the experiments. The cells were exposed to particles at 1–100 µg/mL for up to 20 min.

**Western blot analysis.** After the exposure, the cells were washed once with ice-cold phosphate-buffered saline (PBS) and then lysed with RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS, pH 7.4) containing 0.1 mM VOSO4 and protease inhibitors (0.5 mg/mL aprotinin, 0.5 mg/mL E-64, 0.5 mg/mL pepstatin, 0.5 mg/mL bestatin, 10 mg/mL chymostatin, and 0.1 ng/mL leupeptin). We then centrifuged the cell lysates at 3,000g for 10 min at 4°C. Protein concentration of supernatant was measured with Bio-Rad protein assay reagent.

Cellular proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane. The blot was blocked with 5% milk in PBS with 0.05% Tween-20 for 1 hr at room temperature, washed briefly, and then probed with primary antibodies against phospho-p38 or phospho-ERK1/2 overnight at 4°C. This was followed by incubation with HRP-conjugated secondary antibodies. We detected bands using ECL and films. We then stripped the blot and reprobed with antibodies against total p38 or total ERK1/2 and appropriate HRP-conjugated secondary antibodies.

**Statistical analysis.** Data are reported as mean ± SE. Data from the blocking ring experiments were analyzed by the repeat-measures analysis of variance (ANOVA). Data from the cell experiments were analyzed by ANOVA followed by the Scheffe’s test for post hoc comparisons. The statistical analysis was performed using commercially available software (Statview, version 5.0.1; SAS Institute Inc., Cary, NC). We considered a p-value < 0.05 as statistically significant.

**Results.**

**Effects of losartan on UP-induced pulmonary artery constriction.** Treatment of isolated rat pulmonary artery rings with UPs produced a dose-dependent increase in vasoconstriction (Figure 1A). At 100 µg/mL, the ring tension was approximately 25% of that produced by 1 µM phenylephrine. UP-induced pulmonary vasoconstriction was inhibited by losartan (0.2 µM), an AT1R receptor antagonist (Figure 1B).

**Effects of UPs on ERK1/2 and p38 phosphorylation.** Figure 2A shows the time-dependent increase in phospho-ERK1/2 induced by UPs. The intensity of phosphorylated ERK1/2 peaked at 5 min and gradually decreased. The phosphorylation of ERK1/2 was enhanced significantly by 1–100 µg/mL UPs (Figure 2B). The phosphorylated p38 increased with time after UP treatment (Figure 2C). There was also a dose-dependent increase in the intensity of phospho-p38 (Figure 2D). The UP-induced phosphorylation of ERK1/2 and p38 was completely inhibited by PD98059, an ERK1/2 MAPK inhibitor, and SB203580, a p38 MAPK inhibitor (Figure 3A,B). The UP-induced constriction of rat pulmonary artery was also attenuated by PD98059 and SB203580 (Figure 3C,D), indicating that activation of ERK1/2 and p38 may mediate UP-induced pulmonary vasoconstriction.

**Effects of losartan on UP-induced ERK1/2 and p38 phosphorylation.** We then investigated the role of losartan in UP-induced ERK1/2 and p38 phosphorylation. HPAECs were pretreated with losartan (0.2 µM) for 30 min before incubation with 10 µg/mL UPs for 10 min. Figure 4 shows that the UP-induced ERK1/2 and p38 phosphorylation was completely inhibited by losartan, indicating that AT1R mediates the activation of ERK1/2 and p38 MAPK induced by UPs.

**Effects of particle components on ERK1/2 and p38 phosphorylation.** Because the soluble fraction of the particles may better penetrate the alveolar–capillary membrane and be in contact with the pulmonary vessels than the whole particles when inhaled, we determined whether the soluble fraction of UPs could activate ERK1/2 and p38 MAPK. The watersoluble fraction of UPs was capable of
increasing the phosphorylation of ERK1/2 and p38 (Figure 5A,B). The insoluble fraction was equally effective. Similar to the whole UP, the soluble-fraction–induced activation of ERK1/2 and p38 was inhibited by losartan (Figure 5C,D).

**Effects of UP-associated soluble metals on pulmonary artery constriction.** To determine which water-soluble metal components of UPs may be responsible for the activation of ERK1/2 and p38 MAPK, we tested the vasoconstrictor effects of soluble metals contained in UPs. We found that Cu and V induced significant vasoconstriction (Figure 6). Other metals, including Ni, Fe, Mn, Zn, and Al, produced no or weak vasoconstriction (data not shown).

**Effect of Cu and V on ERK1/2 and p38 phosphorylation.** Because only Cu and V showed significant vasoconstrictor activity, we determined whether or not Cu and V could activate ERK1/2 and p38 MAPK. Figure 7 shows that CuSO₄ and VOSO₄ increased phosphorylation of ERK1/2 and p38 in a dose-dependent manner.

**Effects of losartan on Cu- and V-induced phosphorylation of ERK1/2 and p38.** We further determined whether or not Cu- and V-induced activation of ERK1/2 and p38 was inhibitable by losartan. Figure 8 shows that Cu- and V-induced phosphorylation of p38 was inhibited by losartan. The phosphorylation of ERK1/2, however, was not inhibited by losartan (data not shown).

**Effects of captopril on UP-induced ERK1/2 and p38 phosphorylation.** Because angiotensin II, the ligand for AT₁R, is a metabolic product of angiotensin-converting enzyme (ACE), we further determined whether or not UP-induced AT₁R-mediated MAPK activation required ACE. We pretreated the cells with captopril (100 µM), an ACE inhibitor, for 30 min before the addition of UPs. Figure 9 shows that the UP-induced ERK1/2 and p38 phosphorylation was inhibited by captopril.

**Discussion**

In this study, we have shown that UPs induced vasoconstriction in rat pulmonary arterial rings. Thus, UPs have joined a list of pollutant particles that have been shown to cause vasoconstriction, including CAPs (Batalha et al. 2002), residual oil fly ash (Huang et al. 2002), and diesel particles (Tzeng et al. 2003). We further have shown that the UP-induced pulmonary vasoconstriction could be inhibited by losartan, an AT₁R antagonist, indicating that the renin–angiotensin system may play an important role. PM has been shown to activate several vasoactive pathways, including the release of endothelins (Bouthillier et al. 1998; Thomson et al. 2004), the activation of EGFR (Huang et al. 2002), and the inhibition of NO production (Bai et al. 2001; Bouthillier et al. 1998; Huang et al. 2002; Ikeda et al. 1995). Compared with these vasoactive pathways, the angiotensin II–AT₁R pathway has the following advantages: a) the activation of AT₁R-mediated signaling by particles occurs earlier than that of endothelins; b) angiotensin II is a much more potent vasoconstrictor than EGF; and c) activation of AT₁R leads to vasoconstriction rather than the loss of vasodilator activity as a result of inhibition of NO production. In these aspects, the AT₁R pathway may be a more clinically relevant mechanism for the acute cardiovascular events associated with ambient PM demonstrated in the epidemiologic and clinical studies, especially in patients with compromised heart function.

Our study also showed that both the water-soluble and -insoluble fractions of UPs...
were equally effective in increasing ERK1/2 and p38 phosphorylation. The effects by the water-soluble fraction would be more biologically relevant because components in this fraction are more likely than the whole particles or the insoluble fraction to gain access to the pulmonary vessels quickly by diffusion after the particles are inhaled into the lung. Many components are present in the water-soluble fraction of UPs. Among them are transition metals. In this study, we found that Cu and V produced strong vasoconstriction and activated ERK1/2 and p38. Thus, Cu and V may be among the active components for PM-induced vasoconstriction. Residual oil fly ash, a pollutant dust containing abundant V, also produces vasoconstriction and MAPK activation (Huang et al. 2002; Silbajoris et al. 2000). Cu and V may reach the vasculature via specialized membrane transporters (Chasteen et al. 1986; Eisses and Kaplan 2002). V appeared to be a stronger inducer for ERK1/2 and p38 phosphorylation than did Cu, but its vasoconstrictor property was weaker. This indicated that V, a nonspecific protein tyrosine phosphatase inhibitor, may also activate other signaling proteins that counteract vasoconstriction. Our results do not exclude other mechanisms that are metal independent. Diesel particles, which contain little metal, impair endothelium-dependent vasorelaxation (Ikeda et al. 1995) possibly via the production of reactive oxygen species inactivating NO. Ultrafine particles (PM with a mass median aerodynamic diameter < 100 nm), which can more effectively penetrate the distal lung regions, may produce cardiovascular adverse effects via more intense lung inflammation (Brown et al. 2000; Utell and Frampton 2000). Previous studies have also shown that some components of ultrafine particles deposited in the respiratory tract could enter the circulation exerting their adverse effects on the cardiovascular system (Takemaki et al. 2001).

The UP-induced activation of ERK1/2 and p38 could be inhibited by an antagonist of AT1R, losartan. Similar inhibition by losartan was also seen when activation was induced by the water-soluble fraction. This is the first demonstration that PM-induced vascular effects may be mediated by the angiotensin signaling. AT1R is one of the four G protein-coupled receptors that mediates intracellular signaling induced by angiotensin II (Touyz and Berry 2002). AT1R activation leads to cell growth, vascular contraction, inflammatory responses, and salt and water retention (Touyz and Berry 2002). These effects are regulated by complex intracellular signaling pathways including MAPKs, phospholipase C, protein kinase C, and phospholipase A2. Our results that inhibitors of ERK1/2 and p38 MAPKs attenuated UP-induced vasoconstriction support the established role of these MAPKs in angiotensin II–induced vasoconstriction (Ishihata et al. 2002; Massett et al. 2002; Melloch et al. 2000; Touyz et al. 1999). Phosphorylation of MAPKs may further activate downstream MAPK–activated protein kinase-2 and heat shock protein 27 with or without phosphorylation of myosin light chain (Melloco et al. 2000; Roberts 2004). Occupancy and activation of AT1R also stimulate many intracellular nonreceptor tyrosine kinases, such as Src, Pyk2, p130Cas, FAK, and JAK/STAT (Eguchi and Inagami 2000). In the cardiovascular system, alterations of these highly regulated pathways underlie the pathologic processes such as hypertension and atherosclerosis, conditions that also have been linked to PM exposure (Ibald-Mulli et al. 2001; Suwa et al. 2002). That losartan only inhibited p38 activation induced by V and Cu indicates that other components of PM may also be involved in PM-induced ERK1/2 MAPK activation.

The mechanisms by which UPs activate AT1R are unclear. Because the UP-induced phosphorylation of ERK1/2 and p38 could be inhibited by captopril, an ACE inhibitor, the ACE activity appeared required for these effects. The vascular endothelial cells contain a large amount of ACE and can synthesize angiotensins via the local renin–angiotensin system (Kifor and Dravc 1987). Such a local renin–angiotensin system is likely the more important source of angiotensin II in our cultured HPAECs than is the extracellular angiotensin I contained in the culture medium. One might also consider whether or not UPs may act as an AT1R agonist. At least one nonpeptide agonist of AT1R has been described: a biphenylimidazole derivative (L-162313), whose biologic effects could be inhibited by AT1R antagonist (Perlman et al. 1995). Binding of nonpeptide agonists of AT1R may lead to conformational modifications that affect the preferential binding of agonists or antagonists (Costa-Neto et al. 2002).

Conclusions

The renin–angiotensin system plays an important role in many types of inflammatory and cardiovascular diseases. Angiotensin II via AT1R signaling has been shown to promote cell growth and regulate the expression of bioactive substances such as vasoconstrictor hormones, growth factors, cytokines, aldosterone, and extracellular matrix components (Jacoby and Rader 2003; Schiffrin and Touyz 2004). Our in vitro results indicate that the angiotensin–AT1R signaling pathway may be important in mediating vascular effects induced by PM. Further studies are needed to determine the role of the circulating and local renin–angiotensin systems in PM-induced adverse cardiovascular effects.

Figure 9. Effects of captopril (Cap) on UP-induced ERK1/2 (p-ERK1/2; A and C) and p38 phosphorylation (p-p38; B and D). Cells were pretreated with captopril (Cap; 100 μM) for 30 min before incubation with UPs (10 μg/mL) for 10 min. Results shown are representative Western blots and densitometry results. n = 4 independent experiments.

*p < 0.05 compared with control. #p < 0.05 compared with UPs.

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