Pulmonary Expression of PreproET-1 and PreproET-3 mRNAs Is Altered Reciprocally in Rats After Inhalation of Air Pollutants

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Perturbation of vascular homeostasis is an important mechanism related to the acute health effects of inhaled pollutants. Inhalation of urban particulate matter and ozone by rats has been shown to result in increased synthesis of the potent vasoactive peptide endothelin (ET)-1 in the lungs, with spillover into the circulation. In the present work, we have analyzed the interrelationships between responses of the three major endothelin isoforms, ET-$1_{[1–21]}$, ET-$2_{[1–21]}$, and ET-$3_{[1–21]}$, to inhaled pollutants at the peptide and gene expression levels. Fisher-344 rats were exposed for 4 hrs by nose-only route to clean air, urban particles EHC-93 (0, 50 mg/m^3), ozone (0, 0.8 ppm), or ozone and particles together. Circulating levels of both the ET-$1_{[1–21]}$ and ET-$3_{[1–21]}$ peptides were increased immediately after exposure to particulate matter or ozone. While expression of preproET-1 mRNA in the lungs increased, expression of preproET-3 mRNA decreased immediately after exposure. PreproET-2 mRNA was not detected in the lungs, and exposure to either pollutant did not affect plasma ET-2 levels. Co-exposure to ozone and particles, while altering lung preproET-1 and preproET-3 mRNA levels in a fashion similar to ozone alone, did not cause changes in the circulating levels of the two corresponding peptides. Thus, *de novo* synthesis of ET-3 in the lungs is not responsible for the increase of circulating plasma ET-3 after inhalation of pollutants, which implies regulation of preproET-3 at a remote site and, hence, systemic impacts of the pollutants. Upregulation of preproET-1 coupled with down-regulation of preproET-3 in the lungs of animals exposed to air pollutants implies a mismatch of local ET-1/ET_A receptor–mediated vasoconstriction and ET-3/ET_B receptor–mediated vasodilation. Exp Biol Med 231:979–984, 2006

Key words: endothelin (ET)-1; ET-2; ET-3; lung; gene expression; real-time polymerase chain reaction (PCR); particulate matter; ozone; air pollution

Introduction

Air pollution levels correlate with respiratory and cardiovascular morbidity and mortality (1–3). Several recent epidemiologic studies have reported increased myocardial infarction and decreased heart rate variability within a few hours of increased ambient ozone or respirable particulate matter concentrations (4, 5). Controlled exposure studies involving animal models and humans have generally supported the epidemiologic data by validating potential biologically plausible mechanisms for these effects. For example, inhalation of an ambient particulate matter preparation by rats rapidly activates lung endothelin system genes (6, 7) and causes a shift in circulating levels of the vasoactive peptide endothelin (ET)-1 (8). The increase in plasma ET-1 after exposure to particles is associated with an increase in systemic blood pressure in healthy rats (9) and cardiotoxicity in a rat model of myocardial infarction (10). In line with these animal studies, healthy humans exposed to ambient fine particulate matter and ozone exhibit vasoconstriction (11). Children living in southwest Mexico City have higher ET-1 plasma levels than children living in a less-polluted city (12).

Although preproET-1 is the predominant endothelin mRNA in the lungs, preproET-3 mRNA has also been detected, while preproET-2 mRNA appears to be absent or poorly expressed (13, 14). ET-2 differs from ET-1 by only two amino acids (Trp^6, Leu^7) and exerts effects similar to ET-1 through the endothelin-A (ET_A) and endothelin-B (ET_B) receptors (15). ET-3 differs by six amino acids (Thr^2, Phe^4, Thr^5, Tyr^6, Lys^7, and Tyr^{14}) and has a lesser affinity for the ET_A receptor but has been shown to induce a biphasic response in the pulmonary circulation, exerting

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potent nitric oxide–dependent vasodilation at lower levels and vasoconstriction at higher levels (16). Little is known about the regulation of these peptides in the lungs. Recent studies indicate that exposure to air pollution, in addition to increasing spillover of ET-1 into circulation, also increases circulating levels of ET-2 and ET-3. Plasma ET-2 was elevated in aged rats after exposure to highway air pollution (17), and plasma ET-3 levels rapidly increased after inhalation exposure to particulate matter (9). The sources of the excess circulating ET-2 and ET-3 in response to inhaled pollutants are not known.

Real-time polymerase chain reaction (PCR) is the current benchmark for sensitive and accurate quantification of mRNA levels. In the present work we set out to validate a real-time PCR system for the endothelin genes. We then investigated the interrelationships between circulating levels of the three ET isoforms and expression of their corresponding mRNAs in the lungs of rats after inhalation of ozone and urban particles.

Materials and Methods

Animals. Specific-pathogen-free Fischer-344 male rats (200–250 g) obtained from Charles River (St. Constant, Canada) were housed in individual Plexiglas cages on wood-chip bedding under HEPA-filtered air and were held to a 12:12-hr dark:light cycle. Food and water were provided *ad libitum*. All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada.

Inhalation Exposure to Air Pollutants. The ambient urban particles EHC-93 consist of total suspended particulate matter recovered from filters of the single-pass air-purification system at the Environmental Health Centre (Tunney's Pasture, Canada) and were mechanically sieved using a 36-μm mesh filter. The chemical composition, biological reactivity of the particles in cell culture models, and applications in inhalation studies have been described elsewhere (8, 9, 18, 19). Rats ($n = 4$–12) were trained in nose-only exposure tubes over five consecutive days and were then exposed for 4 hrs to clean air or to combinations of the individual pollutants EHC-93 (0, 50 mg/m^3) and ozone (0, 0.8 ppm) using a nose-only exposure system, essentially as described previously (7, 19). Animals were euthanized immediately after exposure or following 24 hrs recovery in filtered air.

Biological Samples. Rats were anaesthetized by administration of sodium pentobarbital (60 mg/kg, ip). Blood was collected from the abdominal aorta into vacutainer tubes containing the sodium salt of EDTA at 10 mg/ml and phenylmethylsulfonylfluoride at 1.7 mg/ml, mixed gently, and placed on ice (20). Plasma was isolated by centrifugation (2000 rpm for 10 mins), aliquoted, and frozen at –80°C. The lungs were washed by bronchoalveolar lavage with warm saline (37°C) at 30 ml/kg body wt, then flash-frozen in liquid nitrogen and stored at –80°C. Frozen lung samples were homogenized in TRIzol reagent (Invitrogen Canada, Inc., Burlington, Canada), and total RNA was isolated according to the manufacturer's instructions. RNA was quantified using the RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, OR), and total RNA was reverse-transcribed using MuLV reverse transcriptase and random hexamers (Applied Biosystems, Mississauga, Canada), according to the manufacturer's instructions.

Analysis of Plasma Endothelins. Plasma ET-$1_{[1–21]}$, ET-$2_{[1–21]}$, and ET-$3_{[1–21]}$ were analyzed by high-performance liquid chromatography (HPLC)–fluorescence in a subset of the animals immediately after exposure, as previously described (20).

Real-Time PCR Analysis of Lung Gene Expression. Primers for preproET-1 (Sense: GAC AAG GAG TGT GTC TAC TTC TGC; Antisense: GGC TTC CTA GTC CAT ACG GG), preproET-2 (Sense: CAA CTC CTG GCT TGA CAA GG; Antisense: TAG GGA GCT GTC TGT CCT GC), and preproET-3 (Sense: CTG TCC AAC CAC AGA GGA AGC; Antisense: TGT CTG TGG AGA AGA CTG GG) genes, and a reference gene, β-actin (Sense: CAC TAT CGG CAA TGA GCG GTT CC; Antisense: CTG TGT TGG CAT AGA GGT CTT TAC GG), were designed to have 50%–60% GC content, an optimal annealing temperature of 60°–62°C, and to yield PCR products of 75–150 base pairs in length using Vector NTI software (InforMax, Frederick, MD). Primers and predicted amplicons were evaluated for secondary structure using online m-fold software (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/). Double-desalted primers were purchased from Invitrogen. Optimal annealing temperatures were determined to be 60°C (ET-2) and 62°C (ET-1 and ET-3), with either temperature resulting in high-efficiency amplification of β-actin. Evaluation of product purity by melt curve analysis revealed no additional products. Since primer sets amplified rat cDNA with similar high efficiency over a range of cDNA dilutions, preproET-1, preproET-2, and preproET-3 could be compared relative to β-actin to allow the determination of relative gene expression. Master mixes of the reagents were prepared to minimize differences in reagent composition. Twenty nanograms of sample cDNA were incubated with 25 μl iQ SYBR Green Supermix (Bio-Rad Laboratories [Canada] Ltd., Mississauga, Canada) and 200 n*M* of each primer, and the reagent mixture was brought up to 50 μl with DNase/RNase-free water. All reactions were performed in duplicate on 96-well plates in a spectrofluorometric thermal cycler (iCycler iQ, Bio-Rad). PCR runs were initiated by incubation at 95°C for 3 mins to activate the iTAQ polymerase followed by 40 cycles of 95°C for 15 secs, the appropriate annealing temperature for 15 secs, and 72°C for 30 secs. Fluorescence was monitored at every cycle during the elongation step. Negative reverse transcription controls (samples for which the reverse transcriptase was omitted from the reverse transcription reaction mix) were included in each run to test for genomic

DNA contamination, and a melt curve was conducted following each run to verify product purity. Expression was calculated relative to β-actin using the delta-delta Ct method (21) and was expressed as fold change relative to air control samples.

Statistical Analyses. Data are expressed as mean ± standard error of the mean (SEM). The effects of ozone and the particles EHC-93 on mRNA levels were tested for statistical significance by three-way analysis of variance (ANOVA) with OZONE (0, 0.8 ppm O_3), EHC (0, 50 mg EHC-93/m^3), and TIME (0, 24 hrs postexposure) as factors. Plasma ET data immediately after inhalation exposures were analyzed by two-way ANOVA with OZONE (0, 0.8 ppm O_3) and EHC (0, 50 mg EHC-93/m^3) as factors. Tukey's multiple comparison procedure was applied to elucidate the patterns of significant effects ($\alpha = 0.05$). Significance of the linear regression correlation coefficient was determined using the Student's t test ($\alpha = 0.05$). Statistical analyses were performed using the Sigma-Stat software (Sigma-Stat 3.0, Chicago, IL).

Results

In contrast to the increase in preproET-1 mRNA expression in the lungs immediately after exposure to the pollutants (Fig. 1A; OZONE × TIME factor interaction, $P < 0.001$), preproET-3 mRNA was transiently decreased (Fig. 1E; OZONE × TIME factor interaction, $P < 0.001$). Rats exposed to particulate matter exhibited a sustained decrease in preproET-3 mRNA expression in the lungs (EHC main effect, $P = 0.025$). At the experimental concentrations used, ozone was more potent than the particles in elevating expression of preproET-1 mRNA, but in contrast to the sustained 24-hr increase in preproET-1 mRNA by particles, the response to ozone was transient. The circulating levels of ET-$1_{[1–21]}$ (Fig. 1B; EHC × OZONE factor interaction, $P < 0.001$) and ET-$3_{[1–21]}$ (Fig. 1F; EHC × OZONE factor interaction, $P = 0.099$) peptides increased immediately after inhalation exposure of the animals to either particles or ozone, while plasma ET-$2_{[1–21]}$ levels remained unchanged (Fig. 1D). Coexposure to ozone plus particles did not result in statistically significant changes of the three endothelin peptides in plasma; in fact, levels of all three peptides were lower in the coexposure group than in the animals exposed to either pollutant. Overall, while plasma levels of ET-1 and ET-3 were positively correlated (Fig. 2A; $r = 0.60$, $P = 0.014$), lung levels of preproET-1 and preproET-3 mRNA were negatively correlated (Fig. 2B; $r = –0.66$, $P < 0.0001$).

Discussion

With appropriate controls to verify the absence of genomic DNA contamination, a SYBR Green I dye-based real-time PCR approach is a simple, relatively inexpensive, and reproducible system for gene expression analysis. We have validated a SYBR Green I dye-based real-time PCR assay for the determination of preproET-1, preproET-2, and preproET-3 mRNA expression in rats. In combination with primers for rat ECE-1, ET_A and ET_B receptors, and endothelial and inducible nitric oxide synthase (7), these primers allow investigation of endothelin-nitric oxide system gene expression. Using these primers we confirmed that in our animals, preproET-1 was the dominant endothelin mRNA in the lungs, followed by preproET-3 mRNA, while pulmonary preproET-2 mRNA expression was negligible, a result that is consistent with previous reports (13, 22).

Increased endothelin production is a plausible mechanism to explain the association between episodic variations of ambient air pollutants and acute cardiovascular morbidity and mortality in susceptible individuals (6–9). An imbalance in the production of endothelium-derived constriction and dilation factors in the pulmonary vascular bed contributes to the alteration of vascular tone. Such endothelial dysfunction and increased vascular resistance may contribute to the progression of cardiovascular disease or cause acute adverse effects, such as increasing cardiac afterload and myocardial ischemia (23). Inhalation of either particulate matter or ozone has been shown to cause concurrent increases of preproET-1 and ECE-1 mRNA in the lungs and increased plasma steady-state concentrations of the ET-1 peptide in circulation (7). Exposure of rats to urban particles also resulted in an immediate and sustained (>48 hrs) 2-fold increase of the steady-state levels of plasma ET-3, revealing a major impact on regulation of the endothelin system (9). Here we report that while plasma ET-3 tends to increase immediately after inhalation of particulate matter and ozone in a pattern similar to that observed for the ET-1 peptide, preproET-3 mRNA expression in the lung actually decreases. Therefore, the data indicate that the lungs are most probably not the site of *de novo* ET-3 synthesis responsible for the sustained elevation of plasma ET-3 after inhalation of urban particles. Neuronal cells in the lungs possess secretory granules that can contain endothelin (24), and it is conceivable that EHC-93 may act as a trigger to cause the release of ET-3. However, since ET-3 has a half-life of only 1–2 mins in plasma (25), the sustained increase in steady-state plasma ET-3 levels over 48 hrs after inhalation of EHC-93 (9) should require continuous *de novo* peptide production, or a sustained reduction in clearance. Since lung ET_B receptor mRNA initially increases immediately after exposure to either ozone or particles (7), decreased receptor expression and reduced receptor-mediated clearance of the peptide is not a likely mechanism to explain the rapid increase of circulating ET-3. ET-3 is a neurotransmitter, and the pituitary is a site of ET-3 synthesis (26). Other sites of importance in the regulation of circulating ET-3 levels are the vascular beds of the spleen and kidney, which have been shown to clear ET-3 from circulation (27). The data indicate that these sites are potentially affected after inhalation of air pollutants, and

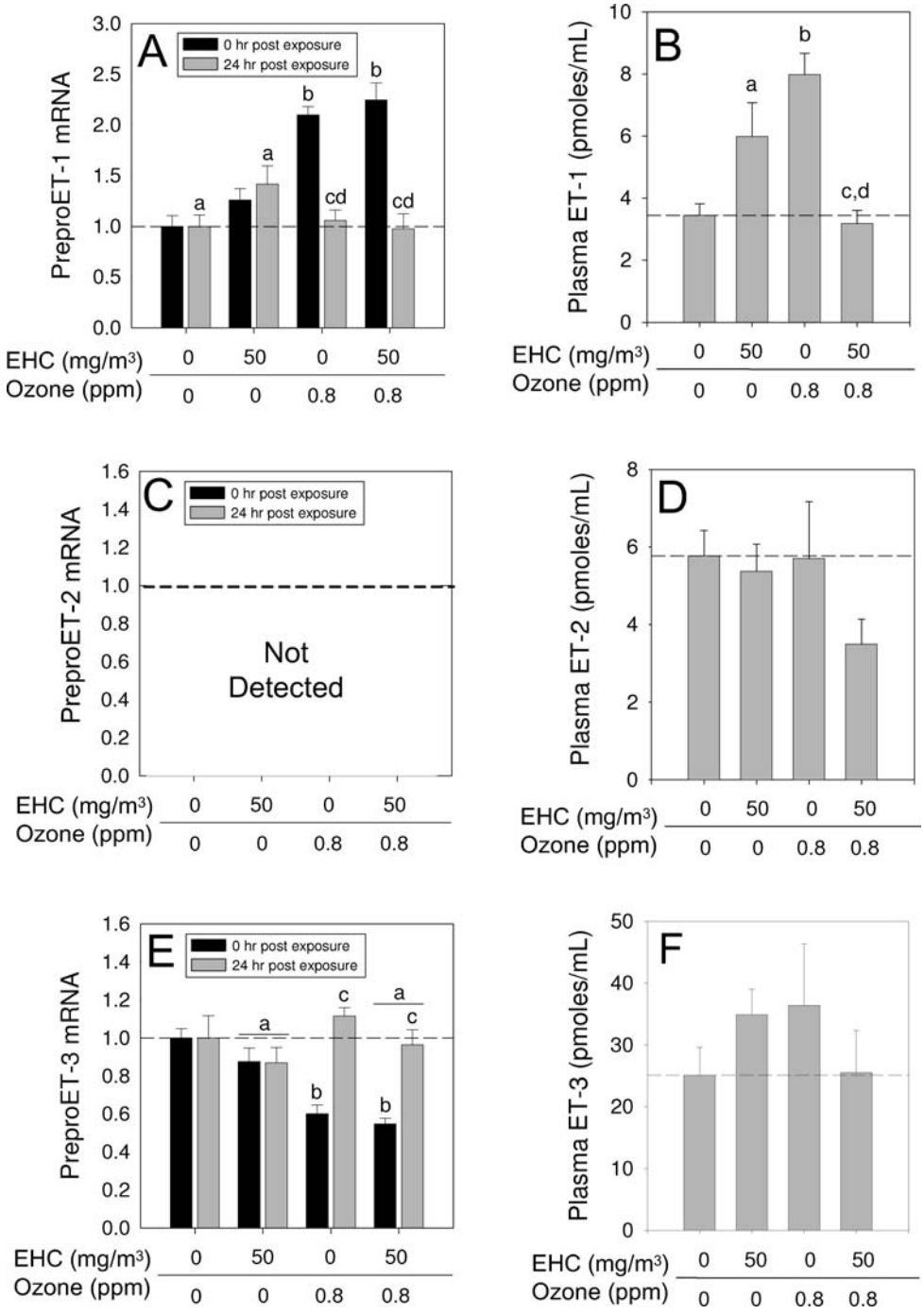

Figure 1. Effects of inhalation of particulate matter and ozone on preproET mRNA levels in the lungs 0 to 24 hrs. postexposure (A, C, E) and circulating endothelin levels in plasma measured immediately postexposure (B, D, F). Results are expressed as mean ± SEM ($n = 4$–12 animals/group). Letters over bars indicate statistical significance (Tukey, $P < 0.05$). (A) PreproET-1 mRNA. Three-way ANOVA, OZONE × TIME factor interaction, $P < 0.001$. (a) 0 versus 24 hrs within 0 ppm O_3; (b) 0 versus 0.8 ppm O_3 within 0 hrs; (c) 0 versus 0.8 ppm ozone within 24 hrs; (d) 0 versus 24 hrs within 0.8 ppm O_3. (B) Plasma ET-1 peptide. Two-way ANOVA, EHC × OZONE factor interaction, $P < 0.001$. (a) 0 versus 50 mg/m^3 EHC within 0 ppm O_3; (b) 0 versus 0.8 ppm O_3 within 0 mg/m^3 EHC; (c) 0 versus 0.8 ppm O_3 within 50 mg/m^3 EHC; (d) 0 versus 50 mg/m^3 EHC within 0.8 ppm O_3. (C) PreproET-2 mRNA. No detectable levels in lung tissue. (D) Plasma ET-2 peptide. No significant effects. (E) PreproET-3 mRNA. Three-way ANOVA, OZONE × TIME factor interaction, $P < 0.001$; EHC main effect, $P = 0.025$. (a) 0 versus 50 mg/m^3 within EHC; (b) 0 versus 0.8 ppm O_3 within 0 hrs postexposure; (c) 0 versus 24 hrs postexposure within 0.8 ppm O_3. (F) Plasma ET-3 peptide. Two-way ANOVA, EHC × OZONE factor interaction, $P = 0.099$. Panels A and B reproduced from Thomson *et al.* (7) by permission of the Society of Toxicology.

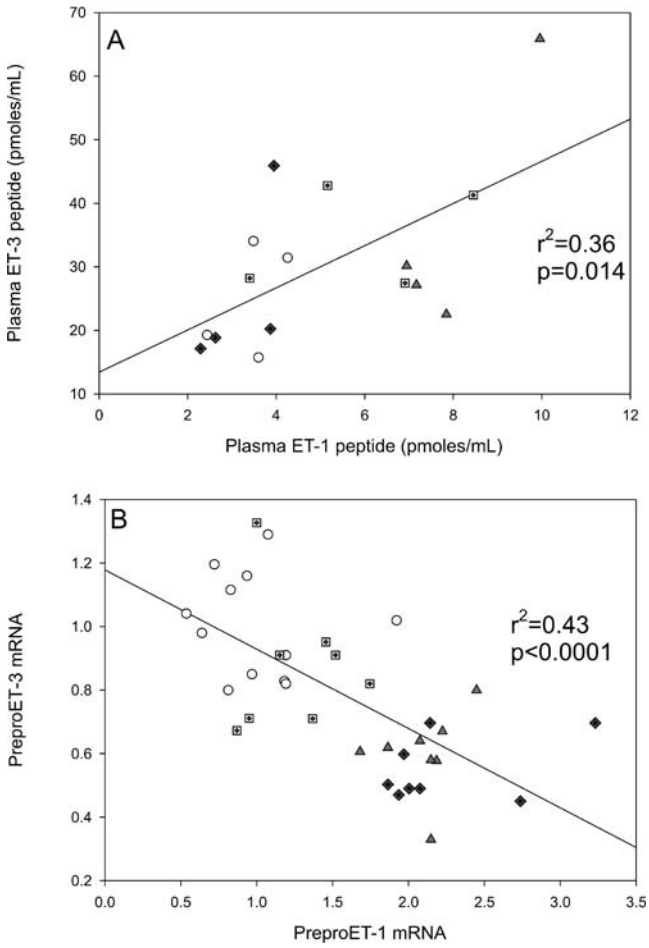

Figure 2. Linear regressions of plasma ET-1 and ET-3 peptides (A) and lung tissue preproET-1 and preproET-3 mRNA levels (B) for individual animals exposed to air (open circle), EHC-93 particles (square), ozone (triangle), or ozone plus particles (diamond).

organ-specific expression analyses should help resolve this issue.

It is unclear what is prompting the decrease in preproET-3 mRNA in the lungs. PreproET-3 mRNA was altered reciprocally to preproET-1 mRNA in the lungs after exposure to the pollutants. Since ET-1 and ET-3 may induce similar effects when expressed at high levels, it is conceivable that our findings reflect a feedback mechanism whereby ET-1 released as a result of increased preproET-1 mRNA expression acts locally to depress preproET-3 mRNA expression. There is some evidence to support this possibility. While initial studies of ET-1 knockout mice found no change in ET-2 or ET-3 mRNA and peptide levels in the lungs, intestines, or brains (22), preproET-3 mRNA has been shown to be overexpressed in ET-1–null astrocytes derived from these animals (28), indicating that expression of preproET-3 is normally repressed by ET-1. In a rat model of diabetes, preproET-1 mRNA increased, while preproET-3 mRNA decreased in the adrenal glands, indicating that the two genes are regulated by different mechanisms (29). Along the same lines, preproET-1 and preproET-2 are reciprocally modulated in the failing heart and during ischemia of cardiomyocytes (30). Clearly, additional work is required to identify the specific cells or areas in the lungs that are responsible for the increased preproET-1 mRNA and decreased preproET-3 mRNA and to examine regulation of the respective genes in these cells. This mismatch between expression of preproET-1 and preproET-3 mRNAs in the lungs after inhalation of the pollutants could imply increased local ET-1 and ET_A receptor–mediated vasoconstriction, coupled with decreased ET-3 and ET_B receptor–mediated vasodilation.

When inhaled individually, particulate matter and ozone increased the circulating levels of ET-1 and ET-3 measured immediately after exposure. However, after coexposure of the animals to both pollutants, the plasma levels of the two peptides were not affected, despite clear evidence of alteration of gene expression in the lungs. We have reported previously that coexposure to particulate matter and ozone provokes an increase of MMP-2 activity in the lungs (7), an enzyme that has been shown to cleave bigET-1 to ET-$1_{[1–32]}$ *in vitro* (31), and we proposed that the excess bigET-1 produced in the injured lungs may be diverted toward ET-$1_{[1–32]}$ production. Sources of circulating ET-3 peptide and the mechanistic explanation for the apparent ($P = 0.099$) interaction of the two pollutants with respect to plasma levels of ET-3 remain to be determined. Nevertheless, if regulation of preproET-3 at a remote site after inhalation exposure of ozone and particles is dependent on elevated systemic ET-$1_{[1–21]}$ levels from increased *de novo* synthesis and spillover from the pulmonary capillary bed, then the pattern of response of plasma ET-3 is entirely consistent with that of ET-1.

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