

# Brief Secondhand Smoke Exposure Depresses Endothelial Progenitor Cells Activity and Endothelial Function

## Sustained Vascular Injury and Blunted Nitric Oxide Production

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- Objectives** This study sought to analyze the effects of acute secondhand smoke (SHS) exposure on the number and function of endothelial progenitor cells (EPCs) over 24 h.
- Background** Secondhand smoke increases the risk of vascular disease and is a major public health concern, but the mechanism(s) of action are not fully understood.
- Methods** Healthy nonsmokers (age SEM  $30.3 \pm 1.3$  years,  $n = 10$ ) were exposed to 30 min of SHS yielding cotinine levels commonly observed in passive smokers and to smokefree air on 2 separate days. Measurements were taken before exposure (baseline), immediately after (0 h), and at 1 h, 2.5 h, and 24 h after. The EPCs (CD133<sup>+</sup>/KDR<sup>+</sup>, CD34<sup>+</sup>/KDR<sup>+</sup>) and endothelial microparticles (EMPs: CD31<sup>+</sup>/CD41<sup>-</sup>, CD144<sup>+</sup>, CD62e<sup>+</sup>) were determined in blood using flow cytometry. The EPC chemotaxis toward vascular endothelial growth factor was measured. Endothelial function was assessed as flow-mediated dilation (FMD) using ultrasound.
- Results** Secondhand smoke exposure increased EPCs and plasma vascular endothelial growth factor and completely abolished EPC chemotaxis during 24 h after exposure. Secondhand smoke increased EMPs and decreased FMD. Although FMD returned to baseline at 2.5 h, EMPs and vascular endothelial growth factor levels remained elevated at 24 h, suggesting endothelial activation and injury with functional impairment of the vascular endothelium. Exposure to smokefree air had no effect. Incubation of EPCs from nonexposed subjects with plasma isolated from SHS-exposed subjects in vitro decreased chemotaxis by blockade of vascular endothelial growth factor–stimulated nitric oxide production.
- Conclusions** Brief exposure to real-world levels of SHS leads to sustained vascular injury characterized by mobilization of dysfunctional EPCs with blocked nitric oxide production. Our results suggest that SHS not only affects the vascular endothelium, but also the function of EPCs. (J Am Coll Cardiol 2008;51:1760–71) © 2008 by the American College of Cardiology Foundation

Exposure to secondhand smoke (SHS) accounts for about 50,000 deaths annually in the United States, mostly from heart disease (1). The effects of SHS on the cardiovascular system are remarkably similar in magnitude to those caused

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by chronic active smoking (2). Although the mass dose of smoke delivered during exposure to SHS is 10 to 100 times

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Cardiac Stem Cell Foundation to Dr. Yeghiazarians. The funding agencies played no role in the conduct of the research or preparation of the article. Drs. Heiss and Amabile contributed equally to this work. Dr. Heiss is currently affiliated with the University RWTH Aachen, Medizinische Klinik 1, Aachen, Germany.

Manuscript received October 10, 2007; revised manuscript received December 12, 2007, accepted January 14, 2008.

lower than that delivered during active smoking, the relative risk of coronary artery disease is 1.31 in passive smokers (2) compared with 1.78 in active light smokers (3). Laws mandating smoke-free workplaces and public places are associated with immediate reductions in acute myocardial infarction in both U.S. and Italian communities (4–7).

The pathophysiology of SHS (2) and active cigarette smoking-induced (8) toxicity is similar and includes arterial endothelial dysfunction, platelet activation, oxidation of low-density lipoprotein (LDL) cholesterol, and increased insulin resistance (2). Acute (9–11) and chronic (9) exposure of nonsmokers to SHS decreases endothelial function to near the levels observed in chronic active smokers (9–12). In vitro, cigarette smoke causes generation of reactive oxygen species, impairs nitric oxide (NO) production, and causes apoptosis and activation of endothelial cells, all of which may contribute to SHS-induced toxicity (13,14).

Smoking impairs not only endothelial function, but also endothelial regeneration or maintenance. The regeneration process is mediated in part by endothelial progenitor cells (EPCs) circulating in peripheral blood. There is an association between the number and functional activity of EPCs and the health of the endothelium (15,16). The chemotactic capacity of EPCs determines the degree of functional recovery when EPCs are used as a cell based therapy (17). Because baseline levels of circulating EPCs and their functional activities *ex vivo* are impaired in smokers, increase rapidly toward control values during smoking cessation, and decrease again with resumption of smoking (18), we tested the hypothesis that the number and function of these cells might also be adversely affected by acute brief SHS exposure in nonsmokers (19,20).

## Methods

### Study Design and Protocol

To study the effect of SHS exposure on EPCs and vascular endothelial integrity, we exposed healthy, young nonsmokers ( $n = 10$ ) to 30 min of SHS at levels comparable to those present in public smoking areas. We measured the number and function of EPCs (21), cotinine levels as a marker for SHS exposure, and several markers of endothelial injury including circulating endothelial microparticles (EMPs), plasma vascular endothelial growth factor (VEGF) levels, and flow-mediated dilation (FMD) (22) before, and immediately (0 h), 1 h, 2.5 h, and 24 h after SHS exposure. In control experiments, 7 of the individuals were also exposed to smoke-free air. The study visits were separated by 19 days on average (3 SHS first, 4 control air first). The remaining 3 subjects dropped out of the study after the first session. Because cardiovascular risk factors are associated with impaired number and function of EPCs (15,20), exclusion criteria consisted of hypertension, diabetes mellitus, smoking, and hypercholesterolemia. All study subjects were lifelong nonsmokers and reported no recent exposure to SHS. To substantiate the *in vivo* findings, we performed

additional *in vitro* experiments incubating EPCs isolated from nonsmokers with plasma isolated from exposed subjects before and after SHS exposure on VEGF stimulated NO production, chemotaxis, and proliferation. The study protocol was approved by the University of California at San Francisco Committee on Human Research, and all subjects gave written informed consent. Analyses were performed by blinded investigators.

### SHS Exposure

The SHS exposures were conducted in a custom-built steel and glass exposure chamber (15 m<sup>3</sup>) with an average airflow rate of 11 m<sup>3</sup>/min (380 cfm) as described (23). The temperature (21°C ± 2°C) and relative humidity (55 ± 7%) were controlled inside the chamber. Sidestream smoke from Marlboro Red filtered cigarettes was generated according to the FTC method of one 35-ml puff of 2 s duration per minute using an adapted Borgwaldt RM1/G smoking machine located approximately 1 meter from the subject's breathing zone (24). Mainstream smoke was exhausted directly from the exposure chamber. Average total suspended particulate matter and airborne nicotine were 367 ± 43 μg/m<sup>3</sup> and 67 ± 9 μg/m<sup>3</sup>, respectively, in the breathing zone of the subjects. These are levels typically seen in smoking areas of restaurants and bars (25).

**Plasma cotinine.** Plasma cotinine concentrations were measured using gas chromatography via nitrogen phosphorus detection, modified by use of a capillary column, as described previously to provide biomarkers of exposure (26).

### EPCs

**EPC numbers in blood.** The number of EPCs in whole blood was measured as CD133/KDR and CD34/KDR double-positive cells using fluorescence-activated cell sorting (FACS). Staining was performed after washing with phosphate-buffered saline (PBS) and F<sub>c</sub>-receptor blocking (immunoglobulin G 1 mg/ml, Zymed, San Francisco, California). Phycoerythrin (PE) and allophycocyanin-conjugated antihuman mouse antibodies (CD34-PE and KDR-allophycocyanin, Pharmingen, San Diego, California; CD133-PE, Miltenyi Biotech, Auburn, California) or isotype controls were incubated with 100 μl of 10<sup>6</sup> /ml cell suspension using FACS-buffer (1% bovine serum albumin [BSA]/PBS) for 20 min. After lysing red blood cells and washing with FACS-buffer twice, cells were fixed with 1% formaldehyde/PBS and stored at 4°C until analysis; 100,000

### Abbreviations and Acronyms

<b>BrdU</b>	= bromodeoxyuridine
<b>BSA</b>	= bovine serum albumin
<b>DAF</b>	= diaminofluorescein
<b>EMP</b>	= endothelial microparticle
<b>EPC</b>	= endothelial progenitor cell
<b>FMD</b>	= flow-mediated dilation
<b>L-NMMA</b>	= L <sup>α</sup> -monomethyl-arginine
<b>NO</b>	= nitric oxide
<b>PE</b>	= phycoerythrin
<b>SHS</b>	= secondhand smoke
<b>UEA</b>	= <i>Ulex europaeus</i> agglutinin
<b>VEGF</b>	= vascular endothelial growth factor

events were collected in the lymphocyte and monocyte gate (FACSCalibur, Becton Dickinson, San Diego, California). **EPC characterization after ex vivo expansion.** The EPCs were differentiated ex vivo from peripheral blood mononuclear cells, which were isolated based on the Ficoll method (Vacutainer CPT, Becton Dickinson, Franklin Lakes, New Jersey). The cells were pre-plated on fibronectin-coated culture plates for 1 day in endothelial basal medium (EBM)-2 MV supplemented with Singlequots (Cambrex, East Rutherford, New Jersey) and 20% fetal bovine serum (HyClone, Logan, Utah). The adherent cells were discarded and the nonadherent cells were moved to a new dish and cultured for another 6 days, during which time many cells became newly adherent.

To confirm the endothelial phenotype and survival of ex vivo differentiated EPCs at day 7, we performed fluorescent staining to detect lectin binding and uptake of acetylated low-density lipoprotein (acLDL). Adherent EPCs on fibronectin-coated glass slides (Nalgen NUNC, Naperville, Illinois) were incubated for 1 h with 2  $\mu\text{g}/\text{ml}$  DiI-acLDL (Invitrogen, Carlsbad, California) in EBM-2 MV, washed twice with PBS, and fixed in 2% formaldehyde/PBS. After blocking (2% goat serum/PBS 1 h), cells were washed and incubated with 23  $\mu\text{g}/\text{ml}$  fluorescein isothiocyanate (FITC)-conjugated *Ulex europaeus* agglutinin (UEA)-1 (Sigma, St. Louis, Missouri). The nuclei were stained with 125 ng/ml Hoechst 33258 (Invitrogen) and observed using a Nikon E800 fluorescence microscope and Openlab software (Improvision, Lexington, Massachusetts). Almost all adherent cells were double positive for UEA-1 and acLDL-uptake confirming endothelial phenotype ( $95 \pm 10\%$  adherent cells).

**Chemotaxis assay.** Cell migration was quantified using a modified Boyden chamber (15). Cells ( $2 \times 10^4$ ) were plated in the upper of 2 chambers divided by a membrane with 8- $\mu\text{m}$  pores (Corning Transwell, Corning, New York). Chemoattractant VEGF (Sigma, 50 ng/ml in EBM-2, 0.5% BSA) or medium alone was added to the lower chamber. For in vitro experiments with EPCs isolated from nonexposed non smokers, L<sup>G</sup>-monomethyl-arginine (L-NMMA) (100  $\mu\text{M}$ ), plasma (1% v/v added to EBM-2 0.5% BSA at baseline, 0 h, and 24 h), and cotinine (0.01 to 10 ng/ml) were added both lower chambers and cells suspension before addition to the upper chamber. After 6 h, the membranes were washed twice in PBS and fixed in 4% formaldehyde. Cells on the lower side of the membrane were counted. Each experimental condition was performed in triplicate, and the number of migrated cells was determined on 5 random 100 $\times$  fields (0.998 mm<sup>2</sup>) per membrane. Chemotaxis was calculated as number of migrated cells in wells with VEGF minus number of cells in control wells without VEGF (randomly migrated cells). We have established that this assay quantitates directional migration toward a chemokine gradient (chemotaxis) as opposed to random cell movement in the presence of a chemokine (chemokinesis). Furthermore, we confirmed in this system

that a VEGF gradient remains throughout the experimental period by enzyme-linked immunosorbent assay. Chemoattractant VEGF (50 ng/ml) was added only to the lower chamber. Parallel experiments were performed without VEGF to measure random cell movement.

#### Plasma VEGF Level

The expression level of VEGF, which is an important regulator of EPC mobilization in response to injury, was measured in plasma (subset  $n = 5$ ) by a high-sensitive ELISA assay (R&D Systems, Minneapolis, Minnesota).

#### Circulating Endothelium-Derived Microparticles (EMPs)

**Preparation of platelet-free plasma.** Endothelium-derived microparticles were measured as previously described (27). Platelet-free plasma was obtained by successive centrifugation of citrated whole blood (500 g, 15 min; 9,500 g, 5 min; room temperature) and stored at  $-80^\circ\text{C}$  until analysis.

**EMP characterization.** The method for EMP phenotype analysis is based on size characterization and fluorescence measurement and has been previously described (27). Events with a 0.1- to 1- $\mu\text{m}$  diameter were identified in forward and side scatter intensity dot representation and then plotted on 1-color or 2-color fluorescence histograms (FACSCalibur, Becton Dickinson). Different phenotypes of EMPs were determined as previously reported (27,28): CD31<sup>+</sup>/CD41<sup>-</sup> (PECAM/glycoprotein IIb), CD144<sup>+</sup> (VE-cadherin) (Beckman Coulter), and CD62e<sup>+</sup> (E-selectin) (Becton Dickinson). The platelet-free plasma samples were incubated for 30 min with different fluorochrome-labeled antibodies or matching isotype controls; a known amount of Flowcount calibrator beads (Beckman Coulter; 20  $\mu\text{l}$ ) was added to the samples just before performing flow cytometry analyses.

#### FMD

Endothelium-dependent dilation of the brachial artery was measured by ultrasound (Sonosite Micromax, Bothell, Washington) in combination with an automated analysis system (Brachial Analyzer, Medical Imaging Applications, Iowa City, Iowa). Baseline data for diameter and blood flow velocity of the brachial artery were quantified after 10 min of supine rest in a 21 $^\circ\text{C}$  room. A forearm blood pressure (BP) cuff was placed distal to the antecubital fossa and inflated to 250 mm Hg for 5 min. Diameter was measured immediately after cuff deflation, at 20 s, 40 s, 60 s, and 80 s. The FMD was expressed as:  $(\text{diameter}_{\text{max}} - \text{diameter}_{\text{baseline}}) / \text{diameter}_{\text{baseline}}$ . Endothelium-independent vasodilation was not assessed in this study because the application of nitroglycerin affects consecutive measurements and studies have shown that nitroglycerin response is not altered by SHS exposure (11) and is not significantly different between age-matched groups of smokers and nonsmokers (29).

### Mechanistic In Vitro Experiments

To substantiate the in vivo findings, we measured the effect of plasma isolated from study subjects at baseline (before exposure), 0 h, and 24 h after SHS exposure on NO production, chemotaxis, and proliferation of EPCs isolated from nonexposed subjects. Plasma cotinine concentrations were nondetectable (baseline), 0.1 ng/ml (0 h), and 0.3 ng/ml (24 h). Plasma was added at 1% (v/v). To test whether cotinine is a mere biomarker of smoke exposure or whether it may directly contribute to the observed effects on EPCs, we performed parallel EPC experiments after adding pure cotinine (Sigma) at concentrations observed in plasma after SHS exposure (0.01 to 10 ng/ml in PBS).

**NO production.** We incubated EPCs with an NO-sensitive fluorescent dye 4,5-diaminofluorescein (DAF-2) diacetate (Sigma). The DAF-2 diacetate is membrane-permeable and is deacetylated by intracellular esterases to DAF-2. However, DAF-2, remains essentially nonfluorescent until it reacts with the nitrosonium cation (produced by spontaneous oxidation of NO) to form a fluorescent heterocycle, which becomes trapped in the cell's cytoplasm (30) (excitation 492 nm, emission 515 nm). The EPCs cultured on fibronectin-coated glass slides were serum starved with 0.5% BSA/EBM-2 for 2 h. Cells were pre-incubated with L-NMMA (100  $\mu$ M), 2-(4-Carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO) (100  $\mu$ M), plasma (baseline, 0 h, and 24 h; 1% and 10% v/v added to EBM-2 0.5% BSA), cotinine (Sigma, 0.01 to 1 ng/ml), and DAF-2 diacetate (10  $\mu$ M) for 20 min. The VEGF (50 ng/ml) was added for another 20 min to stimulate NO production. Cells were washed with PBS once and fixed in 2% formaldehyde/PBS for 15 min. Cells were washed with PBS, nuclei stained with Hoechst, and cells mounted. Fluorescence images were taken using the identical exposure settings for all conditions. Image-Pro Plus 6.0 software, version 6.0, 2007 (Media Cybernetics, Inc., Bethesda, Maryland), was used to quantify fluorescence intensity.

**Proliferation.** The bromodeoxyuridine (BrdU) incorporation assays were performed following the manufacturer's protocol (Cell Proliferation BrdU Assay, Roche). The EPCs were detached after 7 days in culture, resuspended in EBM-2 supplemented with 0.5% BSA, and plated at  $2 \times 10^4$  /well in 96-well cell culture plates (Corning) coated with fibronectin (100  $\mu$ g/ml). The cells were pre-incubated with the following test mitogens for 24 h: VEGF (50 ng/ml), L-NMMA (100  $\mu$ M), plasma (1% v/v added to EBM-2 0.5% BSA; baseline, 0 h, and 24 h). The BrdU was added and cells were incubated for another 2 h. The BrdU incorporation was determined colorimetrically after incubation with anti-BrdU antibodies conjugated with horseradish peroxidase.

### Statistical Analyses

Results are expressed as means  $\pm$  SEM. The primary test for an effect was a test of the interaction in a 2-way repeated measures analysis of variance (where the 2 factors were SHS

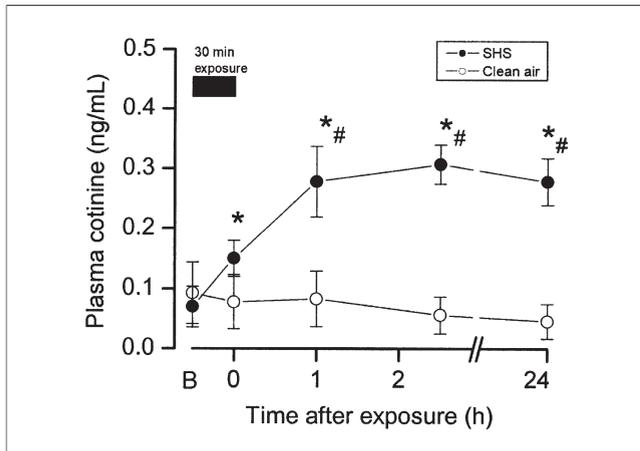
vs. clean air and time). We used a general linear model implementation of a repeated measures analysis of variance (31), which does not require dropping cases when there are incomplete data. Thus, we were able to use all available data for the 10 subjects (even though the data were incomplete for 3 subjects). The following family of 13 pairwise comparisons were conducted using the Holm-Sidak method: observed values compared with baseline within the SHS and clean air groups (4 comparisons each) and secondhand smoke versus clean air at each time (5 comparisons). The analysis of variance and unadjusted Holm-Sidak p values were computed with SigmaStat, version 3.5, 2006 (Systat Software Inc., San Jose, California), and the critical p values computed manually based on the actual family of 13 pairwise comparisons that we performed. Statistical power analyses were performed post hoc using PS Power and Sample Size Calculations 2.1.30 (32) and showed that at  $\alpha = 0.05$  and power = 0.80 a change in CD133<sup>+</sup>/KDR<sup>+</sup>, CD34<sup>+</sup>/KDR<sup>+</sup> EPCs, and FMD of >0.0298%, >0.0597%, and >0.8%, respectively, can be detected. We tested for a linear relationship between biological effects and cotinine levels using a 2-level random effects linear regression model to account for both within-person and between-person variation in the association of cotinine levels on the different biological outcomes using MLwinN 2.02 (Centre for Multilevel Modeling, University of Bristol, Bristol, England).

### Results

**Baseline characteristics of study population.** The clinical baseline characteristics (body mass index, mean arterial pressure, total cholesterol, LDL, high-density lipoprotein, and fasting glucose) were within normal limits (Table 1). The baseline FMD was 6.9%  $\pm$  0.8%, suggesting normal endothelial function (15). Baseline FMD correlated significantly with CD34<sup>+</sup>/KDR<sup>+</sup> EPCs ( $r = 0.65$ ,  $p = 0.005$ )

**Table 1** Baseline Characteristics of Study Subjects (n = 10)

	Mean $\pm$ SEM
Age (yrs)	30.3 $\pm$ 1.3
Gender (M/F)	7/3
Body mass index (kg/m <sup>2</sup> )	24.3 $\pm$ 1.1
Glucose (mg/dl)	73 $\pm$ 3
Cholesterol (mg/dl)	179 $\pm$ 7
Triglycerides (mg/dl)	77 $\pm$ 9
High-density lipoprotein (mg/dl)	51 $\pm$ 5
Low-density lipoprotein (mg/dl)	121 $\pm$ 9
White blood cells (per $\mu$ l)	5,822 $\pm$ 236
Polymorph nucleated cells (per $\mu$ l)	3,373 $\pm$ 295
Hemoglobin (g/dl)	14 $\pm$ 1
Lymphocytes (per $\mu$ l)	1,818 $\pm$ 104
Monocytes (per $\mu$ l)	427 $\pm$ 32
Platelets (per $\mu$ l)	276,222 $\pm$ 13,973
Cotinine (ng/ml)	0.08 $\pm$ 0.03
Mean arterial pressure (mm Hg)	74.3 $\pm$ 2.7
Heart rate (beats/min)	64 $\pm$ 3
Flow-mediated dilation (%)	6.8 $\pm$ 0.3



**Figure 1** Time Course of Plasma Cotinine

Cotinine levels significantly increased immediately after SHS exposure and remained significantly elevated until 24 h. No increase was seen after exposure to clean air in the chamber. Symbols are mean  $\pm$  SEM. \* $p < 0.05$  versus baseline of same day (exposure condition). # $p < 0.05$  comparing SHS and clean air at the same time. B = baseline; SHS = secondhand smoke.

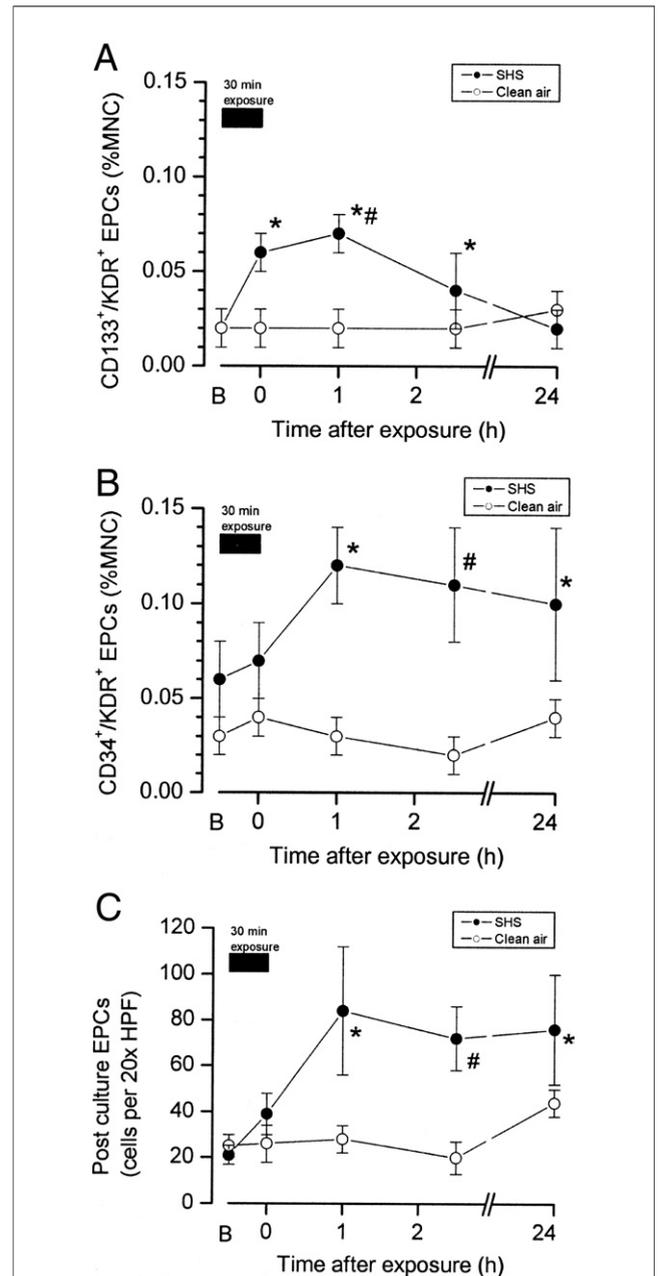
and inversely with LDL ( $r = -0.833$ ,  $p = 0.005$ ). Baseline plasma cotinine levels were very low,  $0.08 \pm 0.03$  ng/ml, consistent with nonsmokers not exposed to secondhand cigarette smoke (33). There were no correlations between baseline cotinine levels and EPC numbers or chemotaxis, EMPs, plasma VEGF level, or FMD.

**Exposure levels/plasma cotinine.** Plasma cotinine levels significantly increased after SHS exposure but not after smoke-free air (Fig. 1).

**Increased numbers of EPCs in blood and after culture.** We observed a sequential increase in early less differentiated CD133<sup>+</sup>/KDR<sup>+</sup> EPCs that was followed by a significant and sustained increase in committed CD34<sup>+</sup>/KDR<sup>+</sup> EPCs. The number of CD133<sup>+</sup>/KDR<sup>+</sup> EPCs significantly increased immediately after SHS exposure and remained elevated until 2.5 h later, then returned to baseline by 24 h (Fig. 2A). The CD34<sup>+</sup>/KDR<sup>+</sup> EPCs were significantly increased at 1 h, 2.5 h, and 24 h compared with pre-SHS baseline as well as with measurements taken at the same time points after clean air exposure (Fig. 2B). To confirm EPC numbers and to confirm that SHS did not change the cell phenotype, cells were also characterized after culture. Almost all adherent cells were double positive for UEA-1 and acLDL uptake confirming endothelial phenotype ( $95\% \pm 10\%$  adherent cells). We observed significantly greater numbers of EPCs in samples from SHS-exposed subjects at 1 h to 24 h (Fig. 2C). This pattern mimicked the time course of CD34<sup>+</sup>/KDR<sup>+</sup> EPCs in blood.

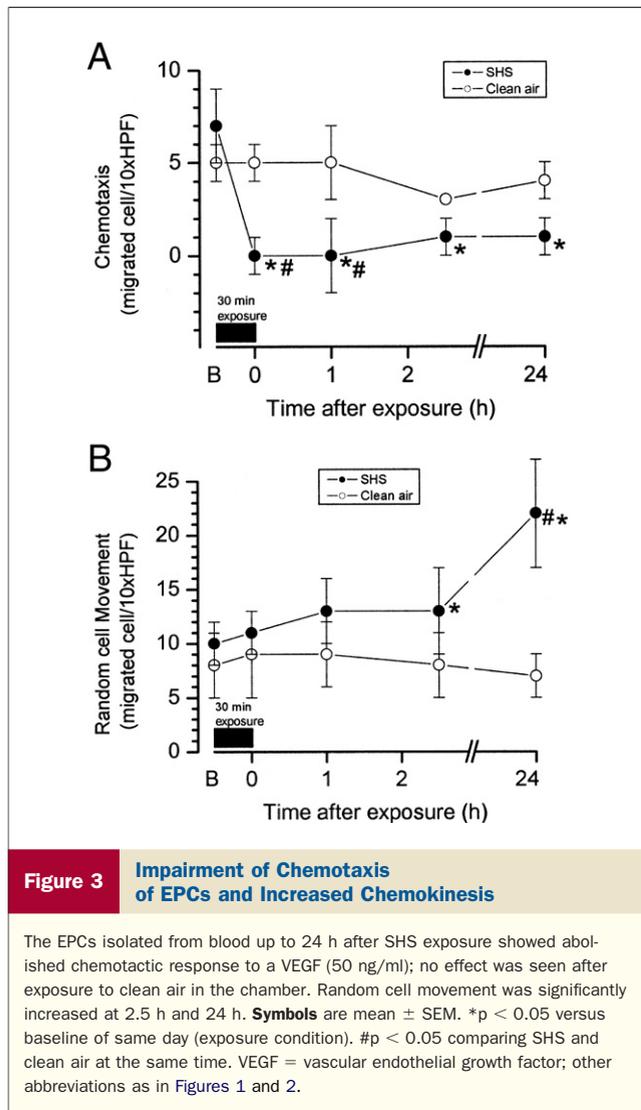
**SHS exposure blocks chemotaxis in EPCs.** Chemotaxis to VEGF was completely absent in EPCs isolated from blood samples immediately after SHS exposure, and this effect persisted for 24 h after SHS exposure (Fig. 3A), Random cell movement was not impaired at 0 h to 1 h and significantly increased at 2.5 h and 24 h (Fig. 3B).

**Increase in plasma VEGF.** Vascular endothelial growth factor, an important regulator of EPC mobilization in response to injury, was significantly increased after SHS exposure at all time points (0 h, 1 h, 2.5 h, and 24 h)



**Figure 2** Mobilization of EPCs in Blood and EPC Survival After Culture

(A) Early less differentiated CD133<sup>+</sup>/KDR<sup>+</sup> EPCs and (B) committed CD34<sup>+</sup>/KDR<sup>+</sup> EPCs in blood were sequentially and significantly increased after SHS exposure but not after exposure to clean air only. (C) The survival of EPCs as determined by UEA-1<sup>+</sup>/acLDL<sup>+</sup> after 7 days of culture was also significantly increased after SHS, mimicking the time course of CD34<sup>+</sup>/KDR<sup>+</sup> EPC. Symbols are mean  $\pm$  SEM. \* $p < 0.05$  versus baseline of same day (exposure condition). # $p < 0.05$  comparing SHS and clean air at the same time. acLDL = acetylated low-density lipoprotein; EPC = endothelial progenitor cell; MNC = mononuclear cells; UEA-1 = *Ulex europaeus* agglutinin-1; other abbreviations as in Figure 1.



compared with baseline levels and respective time points of the control exposure (Fig. 4A).

**EMP generation.** The EMP plasma concentrations increased immediately after SHS exposure and remained elevated 24 h after SHS (Figs. 4B to 4D). Whereas levels of CD31<sup>+</sup>/CD41<sup>-</sup> EMPs were significantly increased over baseline and over the respective air control at 1 h, the subpopulations of CD144<sup>+</sup> and CD62e<sup>+</sup> EMPs were increased throughout the observation period after SHS exposure and remained significantly elevated at 24 h. The EMPs values remained constant during control clean air exposure.

**Impaired endothelium-dependent vasomotor function.** Exposure to SHS led to an immediate reduction in FMD, followed by recovery at 2.5 h and an elevation in FMD at 24 h (Fig. 4E). Average FMD values measured immediately after 30 min of exposure to SHS (0 h time point) were 3% lower than the values observed during exposure to smoke-free air. The FMD remained significantly depressed 1 h

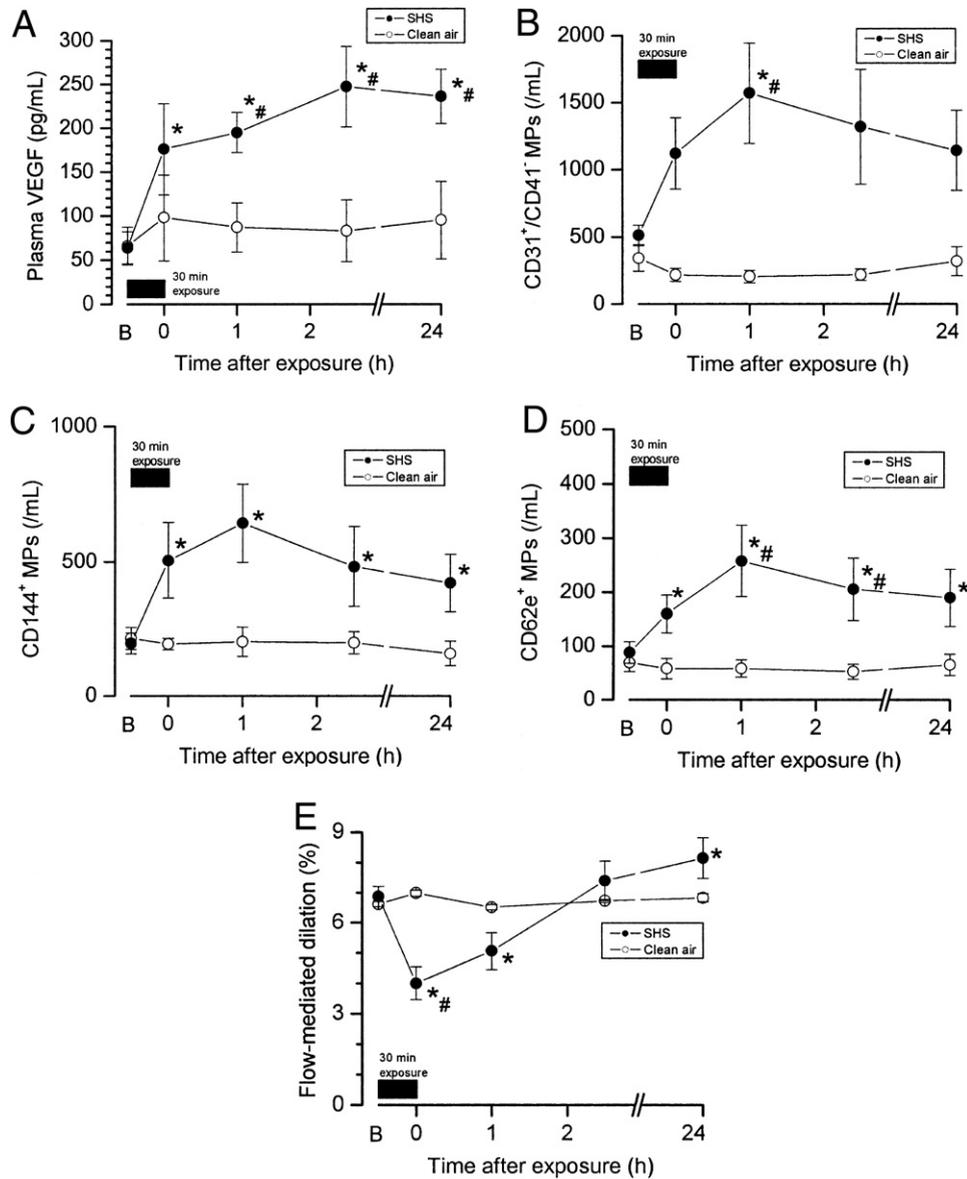
after SHS exposure, then returned to normal 2.5 h after exposure. The average FMD measured 24 h after SHS exposure was significantly higher than baseline and fresh air values. The FMD remained unchanged over time during exposure to smoke-free air.

**Increase in systolic BP.** Systolic BP significantly increased by  $8.7 \pm 1.6$  mm Hg at 2.5 h and by  $9.2 \pm 1.5$  mm Hg at 24 h after SHS smoke exposure. Heart rate and diastolic BP remained unchanged at these time points. Baseline BP and heart rate were not significantly different between study days (average  $104 \pm 3/61 \pm 3$  mm Hg,  $62 \pm 3$  beats/min), and smoke-free air did not affect systolic and diastolic BP or heart rate.

**Linear relationship between plasma cotinine and biological effects.** To estimate the linear association between plasma cotinine levels and the observed biological effects (dependent variables), we performed multilevel random-effects linear regression analyses to account for between-subject effects (Table 2). Three models were tested: all data (baseline to 24 h), early exposure phase (baseline–1 h), and late exposure phase (baseline and 24 h). These 3 models were considered a family of 3 comparisons for each variable, and the individual test  $p$  values compared with critical levels using the Holm procedure. Using all available data points, the analysis showed significant linear relationships between cotinine and chemotaxis, CD31<sup>+</sup>, CD144<sup>+</sup>, CD62e<sup>+</sup> EMPs, and systolic BP. In the early acute phase (baseline–1 h post-exposure), there was a significant linear relationship between cotinine and chemotaxis, VEGF, FMD, CD31<sup>+</sup>, CD144<sup>+</sup>, and CD62e<sup>+</sup> EMPs. In the late phase (baseline and 24 h), there was a significant linear relationship between cotinine and CD144<sup>+</sup>, and CD62e<sup>+</sup> EMPs.

**Incubation of EPCs with post-exposure plasma leads to decreased NO production, decreased chemotaxis, and increased proliferation.** To substantiate the in vivo findings and gain mechanistic insight, we measured the effect of plasma isolated from study subjects at baseline (before exposure), at 0 h, and 24 h after SHS exposure in vitro on EPCs isolated from nonexposed nonsmokers (Figs. 5 and 6). Whereas baseline plasma had no significant effect on VEGF-stimulated NO production and chemotaxis toward VEGF, post-exposure plasma (isolated at 0 h and 24 h) completely inhibited NO production and chemotaxis. The DAF-2 fluorescence was abolished by L-NMMA, a competitive NO synthase inhibitor, and PTIO, a NO scavenger, showing the specificity of this assay. Although chemotaxis was inhibited by L-NMMA, proliferation was enhanced by L-NMMA. Post-exposure plasma stimulated EPC proliferation like L-NMMA did. Although plasma induced significant chemokinesis, L-NMMA had no effect.

To determine whether cotinine is only a biomarker for SHS smoke exposure or may also contribute to biological effects, we incubated EPCs with cotinine levels equivalent to the maximal plasma values measured in our subjects (0.1 to 10 ng/ml). We observed that cotinine dose-dependently



**Figure 4** Vascular Injury: Increased Plasma VEGF, Acute Sustained MP Generation, and Acute Reversible Endothelial Dysfunction

(A) Plasma VEGF levels were increased up to 24 h after SHS exposure. (B to D) The microparticles (MPs) in plasma increased significantly immediately (0 h) after SHS exposure and remained elevated throughout the observation period at 24 h, but remained unchanged after exposure to clean air in the chamber. The effect was observed in the 3 subgroups of endothelial MPs studied [CD31<sup>+</sup>/CD41<sup>-</sup> MPs (B), CD144<sup>+</sup> MPs (C), and CD62e<sup>+</sup> (D)]. After 30 min of SHS exposure, flow-mediated dilation (E) was significantly decreased immediately (0 h) and at 1 h after, but was increased at 24 h; FMD remained unchanged after clean air control. **Solid symbols** indicate SHS exposure and **open symbols** depict clean air controls. **Symbols** are mean  $\pm$  SEM. \* $p < 0.05$  versus baseline of same day (exposure condition). # $p < 0.05$  comparing SHS and clean air at the same time. FMD = flow-mediated dilation; other abbreviations as in Figures 1 and 3.

decreased NO production and chemotaxis and increased proliferation and chemokinesis.

## Discussion

Our results show that brief exposure of nonsmokers to real-world levels of SHS leads to increased numbers of circulating dysfunctional EPCs in response to acute vascular injury that persists for at least 24 h. Mechanistically, we show in vitro that post-exposure plasma blocks chemotaxis

and increases EPC proliferation by inhibiting NO production. Furthermore, pure cotinine had a similar yet independent effect in vitro at levels observed in vivo.

Our study is the first to test the vascular effects of brief (30 min) controlled exposure to SHS on EPCs in healthy nonsmokers. The mean airborne nicotine concentration in this study was  $67 \mu\text{g}/\text{m}^3$  (total suspended particulate matter  $367 \mu\text{g}/\text{m}^3$ ), which is approximately the level seen in bars that permit smoking (25). The peak post-exposure level of

**Table 2** Linear Regression of Plasma Cotinine (Independent, ng/ml) and Biological Parameters (Dependent)

	All Data (Baseline, 0 h, 1 h, 2.5 h, 24 h)			Early Response (Baseline, 0 h, 1 h)			Late Response (Baseline, 24 h)		
	Slope	SE	p Value	Slope	SE	p Value	Slope	SE	p Value
CD133/KDR EPCs (%)	0.04	0.04	0.338	0.11	0.06	0.065	-0.03	0.03	0.823
CD34/KDR EPCs (%)	0.23	0.10	0.020	0.30	0.14	0.029	0.30	0.30	0.320
UEA/acLDL EPCs (per HPF)	-60.8	48.4	0.209	40.7	41.4	0.325	-3.6	88.0	>0.999
Chemotaxis (per HPF)	-8.6	3.4	0.011	-12.7	5.4	0.019	-6.8	6.5	0.294
Random cell movement (per HPF)	6.8	7.5	0.362	16.3	10.0	0.104	-16.5	14.9	0.267
VEGF (pg/ml)	325	150	0.031	434	132	0.001	-143	111	0.198
CD31 EMPs (per ml)	3,873	1,128	0.001	3,959	1,322	0.003	984	814	0.227
CD144 EMPs (per ml)	1,302	511	0.011	1,358	521	0.009	629	277	0.023
CD62e EMPs (per ml)	575	204	0.005	697	243	0.004	540	213	0.011
FMD (%)	-0.3	1.3	0.823	-4.5	2.1	0.030	2.5	1.6	0.110
Blood pressure, systolic (mm Hg)	12.2	4.9	0.013	3.5	7.0	0.617	16.1	8.1	0.047
Blood pressure, diastolic (mm Hg)	5.0	4.4	0.262	3.1	5.8	0.579	4.7	9.1	0.603

acLDL = acetylated low-density lipoprotein; EMP = endothelial microparticles; EPC = endothelial progenitor cell; FMD = flow-mediated dilation; HPF = high power field; UEA = *Ulex europaeus* agglutinin; VEGF = vascular endothelial growth factor;

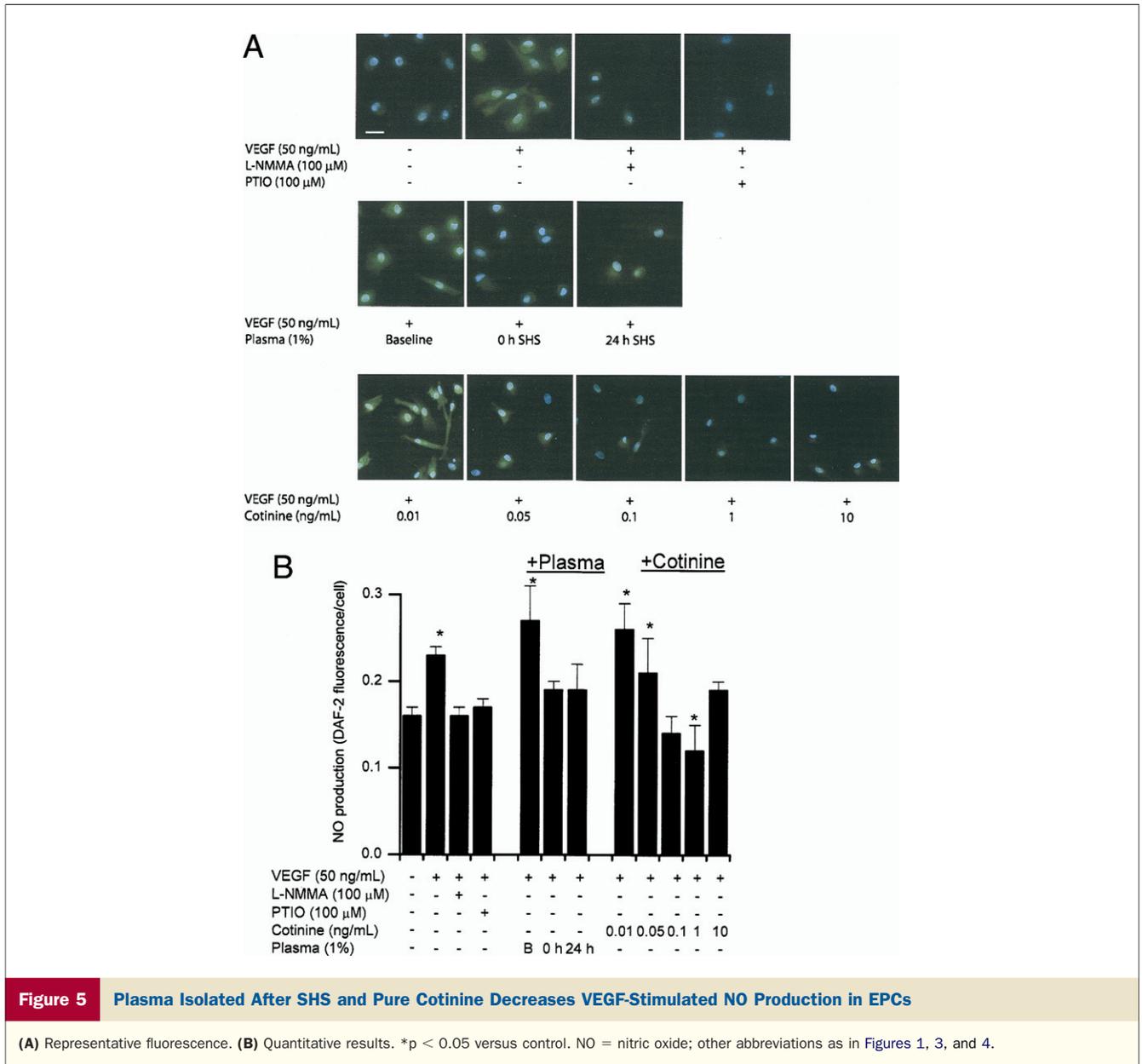
0.31 ± 0.03 ng/ml plasma cotinine at 2.5 h is similar to that reported for nonsmokers exposed to SHS (33). In contrast to previous studies showing persistently decreased levels of EPCs in chronic smokers (16,20,34), SHS exposure led to increased numbers of EPCs in circulating blood in the first 24 h. Levels of immature (CD133<sup>+</sup>/KDR<sup>+</sup>) EPCs were elevated immediately after exposure, remained elevated at 2.5 h, but decreased to pre-exposure values by 24 h. Levels of more mature (CD34<sup>+</sup>/KDR<sup>+</sup>) EPCs were elevated at 1 hr after exposure and remained elevated at 24 h. The sequential appearance of the 2 EPC populations in blood, first immature CD133<sup>+</sup>/KDR<sup>+</sup> then committed CD34<sup>+</sup>/KDR<sup>+</sup> together with the observed UEA-1<sup>+</sup>/acLDL<sup>+</sup> EPCs after culture, suggests that SHS may cause an acute mobilization of EPCs that can still differentiate and proliferate under the influence of growth factors and SHS components while circulating in blood and during culture.

Although there were more circulating EPCs after SHS exposure, these cells showed severe functional impairment. The EPCs did not show chemotaxis toward a VEGF gradient, suggesting either inability to sense and follow a chemokine gradient or decreased general cell motility. Because random cell movement was not impaired, the lack of chemotaxis was likely caused by a decreased ability of EPCs to sense or follow a VEGF gradient.

Nitric oxide seems to play an important regulatory role in endothelial biology and directional cell movement. We have previously shown that NO synthase inhibition leads to decreased chemotaxis and increased proliferation in EPCs (35). In the present study, we show that plasma isolated after SHS exposure decreased NO production and chemotaxis but increased proliferation of EPCs isolated from nonexposed subjects. These observations suggest that SHS may inhibit chemotaxis and stimulate proliferation by blocking NO production. Our results corroborate the findings of Michaud et al. (34) showing that EPCs from

otherwise healthy smokers exhibit impaired functional activities, including cell migration and tube formation. In contrast to the present study in nonsmokers, those investigators observed lower proliferation in smokers' EPCs, suggesting cumulative damage with chronic exposure. In a more recent article, Michaud et al. (19) performed in vitro experiments with endothelial cells. Incubation of human umbilical vein endothelial cells with cigarette smoke extract led to a blockade of the Akt/endothelial nitric oxide synthase (eNOS)/NO pathway and impaired VEGF-induced migration. This suggests that the mechanisms involved in smoke toxicity are similar in endothelial cells and EPCs.

Furthermore, our results suggest that cotinine is not only a biomarker of smoke exposure, but may also affect cell behavior at concentrations observed in vivo. Although qualitatively similar, the effects of cotinine on EPCs in vitro seem to be independent of other more potent toxins in SHS because plasma completely abolished chemotaxis at 100-fold dilution. Thus, the final cotinine concentration was <0.003 ng/ml, which is a concentration that had no effect on EPCs when added alone in vitro. In vivo, the effect of cotinine may be relevant in chronically exposed subjects because cotinine has a long half-life (approximately 15 h), and active smokers' plasma typically contains >15 ng/ml cotinine. Taken together, our results suggest that SHS leads to mobilization of dysfunctional EPCs, potentially involving an impaired NO signaling, which may lead to a sustained impairment of vascular repair. Clinically, impaired migratory capacity of EPCs has been associated with inferior outcome when EPCs were used as a cell-based therapy after myocardial infarction (17). Our results imply that EPCs are highly sensitive to SHS and that SHS exposure should be avoided when EPC function is being evaluated in clinical studies or when EPCs are being used therapeutically for cell-based interventions (36).



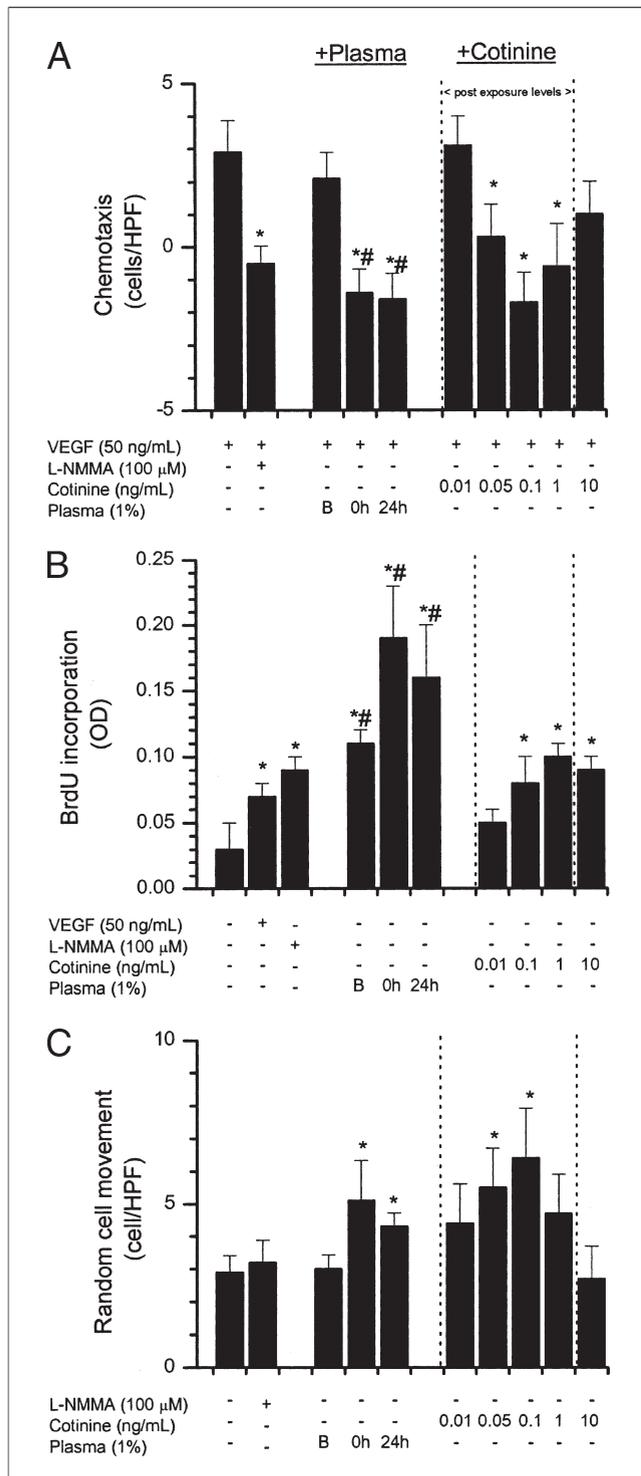
**Figure 5 Plasma Isolated After SHS and Pure Cotinine Decreases VEGF-Stimulated NO Production in EPCs**

(A) Representative fluorescence. (B) Quantitative results. \*p < 0.05 versus control. NO = nitric oxide; other abbreviations as in Figures 1, 3, and 4.

The immediate transient decrease in FMD, a measure of vascular endothelial NO activity, from normal values (15) (approximately 7% at baseline) approached chronic active smoker's values (4% at 0 h) (12,29,37) and is consistent with the results published previously. Kato et al. (11) have shown a similar reduction of FMD in nonsmokers after SHS exposure. Our results suggest that an acute phase of endothelial dysfunction is followed by a rapid recovery and, surprisingly, an overshoot of vascular reactivity with significantly increased FMD at 24 h. This overshoot suggests possible compensatory mechanisms leading to a state of hyperreactivity after SHS exposure potentially involving EPC mobilization or proliferation that may no longer be functional in chronic smokers. Experimental studies suggest potential mechanistic explanations for the observed increase in FMD 24 h after SHS exposure. Barua et al. (14,29) have

shown in vitro that incubation of endothelial cells with serum isolated from chronic smokers with impaired FMD not only leads to uncoupling of eNOS with decreased activity and NO generation, but also increases eNOS expression on the protein level. Both effects were ascribed to H<sub>2</sub>O<sub>2</sub> because they were abolished by addition of superoxide dismutase and catalase. Supporting this notion, markers of oxidative stress, 8-isoprostanes, were significantly increased after SHS exposure in humans (11). Thus, acute SHS-induced oxidative stress may have led to an upregulation of eNOS in the subjects' vascular endothelium. This upregulation would be compatible with increased vasodilation in the absence of further SHS exposure.

To provide further evidence that SHS causes an acute endothelial injury with physical damage on the vascular



**Figure 6** Effects of Post-Exposure Plasma and Cotinine on EPCs In Vitro

Plasma isolated after SHS exposure and pure cotinine decrease chemotaxis to VEGF (A), increase proliferation (B), and increase random cell movement (C) similar to NO synthase inhibition with L-NMMA. The EPCs were isolated from nonexposed subjects (n = 6) and incubated with L-NMMA, plasma isolated before (B, baseline), immediately after (0 h), and 24 h after SHS exposure. **Right sides of graphs** represent cotinine dose response. **Dashed lines** show plasma cotinine range after SHS exposure (approximately 0 to 1 ng/ml). \*p < 0.05 versus control. #p < 0.05 versus baseline plasma. L-NMMA = L<sup>G</sup>-mono-methyl-arginine; other abbreviations as in Figures 1 and 3.

endothelium and the need for cell replacement, we measured circulating EMPs, which are submicron membrane particles shed from endothelial cells during apoptosis, activation (38), or cell death (39,40). Several controversies exist regarding the nature and potential biological effects of circulating EMPs, and there is no consensus on the method for their identification. The phenotype of circulating microparticles varies depending on the cellular origin and the release mode, which may subsequently determine their biological functions (41). The initial decrease in FMD in our study was paralleled by significantly increased numbers of EMPs in plasma, suggesting that even short exposure to SHS causes acute vascular injury/endothelial and structural damage. We measured different subgroups of EMPs because in vitro data suggest that EMPs composition could depend on their production mode: CD62e<sup>+</sup> MPs have been reported to be released during cell activation, whereas CD144<sup>+</sup> MPs might be generated during apoptosis (38,40). Our data show robustly that SHS causes endothelial cell lesions, although the exact underlying ultrastructural mechanisms remain obscure.

Our findings are consistent with previous work reporting associations between EMPs release and endothelial dysfunction in coronary artery disease (42), renal failure (26), or diabetes (41). Unlike the recovery and overshoot seen with FMD, high levels of EMPs still persisted 24 h after exposure. The reasons that EMPs were still elevated at 24 h after SHS exposure whereas FMD returned to pre-exposure levels at 2.5 h remain unknown, because the in vivo circulating microparticles metabolism is largely unknown. An increased half-life of EMPs, a decreased clearance of EMPs, or an endothelial injury or activation process that persists after SHS exposure might explain our findings. Our regression analysis suggests that cotinine may be a contributing factor to ongoing activation at the late time point. Increased circulating EMP levels may be clinically important because EMPs have pro-coagulant activity and may contribute to increased coagulability observed after smoke exposure (22,42). The EMPs not only indicate endothelial damage, but also can cause endothelial dysfunction in vitro (27,43). Thus, the acute release of EMPs during SHS exposure might contribute to sustained deleterious effects of smoke on the vascular wall.

The sustained increase in plasma VEGF levels over 24 h can be interpreted as a rescue signal indicating vascular injury and the need for repair and cell recruitment. Other human studies support this notion by showing that increased VEGF levels are associated with endothelial dysfunction (15,44). The VEGF is both an important marker of endothelial injury and mediator of repair. On one hand, VEGF has been shown to be expressed in severe cases of vascular injury such as ischemia, inflammation, and infarction (45). On the other hand, VEGF stimulates endothelial survival, mobilization, and recruitment of EPCs from the bone marrow (46). Our data corroborate experimental results from Conklin et al. (47), who showed that the

incubation of isolated perfused porcine carotid arteries with cotinine (approximately 17 ng/ml) and nicotine at levels representative of smoker's plasma resulted in a striking upregulation of VEGF mRNA and protein. Several studies in animal models show that SHS can be a potent inducer of angiogenesis and EPC mobilization and that nicotine seems to contribute significantly to this effect (48,49). Zhu et al. (49) have shown that chronic exposure to SHS in mice promoted tumor angiogenesis, mobilized EPCs, and led to increased plasma VEGF. The presented data suggest that the observed increase in VEGF levels after SHS exposure may also be involved in the mobilization of EPCs. Thus, our present data suggest that even short exposure to SHS may cause a systemic and long-lasting proangiogenic response that may also affect concomitant disease states that involve angiogenesis in their pathophysiology, including cancer.

The significant increase in systolic BP at 2.5 h and 24 h after SHS exposure may be a result of increased vascular tone and peripheral resistance and may contribute to cardiovascular effects of SHS exposure. Our results are consistent with those of Mahmud et al. (50), who have reported significantly increased BP after SHS exposure in healthy young men, and McCracken et al. (51) observed that decreased wood smoke exposure during cooking reduced systolic BP in Guatemalan women.

## Conclusions

These results show that brief exposure to real-world levels of SHS leads to a mobilization of dysfunctional EPCs in response to acute vascular injury that persists for more than 24 h. Mechanistically, these effects are linked to an impairment of NO production in EPCs. Taken together, these findings provide further evidence that even a very short period of passive smoke exposure has strong, persistent vascular consequences. The SHS may harm the vasculature not only by directly injuring the vascular endothelium but also by interfering with the vascular repair system, which may lead to chronic damage with recurrent exposures. These results provide further scientific evidence that involuntary SHS exposure constitutes a significant public health risk even at low levels.

## Acknowledgments

The authors thank Dr. Kanu Chatterjee for helpful discussions; Vanessa I. Block, Melanie Taylor, Virginia Scheidel, and William Ong for technical assistance; Dr. Alexis Dinno for computation of random effects regression analyses; and Dr. Neal Benowitz for performing the cotinine measurements.

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