Secondhand Smoking Is Associated With Vascular Inflammation

Tessa Adams, MD; Elaine Wan, MD; Ying Wei, PhD; Romina Wahab, MD; Francesco Castagna, MD; Gang Wang, PhD; Memet Emin, MD; Cesare Russo, MD; Shunichi Homma, MD; Thierry H. Le Jemtel, MD; and Sanja Jelic, MD

BACKGROUND: The relative risk for cardiovascular diseases in passive smokers is similar to that of active smokers despite almost a 100-fold lower dose of inhaled cigarette smoke. However, the mechanisms underlying the surprising susceptibility of the vascular tissue to the toxins in secondhand smoke (SHS) have not been directly investigated. The aim of this study was to investigate directly vascular endothelial cell function in passive smokers.

METHODS: Using a minimally invasive method of endothelial biopsy, we investigated directly the vascular endothelium in 23 healthy passive smokers, 25 healthy active smokers, and 23 healthy control subjects who had never smoked and had no regular exposure to SHS. Endothelial nitric oxide synthase (eNOS) function (expression of basal eNOS and activated eNOS [phosphorylated eNOS at serine1177 (P-eNOS)]) and expression of markers of inflammation (nuclear factor- κ B [NF- κ B]) and oxidative stress (nitrotyrosine) were assessed in freshly harvested venous endothelial cells by quantitative immunofluorescence.

RESULTS: Expression of eNOS and P-eNOS was similarly reduced and expression of NF- κ B was similarly increased in passive and active smokers compared with control subjects. Expression of nitrotyrosine was greater in active smokers than control subjects and similar in passive and active smokers. Brachial artery flow-mediated dilation was similarly reduced in passive and active smokers compared with control subjects, consistent with reduced endothelial NO bioavailability.

CONCLUSIONS: Secondhand smoking increases vascular endothelial inflammation and reduces active eNOS to a similar extent as active cigarette smoking, indicating direct toxic effects of SHS on the vasculature.

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ABBREVIATIONS: eNOS = endothelial nitric oxide synthase; FMD = flow-mediated vasodilation; NO = nitric oxide; NF- κ B = nuclear factor- κ B; P-eNOS = phosphorylated endothelial nitric oxide synthase; SHS = secondhand smoke

AFFILIATIONS: From the Division of Pulmonary, Allergy, and Critical Care Medicine (Drs Adams, Wahab, Castagna, Wang, Emin, and Jelic), the Division of Cardiology (Drs Wan, Russo, and Homma), and the Division of Biostatistics (Dr Wei), Columbia University College of Physicians and Surgeons, New York, NY; and the Section of Cardiology (Dr Le Jemtel), Tulane University School of Medicine, New Orleans, LA.

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CORRESPONDENCE TO: Sanja Jelic, MD, Columbia University College of Physicians and Surgeons, Division of Pulmonary, Allergy, and Critical Care Medicine, PH8 Center, Room 101, 630 W 168th St, New York, NY 10032; e-mail: sj366@columbia.edu

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Passive smoking remains a major public health concern in the United States. Despite efforts to ban smoking, > 40% of nonsmoking children and adults are still exposed to secondhand smoke (SHS).^{1,2} Although the dose of cigarette smoke delivered to passive smokers is < 1% of that delivered to active smokers, the relative risk of coronary artery disease for passive smokers approaches 80% of the risk of active smokers.³⁻⁵ The mechanisms underlying the excessive sensitivity of the vascular tissue to the toxins in SHS remain unclear. Indirect evidence of reduced nitric oxide (NO) availability and soluble markers of inflammation in passive smokers suggests that SHS impairs vascular function.⁶⁻⁹ However, vascular inflammation and oxidative stress, key early steps in the development and progression of cardiovascular diseases, have not been demonstrated directly in passive smokers.

Materials and Methods

Study Sample

We prospectively recruited healthy, young passive smokers (n = 23), active smokers (n = 25), and control subjects who had never smoked or had regular exposure to SHS (n = 23) from the community through advertising. The study participants were recruited by flyers posted at the Columbia University facilities. They all lived in New York City at the time of the recruitment. Smoking burden in passive smokers was determined from self-reported exposure to SHS at home, the workplace, or both for at least 1 h/d for at least 3 years.⁶ The intensity of exposure to SHS was assessed by questionnaire as light (1-3 h daily), moderate (4-6 h daily), or heavy (> 6 h daily).⁶ Smoking burden in active smokers was determined from self-reported smoking history of at least 2 pack-years. One pack-year was defined as 20 cigarettes per day for 1 year or the equivalent.⁶ Plasma cotinine levels were assessed in all participants. The study participants were not diagnosed with any disease and were not receiving medications or nutritional supplements. Active smokers and control subjects were matched to passive smokers for sex, age (within 6 years), and BMI within 15%. All subjects had a normal supine BP and physical examination. This study was conducted in accordance with the amended Declaration of Helsinki. The Columbia University Committee on Human Research approved the study (approval No. IRB-AAAM0703). All study participants gave written informed consent.

Study Protocol

Endothelial cell harvesting, blood sample collection, and flow-mediated dilation (FMD) were performed between 8:00 AM and 10:00 AM while study participants were in a fasting state. The last exposure to cigarette smoke occurred within 12 h in all active and passive smokers. Plasma cotinine, total cholesterol, and glucose levels were measured in commercial laboratory (ARUP Laboratories).

Results

The clinical and laboratory characteristics of the study subjects are presented in Table 1. Age, sex, BMI, and systolic BP were similar in passive and active smokers and control subjects. Diastolic BP was lower in control subjects than in active and passive smokers. The duration

Using a minimally invasive technique of endothelial biopsy, the present study aimed to assess directly whether vascular inflammation and oxidative stress occur in passive smokers and to compare the degree of vascular alterations in passive and active smokers.

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Proteins that regulate basal NO production and inflammation and markers of oxidative stress were quantified in freshly harvested venous endothelial cells. We hypothesized that endothelial inflammation and oxidative stress are increased and NO availability is reduced in healthy active and passive smokers compared with healthy subjects who had never smoked and had no regular exposure to SHS.

Vascular Endothelial Cell Harvesting

A 20-gauge angiocatheter was inserted into a superficial forearm vein. Under sterile conditions, three J-shaped vascular guidewires (Arrow International Inc) were sequentially advanced into the vein up to 10 cm. Endothelial cells were retrieved from wire tips by washing with endothelial cell dissociation buffer.

Immunofluorescence for Protein Expression and Brachial Artery FMD

To assess vascular inflammation and oxidative stress, we measured venous endothelial expression of nuclear factor- κ B (NF- κ B) and nitrotyrosine by quantitative immunofluorescence.¹⁰ To evaluate endothelial NO availability, we measured venous endothelial expression of total endothelial NO synthase (eNOS) and activated eNOS (phosphorylated eNOS at serine1177 [P-eNOS]), as described previously.¹⁰ Details regarding immunofluorescence can be found in e-Appendix 1. Vascular reactivity of the brachial artery was measured in the contralateral arm to the endothelial harvesting site by FMD, according to the guidelines of the International Brachial Artery Reactivity Task Force, as described previously.^{10,11}

Statistical Analysis

The mean levels of FMD and endothelial protein expression were compared among active smokers, passive smokers, and control subjects. To account for the small sample size, exact permutation tests were used to determine the statistical significance.¹² To assess whether endothelial protein expression is associated with the intensity of exposure to cigarette smoke, we performed Spearman correlations between levels of FMD and endothelial protein expression and the number of years and hours of daily exposure to cigarette smoke in passive smokers and the number of cigarettes smoked daily and pack-years in active smokers (SAS version 9.4; SAS Institute Inc).

of passive smoking was 9 ± 6 years (range, 3-39 years). Six passive smokers (26%) had been exposed to SHS throughout childhood. Passive smokers were exposed to SHS at home (light, n = 8; moderate, n = 6; heavy, n = 9) and not in the workplace. Smoking burden in active smokers was 12 ± 12 pack-years (range, 2-60 pack-years),

TABLE 1] Baseline Characteristics of the Study Participants

Characteristic	Active Smokers (n = 25)	Passive Smokers (n = 23)	Control Subjects (n = 23)	P Value
Age, y	34 ± 9	33 ± 9	36 ± 9	.67
Female sex, %	44	48	48	.95
BMI, kg/m ²	26 ± 4	26 ± 6	24 ± 4	.19
Cigarettes/d, No.	13 ± 8	...	...	...
Pack-y, No.	12 ± 12	...	...	...
SHS exposure, h/d	...	6 ± 3	...	...
SHS exposure, y	...	9 ± 6	...	...
Plasma cotinine level, ng/mL				< .0001
Mean	144 ± 99	31 ± 70	0 ± 0	
Median (range)	132 (0.426)	0 (0.236)	0 (0.0)	
Systolic BP, mm Hg	120 ± 17	116 ± 16	118 ± 11	.65
Diastolic BP, mm Hg	73 ± 11	75 ± 10	67 ± 8	.03
Total cholesterol level, mg/dL	170 ± 36	167 ± 16	165 ± 32	.87
Fasting glucose level, mg/dL	91 ± 19	88 ± 13	92 ± 11.98	.68

Data given as mean ± SD unless otherwise indicated. SHS = secondhand smoke.

and the intensity of exposure was 13 ± 8 cigarettes per day (range, 5-40/d). Active smokers had greater plasma cotinine levels than passive smokers and control subjects.

Protein Expression in Venous Endothelial Cells

Expression of eNOS, a main source of basal endothelial NO, and P-eNOS, the activated form of eNOS, was similar in passive and active smokers (Fig 1). Expression of eNOS and P-eNOS was reduced by 37% and 65%, respectively, in passive smokers compared with control subjects ($P = .04$ and $.02$, respectively) (Fig 1). Expression of NF- κ B, a marker of inflammation, was greater in passive smokers than in control subjects and similar in passive and active smokers (Fig 1). Expression of nitrotyrosine, a marker of oxidative stress, was greater in active smokers than control subjects and similar in passive and active smokers (Fig 1). Expression of nitrotyrosine varied widely in both passive and active smokers, with a borderline significant trend toward greater expression in passive smokers compared with control subjects ($P = .05$) (Fig 1). Representative endothelial cell images from the active and passive smokers and control subjects are shown in Figure 2.

Flow-Mediated Dilation

Brachial artery FMD, an indirect marker of endothelial NO-mediated reactivity, was similar in passive and active smokers (Fig 3). FMD was significantly reduced in active and passive smokers compared with control subjects ($P = .003$ and $.02$, respectively) (Fig 3).

Relationship Between Markers of Endothelial Reactivity, Inflammation, and Oxidative Stress and Intensity of Cigarette Smoke Exposure

The association between endothelial protein expression and FMD and the intensity of cigarette smoke exposure is shown in Table 2. Endothelial expression of P-eNOS correlated inversely with pack-years of exposure to cigarette smoke in active smokers ($P = .04$). Correlation between expression of NF- κ B and daily exposure to SHS was borderline significant ($P = .05$), whereas expression of NF- κ B correlated inversely with years of exposure to SHS in passive smokers ($P = .04$). This trend was most pronounced in passive smokers who were exposed to SHS for longer than 15 years (e-Fig 1). Expression of eNOS and nitrotyrosine was unrelated to the intensity of cigarette smoke exposure in both passive and active smokers (Table 2).

Discussion

The present study provides direct evidence that passive and active smoking similarly affect the vascular endothelium by reducing NO availability and promoting inflammation. These findings suggest that involuntary exposure to SHS, even at low levels, presents a significant vascular risk.

Exposure to SHS increases the risk of cardiovascular disease by 30% to 57%, accounting for up to 50,000 deaths annually in the United States. Protection of nonsmokers through smoke-free environments reduces cardiovascular morbidity and mortality.^{4,5,13,14} The relative risk of

Figure 1 – Expression of markers of endothelial reactivity, inflammation, and oxidative stress. Expression of eNOS, P-eNOS, nitrotyrosine, and NFκB was similar in venous endothelial cells harvested from passive (n = 23) and active smokers (n = 25). Expression of eNOS and P-eNOS was reduced whereas expression of NFκB was greater in passive smokers compared with control subjects (n = 23). Expression of nitrotyrosine was borderline significantly different between passive smokers and control subjects. eNOS = endothelial nitric oxide synthase; NFκB = nuclear factor-κB; P-eNOS = phosphorylated endothelial nitric oxide synthase.

cardiovascular disease in passive smokers has been consistently reported to be similar in magnitude to the risk conferred by active cigarette smoking, despite almost a 100-fold lower dose of cigarette smoke inhaled by passive smokers.^{5,15,16} The dissociation between much lower smoke exposure and similar cardiovascular risk may be

explained by the fact that secondhand smoke is unfiltered and, thus, potentially more hazardous than mainstream smoke inhaled by active smokers.^{17,18} Inhaled, fresh, sidestream cigarette smoke is approximately four times more toxic per gram of total particulate matter than mainstream cigarette smoke, and the toxicity of whole sidestream smoke is greater than the sum of the toxicities of its major constituents.¹⁷ Chronic exposure to the highly toxic sidestream cigarette smoke that contains reactive oxygen and nitrogen species and aldehydes is likely responsible for reduced NO availability in endothelial cells harvested from secondhand smokers.

Decreased FMD in chronic passive smokers compared with healthy control subjects in our study is in agreement with previous reports.⁶ Chronic exposure to SHS is associated with a dose-related impairment of NO-dependent vasodilation in healthy young adults who had never smoked, which was not observed in our study, likely due to a relatively small study sample.¹⁹ In addition, the range of FMD observed previously in passive smokers varies widely, with values reported in passive smokers overlapping with values observed in healthy subjects not exposed regularly to SHS.^{6,19-22} Furthermore, endothelial NO availability, as measured by FMD, appears to increase only modestly after smoking cessation, which

Figure 2 – Representative endothelial cell images from active and passive smokers and control subjects. Expression of eNOS and P-eNOS in harvested venous endothelial cells was lower in active and passive smokers than in control subjects, while expression of nitrotyrosine and NFκB was greater. Cell images were analyzed with a fluorescent microscope and captured by digital camera (× 100 magnification). See Figure 1 legend for expansion of abbreviations.

Figure 3 – Brachial artery flow-mediated vasodilation was similar in passive ($n = 23$) and active smokers ($n = 25$) and was significantly reduced in active and passive smokers compared with control subjects ($n = 23$).

contrasts with significant clinical benefits associated with smoking cessation.^{13,14,23} Contrary to the FMD technique, quantification of eNOS and P-eNOS expression in freshly harvested endothelial cells provides direct insight into in vivo endothelial NO production and activity. Our findings of reduced eNOS expression and activity in the human vascular endothelium after exposure to SHS concur with previous reports of reduced eNOS protein and mRNA expression and its enzymatic activity after prolonged exposure to cigarette smoke extract in vitro.^{24,25} Thus, direct characterization of the vascular endothelium may reflect more accurately the extent of reduced NO availability in

passive smokers than indirect assessment of endothelial function by FMD.

Exposure to SHS induces oxidative stress from oxidants contained in cigarette smoke as well as free radicals released endogenously from activated neutrophils.^{26,27} Cigarette smoke extract leads to nitration of tyrosine residues in apolipoprotein B in low density lipoprotein and exposure to SHS increases nitrotyrosine levels in rodent heart tissue.^{28,29} Levels of nitrotyrosine, a marker of oxidative stress, are increased in vascular tissues after exposure to low levels of SHS during gestation to early childhood in the nonhuman primate *Macaca mulatta*.³⁰ Our findings of a similar increase in endothelial nitrotyrosine expression in healthy active and passive smokers provide direct evidence that oxidative stress occurs in the vascular endothelium prior to onset of clinically evident vascular disease in such individuals.

Circulating levels of inflammatory cytokines remain elevated for at least 3 h following SHS exposure, suggesting that chronic, low-grade, systemic inflammation is present in individuals exposed regularly to SHS.³¹ However, increased activity of NF- κ B, a marker of inflammation, has not been consistently observed after exposure to cigarette smoke. Exposure to SHS during the perinatal period suppresses NF- κ B activity in the neonatal nonhuman primate and exposure to mainstream cigarette smoke inhibits NF- κ B in human monocytes.^{32,33}

Conversely, exposure to cigarette smoke increases NF- κ B activity in vitro.³⁴ These observations suggest that cigarette smoke-mediated activation of NF- κ B is tissue specific. Interestingly, despite an overall increase

TABLE 2 Relationship Among Markers of Endothelial Reactivity, Inflammation, and Oxidative Stress and Intensity of Cigarette Smoke Exposure

Marker	Active Smokers		Passive Smokers	
	Cigarette Smoke Exposure		SHS Exposure	
	Cigarettes/d	Pack-y	Hours per Day	Years
FMD	0.29	0.44	-0.1	0.07
eNOS	-0.03	-0.24	0.30	-0.02
P-eNOS	-0.11	-0.42 ^a	0.43	0.07
Nitrotyrosine	0.1	0.09	0.30	-0.06
NF- κ B	0.17	0.12	-0.48 ^a	-0.54 ^a

eNOS = endothelial nitric oxide synthase; FMD = flow-mediated vasodilation; NF- κ B = nuclear factor- κ B; P-eNOS = phosphorylated endothelial nitric oxide synthase. See Table 1 legend for expansion of other abbreviation.

^a $P \leq .05$ using Spearman correlations.

in endothelial NF- κ B expression in passive smokers, we observed that prolonged exposure to SHS (> 15 years) was associated with a less robust increase in expression of NF- κ B than shorter exposure, suggesting the possibility of long-term adaptation to SHS. However, expression of eNOS and P-eNOS remains reduced with prolonged exposure to SHS, strongly suggesting that endothelial function is impaired in passive smokers regardless of the length of exposure.

The molecular mechanisms that mediate atherosclerosis in passive smokers cannot be ascertained from the study of the venous endothelium, which is a clear limitation of our study. Our goal was to investigate whether SHS directly affects the vascular endothelium. Although endothelium-dependent vasomotor function is routinely assessed by arterial FMD and not in the venous compartment, venous endothelium is chronically exposed to the same tobacco toxins as the arterial compartment. Inflammatory and oxidative pathways are similarly activated in venous and arterial segments harvested from patients with atherosclerosis.^{35,36} Expression of eNOS is comparable in human venous and arterial endothelial cells.³⁷ Considering that local biomechanical forces that affect endothelial cells at specific sites appear to play an essential role in determining regional susceptibility to atherosclerosis, endothelial biopsy at specific sites of the arterial vasculature would likely be required to determine the precise mechanisms underlying atherosclerosis in SHS.^{38,39} However, in contrast to arterial cell harvesting,

venous sampling can be performed with minimal hazard, and the discomfort associated with venous endothelial harvesting equals that of phlebotomy. Independently from differences in venous and arterial endothelial cells phenotypes, our data show that SHS toxins directly affect the vascular endothelial environment.

Another limitation of our study is that endothelium-independent, smooth-muscle-dependent brachial vasodilation was not assessed in our study. Endothelial cell harvesting and FMD were performed the same morning within 2 h in all participants. We did not administer nitrates during FMD to avoid possible effects on measured endothelial protein expression in the study participants.

Unrecognized sleep apnea and individual cytokine profile may affect endothelial protein expression. However, this is unlikely in the study participants, who were not diagnosed with any disease, were not taking any medications or supplements, were not overweight or obese, and had no complaints suggestive of sleep apnea. In addition, air pollution may influence endothelial protein expression. However, all study participants were exposed to the same pollution level in the environment and, yet, had different protein expression based on their exposure to cigarette smoke. In conclusion, the direct evidence of impaired vascular endothelial function of similar magnitude in passive and active cigarette smokers strengthens the rationale for strict implementation of tobacco smoke-free environment to reduce vascular risk associated with involuntary SHS exposure.

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Author contributions: S. J. had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. S. J. contributed to the study design; T. A., E. W., R. W., F. C., G. W., M. E., C. R., and S. J. contributed to data acquisition and analysis and manuscript revision; S. H. contributed to FMD data analysis and manuscript revision; Y. W. contributed to statistical analysis and manuscript revision; and T. H. L. J. and S. J. contributed to data interpretation and writing of the manuscript.

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Additional information: The e-Appendix and e-Figure can be found in the Supplemental Materials section of the online article.

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