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Effects of Second-Hand Smoke and Gender on Infarct Size of Young Rats Exposed In Utero and in the Neonatal to Adolescent Period

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Objectives. **We sought to assess the effects of second-hand smoke (SHS) and gender on infarct size in young rats exposed in utero or in the neonatal to adolescent period, or both.**

Background. **We previously demonstrated that exposure to SHS increases infarct size in a rat model of ischemia and reperfusion, with a dose-response relation. These results are consistent with epidemiologic studies demonstrating that SHS increases risk of death from heart disease.**

Methods. **Thirty-one pregnant female rats were randomly divided into two groups: those exposed to SHS and a control group (non-SHS). After 3 weeks, each rat had given birth to 10 to 12 rats. One hundred one neonatal rats were divided into four groups according to exposure to SHS in utero (SHSu) and randomized to SHS exposure in the neonatal to adolescent period (SHSna). After 12 weeks, all rats were subjected to 17 min of left coronary artery occlusion and 2 h of reperfusion.**

Results. **Birth mortality was higher in the SHSu group than in the non-SHSu group (11.9% vs. 2.8%, $p < 0.001$). Body weight of neonatal rats at 3 and 4 weeks in the two SHSu groups was lower than that of rats in the two non-SHSu groups ($p < 0.001$). Exposure to SHSna increased endothelin-1 levels in plasma ($p = 0.001$). In all 70 young rats who survived the neonatal period, infarct size (Infarct mass/Risk area × 100%) was greater in the SHSna groups than in the non-SHSna groups ($p = 0.005$) and in the male groups than in the female groups ($p < 0.001$).**

Conclusions. **Exposure to SHS in the neonatal to adolescent period and male gender increased myocardial infarct size in a young rat model of ischemia and reperfusion. These results are consistent with epidemiologic studies demonstrating that SHS increases the health risk to neonates and adolescents.**

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The health consequences of involuntary exposure to second-hand smoke (SHS) have been a subject of intense scientific and public health concern (1,2). Many studies have shown a relation between maternal smoking during pregnancy and an increased prevalence of acute and chronic respiratory disorders in childhood (3–5). In addition, maternal cigarette smoking during pregnancy has been associated with increased perinatal mortality and low birth weight (6,7). As with active smoking, passive maternal exposure to tobacco smoke (involuntary maternal smoking) during pregnancy is also associated with an increased incidence of asthma and a risk for patterns of negative developmental outcomes (8). Passive smoking and tobacco exposure through breast milk increases the risk of sudden infant death syndrome in infants (9).

Epidemiologic and clinical studies have shown that SHS causes heart disease in nonsmokers (10–12). It has been estimated that SHS contributes to 37,000 deaths from heart disease of the total 53,000 annual deaths, making passive smoking the third leading preventable cause of death, after active smoking and alcohol (10). The American Heart Association has formally concluded that passive smoking is an important risk factor for heart disease in both adults and children (13,14). Of the thousands of chemicals in SHS, those that are suspected to contribute to passive smoking-induced cardiovascular disease include nicotine, carbon monoxide, polycyclic aromatic hydrocarbons and tobacco glycoproteins (10). Passive smoking may exacerbate acute myocardial ischemia by increasing coronary vasoconstriction and reducing coronary blood flow, releasing catecholamines and increasing myocardial oxygen demand, increasing oxygen free radical generation and enhancing thrombosis (10–12).

A previous study from our group (15) showed that exposure to SHS increased myocardial infarct size with a dose-response

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Abbreviations and Acronyms

ANOVA = analysis of variance
LAD = left anterior descending coronary artery
LV = left ventricular
Non-SHS = not exposed to second-hand smoke
SHS = second-hand smoke
SHSna = exposed to SHS in the neonatal to adolescent period
SHSu = exposed to SHS in utero

relation in adult female rats subjected to acute ischemia and reperfusion. The effect of gender on infarct size in this model is unclear. We previously showed that the male sex hormone, testosterone, exacerbates the adverse vascular effects of SHS in atherosclerotic rabbits (16). We therefore hypothesized that the effects of SHS on myocardial ischemia would be greater in male rats. We also investigated whether SHS exposure in utero alone affects infarct size and whether its effect is additive to SHS exposure in the neonatal to adolescent period.

In this study, we evaluated the effects of SHS on male and female rats exposed in utero (3 weeks) and in the neonatal to adolescent period (12 weeks). To further examine potential mechanisms underlying the pro-ischemic effects of SHS, we also measured levels of endothelin-1 and angiotensin-2, agents known to induce coronary vasoconstriction.

Methods

Experimental groups. Thirty-one pregnant Sprague-Dawley rats were randomly divided into SHS and non-SHS groups, as described subsequently. Four Marlboro filter cigarettes were smoked every 15 min for 6 h/day, 5 days/week, and the SHS-exposed rats breathed the SHS that these cigarettes produced. These 96 cigarettes/day were smoked using a smoking machine (RM 1/G, Heinr Borgwald GmbH, Hamburg, Germany), as previously described (17). The rats were housed in separate cages in $1.92 \times 1.92 \times 0.97$ m (3.6 m^3), well mixed SHS exposure chambers (model H 5500, BioClean, Duo Flo, Lab Product Inc.). The exposure chamber had an interior volume of 3.6 m^3, similar to that of an automobile sedan (3.7 m^3). The average concentrations of air nicotine and carbon monoxide, using this protocol, have been previously shown (17) to be about twofold higher than those in heavy smoking environments of humans (12,15). The rats were housed in a room maintained at a constant temperature and kept on a 12-h light–dark cycle. All rats were fed a regular diet. After 3 weeks of exposure in utero, each pregnant rat had given birth to about 10 to 12 neonatal rats. The 101 neonatal rats were divided into four groups—SHS and non-SHS groups—according to SHS exposure in utero (3 weeks before birth) and randomized to exposure in the neonatal (4 weeks after birth) to adolescent (6 to 12 weeks after birth) period. The neonatal and adolescent periods were grouped together because the heart size of neonatal rats was too small to establish a reliable infarct model.

The groups were as follows: *SHSu*/*SHSna* = exposed to SHS in utero for 3 weeks and in the neonatal to adolescent period for 12 weeks; *SHSu*/*non-SHSna* = exposed to SHS in utero for 3 weeks only; *non-SHSu*/*SHSna* = exposed to SHS in the neonatal to adolescent period for 12 weeks only; *non-SHSu*/*non-SHSna* = not exposed in utero or in the neonatal to adolescent period. The rats were then divided into eight groups according to gender, which could be clearly identified at 3 to 4 weeks after birth.

Rat model of acute myocardial ischemia and reperfusion. A rat model of left coronary artery occlusion and reperfusion was used as previously described (18). All of the following procedures were conducted in room air in a laboratory away from the SHS exposure chamber. After induction of anesthesia (pentobarbital, 40 mg/kg body weight intraperitoneally), a tracheostomy was performed and the animal was ventilated on a Harvard Rodent Respirator (model 683, Harvard Apparatus). A reversible coronary artery snare occluder was placed around the proximal left anterior descending coronary artery (LAD) through a midline sternotomy. All four groups were subjected to 17 min of LAD occlusion followed by 120 min of reperfusion. Throughout the study, body core temperature was monitored with a rectal thermometer (Digital Thermometer, Fisher Scientific Inc.). All rats were placed on a heating pad (Deltaphase Isothermal Pad, model 39 DP, Braintree Scientific Inc.) for the duration of the experiment. Body core temperature ranged from 36° to 37°C during the experiment.

Infarct size. Infarct size was measured as described previously (17–19). The left coronary artery was reoccluded, and phthalocyanin blue dye was injected into the left ventricular (LV) cavity, allowing normally perfused myocardium to stain blue. The heart was then excised, rinsed of excess dye and sliced transversely from apex to base into 2-mm-thick sections. The sections were incubated in a 1% solution of triphenyltetrazolium chloride for 10 to 15 min until viable myocardium was stained brick red. Infarct-related myocardium fails to stain with triphenyltetrazolium chloride. The tissue sections were then fixed in a 10% formalin solution and weighed. Color digital images of both sides of each transverse slice were obtained with a videocamera (COHU Y/C 460 HTYL, 768×494 array, Leica) connected to a microscope (Stereo Zoom 6 photo Leica) using the Rasterops Frame Grabber card and Frame Grabber 3.2 software (Rasterops). The regions showing blue-stained (nonischemic), red-stained (ischemic but noninfarcted) and unstained (infarcted) tissue were outlined on each color image and measured using the program NIH Image 1.59 (National Institutes of Health) in a blinded fashion. On each side, the fraction of LV area representing infarct-related tissue (average of two images) was multiplied by the weight of that section to determine the absolute weight of infarct-related tissue. The infarct size for each heart was expressed as:

$$\text{Infarct size/LV mass (\%)} = \frac{\sum \text{Infarct weight in each slice}}{\text{Total LV weight}} \times 100\%,$$

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Risk area/LV mass (%) =

$$\frac{\text{Total weight of unstained section}}{\text{Total LV weight}} \times 100\%,$$

Infarct size as a percentage of risk area was then calculated as

$$\frac{\sum \text{Infarct weight in each slice}}{\sum \text{Risk area weight of each slice}} \times 100\%.$$

Biochemical and hematologic analyses. A subset of the neonatal rats (n = 26; 6 or 7 selected at random from each group) had blood samples withdrawn from the right atrium to measure plasma nicotine, cotinine, endothelin-1 and angiotensin-2 concentrations. The plasma concentrations of nicotine and cotinine were determined by gas chromatography with nitrogen-phosphorus detection. This method has been modified for simultaneous extraction and determination of cotinine by use of capillary gas chromatography (20). The plasma levels of endothelin-1 and angiotensin-2 were measured by radioimmunoassay (Phoenix Pharmaceuticals, Inc.) (21,22).

Statistical analysis. The results are presented as the mean value ± SEM. Data on plasma nicotine and cotinine concentrations in SHS and non-SHS groups were compared using the Mann-Whitney rank-sum test. Differences in birth mortality or mortality during the occlusion and reperfusion period between two and four groups were assessed by the chi-square test. Two-way analysis of variance (ANOVA) was used for most comparisons in the study; the two factors are the presence or absence of SHS exposure in the neonatal to adolescent period (12 weeks) and SHS exposure in utero (3 weeks). For analysis of the effect of gender, we used a three-factor ANOVA, with gender (male or female) as the third grouping factor. All computations were done with the general linear model procedure in Minitab, version 7.2 (Minitab Statistical Software) or Primer of Biostatistics: The Program, version 3.03 (McGraw-Hill). Statistical significance was set at $p < 0.05$.

Results

Mortality and body weight. The birth mortality of the groups exposed to SHS in utero was higher than that of control (non-SHS) groups (11.9% vs. 2.8%, $p < 0.001$) (Fig. 1). The neonatal body weights at 3 weeks and 4 weeks after birth in the same groups exposed to SHS in utero were lower than those in non-SHS exposure groups ($p < 0.001$) (Fig. 2). In addition, SHS exposure during the neonatal period also decreased body weight ($p < 0.001$) (Fig. 2). The gender of the rats could be identified clearly only at 3 to 4 weeks after birth. After 12 weeks of exposure to SHS, the body weight of male rats was higher than that of female rats (308 ± 7 vs. 200 ± 3 g, $p < 0.001$). Exposure to SHS in the neonatal to adolescent period for 12 weeks had no significant effect on body weight ($p = 0.523$). Thirty-one rats died during the occlusion and reperfusion period. The mortality of the male and female rats during the ischemia and reperfusion period was similar—31% and 30%, respectively. The observed mortality in the groups exposed to SHS in the neonatal to adolescent period for 12 weeks was higher than that in the non-SHS groups (37% vs. 24%, $p = 0.273$), but this difference was not statistically significant. Data from the 70 surviving young rats were analyzed for infarct size.

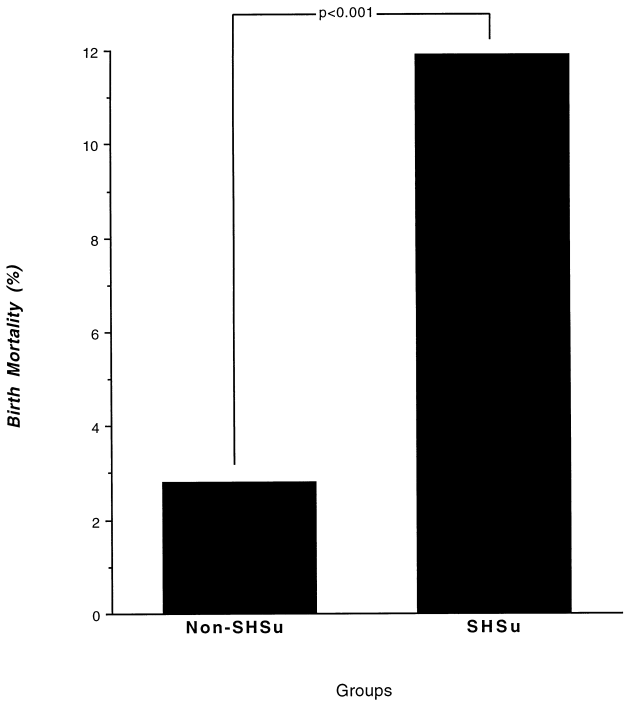


Figure 1. Birth mortality of newborns in the SHS-exposed in utero group (SHSu) is higher than that of newborns not exposed in utero (non-SHSu). The duration of exposure to SHS in utero was about 3 weeks. The p value was derived from the chi-square test.

Myocardial infarct size. Exposure to SHS in the neonatal to adolescent period for 12 weeks significantly increased infarct size (infarct mass/risk area × 100%; $p = 0.005$), especially in female rats (Fig. 3, Table 1). Male gender also increased infarct size in both SHS- and non–SHS-exposed groups ($p < 0.001$) (Fig. 3, Table 1). Exposure to SHS in utero for 3 weeks tended to increase infarct size ($p = 0.082$), especially in female rats (Fig. 3, Table 1). There were no significant interactions (Table 1), indicating that these effects are additive. The results are qualitatively similar for infarct size as a percentage of LV mass, except that there is an interaction between in utero exposure and gender. The risk areas for myocardial infarction were similar among all groups (Table 1).

Biochemical and hematologic analyses. Plasma nicotine and cotinine concentrations in the SHSna groups were higher than those in the non-SHSna groups (39 ± 5 ng/ml vs. undetectable level [i.e., <0.01 ng/ml], $p < 0.0001$ and 437 ± 124 ng/ml vs. undetectable level [i.e., <0.1 ng/ml], $p < 0.0001$, respectively) (Fig. 4). Endothelin-1 levels in SHS-exposed neonatal rats were higher than those in non–SHS-exposed neonatal rats (for SHSna, $p = 0.001$) (Fig. 5). Angiotensin-2 levels in neonatal rats exposed to SHS in utero were lower than

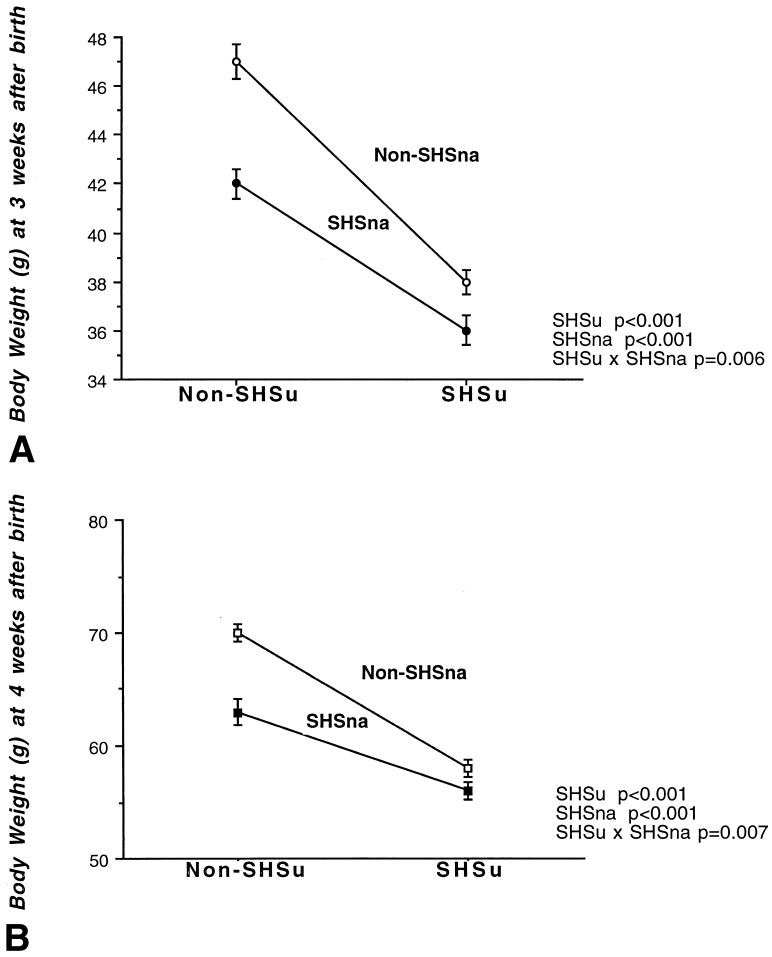


Figure 2. In the four groups, this profile plot shows (**top**) the body weight of neonates at 3 weeks after birth and (**bottom**) the body weight of neonates at 4 weeks after birth. Data are presented as mean value ± SEM. Non-SHSna = newborn rats not exposed to SHS in the neonatal period; SHSna = newborn rats exposed to SHS in the neonatal period; non-SHSu = mother rat not exposed to SHS in utero; SHSu = mother rat exposed to SHS in utero for 3 weeks. The p values were derived from two-way ANOVA.

those in non–SHS-exposed neonatal rats (for SHSu, p = 0.041) (Fig. 6).

Discussion

We hypothesized that the effects of SHS on myocardial ischemia would be larger in male rats and SHS exposure in utero would increase infarct size, an effect additive to SHS exposure in the neonatal to adolescent period.

The salient findings in this study were 1) exposure to SHS in pregnant rats significantly increased birth mortality and decreased neonatal body weight; 2) infarct size in male rats was larger than that in female rats in both SHS- and non–SHS-exposed groups; 3) exposure to SHS in the neonatal to adolescent period increased infarct size in young rats, especially in young female rats; 4) exposure to SHS in the neonatal period increased plasma endothelin-1 levels; and 5) exposure to SHS in utero tended to increase infarct size in young rats.

Birth mortality, body weight and SHS exposure. Clinical studies (6,7) have noted that SHS doubles the risk of delivering a low birth weight baby. Additional consequences to the offspring of involuntary smoke exposure by nonsmoking pregnant women include reduced fetal growth and higher perinatal mortality. The results of our study are consistent with these findings. Only 3 weeks of exposure to SHS in pregnant rats significantly increased birth mortality and decreased body weight in neonatal rats. Although previous studies have linked maternal smoking or exposure to SHS with impairment of newborn pulmonary function (23–25), hematopoietic malignancies (26), brain tumors (27) and DNA damage in the placenta, little is known about the cardiovascular effects of maternal exposure to SHS on the offspring. One clinical study (28) noted that a potent tobacco-related carcinogen, 4-aminobiphenyl, crosses the human placenta and binds to fetal hemoglobin in significantly higher concentrations in smokers than nonsmokers.

Infarct size and SHS exposure. The present study has shown that SHS exposure in utero for 3 weeks tended to increase infarct size in young rats, especially in young female rats. Exposure to SHS in utero tended to increase infarct size (p = 0.082); the failure to reach conventional statistical significance may be because the duration of exposure was too short (only 90 h spread over 3 weeks) and the interval of 12 weeks between exposure in utero to making the infarct was too long. A clinical study also showed that the adverse effect of maternal smoking on lung function was greater on female infants than on male infants (25). The present study also showed that SHS exposure in the neonatal to adolescent

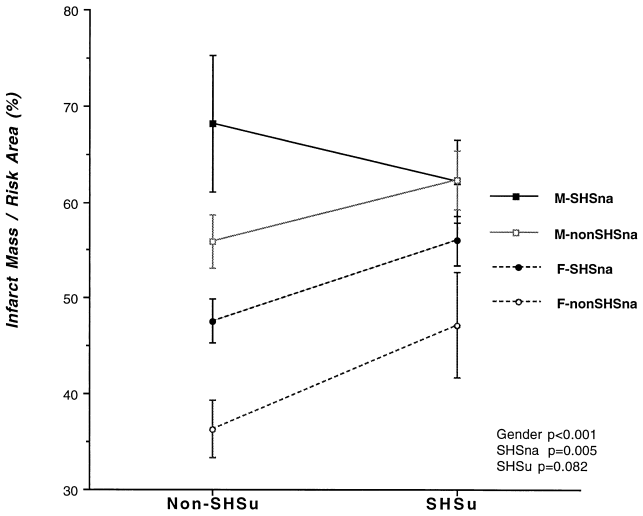


Figure 3. This profile plot shows percent infarct size. M-SHSna and F-SHSna = young male and female rats, respectively, exposed to SHS in the neonatal to adolescent period for 12 weeks; M-non-SHSna and F-non-SHSna = young male or female rats, respectively, not exposed to SHS in the neonatal to adolescent period; SHSu = young rats from mother rats exposed to SHS in utero for 3 weeks; non-SHSu = young rats from mother rats not exposed to SHS in utero. Data are presented as mean value ± SEM. The p values were derived from three-way ANOVA.

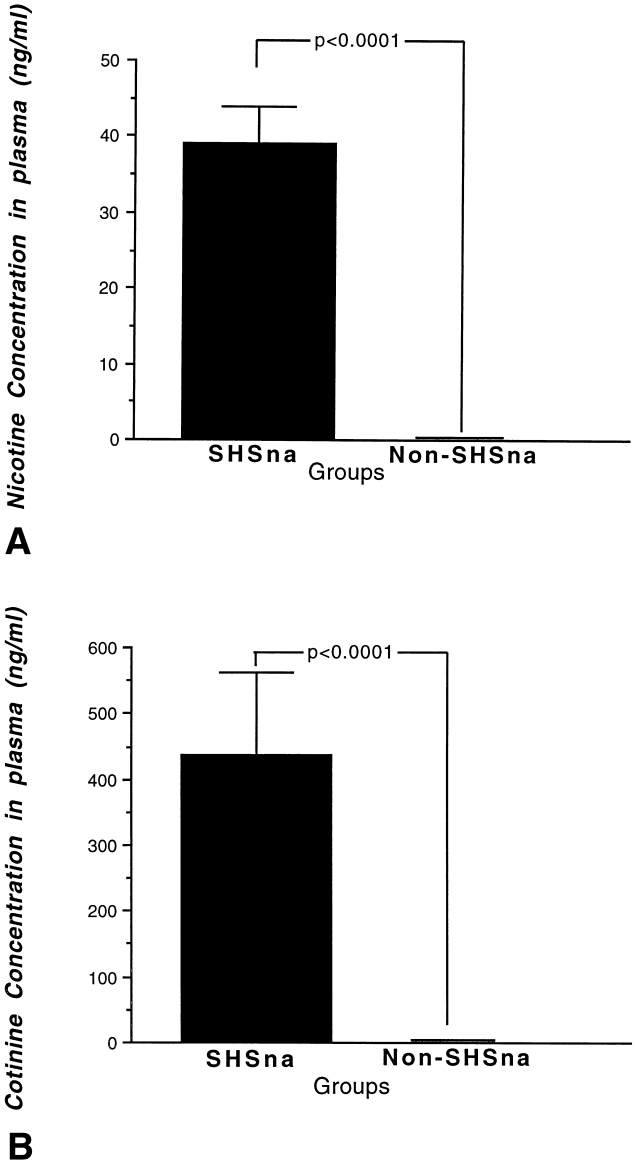
period (12 weeks after birth) significantly increased infarct size, especially in young female rats. This result is consistent with our previous study (15), although there were four differences in the protocols used. First, the occlusion period was reduced from 35 to 17 min in the present study. Second, the SHS exposure duration was increased from 6 to 12 weeks. We increased the SHS exposure duration, because newborn rats were too small to technically achieve a reproducible infarct model. Third, the rats in the present study were younger than those in previous studies. Fourth, we used a color digital imaging system with specialized software to measure infarct size instead of our previous approach of planimetry of photographs. The mechanisms underlying the increase in infarct size induced by neonatal/adolescent exposure to SHS are unclear.

Table 1. Effects of Second-Hand Smoke and Gender on Infarct Size

Groups	No. of Rats	Infarct Mass/ Risk Area (%)	Infarct Size/ LV Mass (%)	Risk Area/ LV Mass (%)
Female				
SHSu/SHSna	8	56 ± 3	33 ± 2	58 ± 2
Non-SHSu/SHSna	11	48 ± 2	24 ± 1	50 ± 2
SHSu/non-SHSna	9	47 ± 6	25 ± 3	53 ± 4
Non-SHSu/non-SHSna	13	36 ± 3	19 ± 2	52 ± 2
Male				
SHSu/SHSna	8	62 ± 4	33 ± 3	53 ± 3
Non-SHSu/SHSna	6	68 ± 7	40 ± 6	57 ± 5
SHSu/non-SHSna	6	62 ± 3	33 ± 3	58 ± 6
Non-SHSu/non-SHSna	9	56 ± 3	34 ± 2	60 ± 3
p Value				
Gender		< 0.001	< 0.001	0.0082
SHSna*		0.005	0.014	0.746
SHSu†		0.082	0.271	0.702
Gender × SHSna		0.469	0.388	0.182
Gender × SHSu		0.093	0.006	0.089
SHSna × SHSu		0.185	0.742	0.505
Gender × SHSu × SHSna		0.377	0.273	0.345

*Exposure to second-hand smoke (SHS) in the neonatal to adolescent period for 12 weeks. †Exposure to SHS in utero for 3 weeks. Data are presented as mean value ± SEM. SHSu/SHSna = exposure to SHS in utero for 3 weeks and in the neonatal to adolescent period of 12 weeks; SHSu/non-SHSna = exposure to SHS in utero for 3 weeks; non-SHSu/SHSna = exposure to SHS in the neonatal to adolescent period for 12 weeks; non-SHSu/non-SHSna = not exposed to SHS (control group).

Figure 4. Nicotine (**top**) and cotinine (**bottom**) concentrations in plasma are higher in SHS-exposed rats. SHSna = young rats exposed to SHS in the neonatal to adolescent period for 12 weeks; non-SHSna = young rats not exposed to SHS in the neonatal to adolescent period. Data are presented as mean value ± SEM. The p values were derived from the Mann-Whitney rank-sum test.



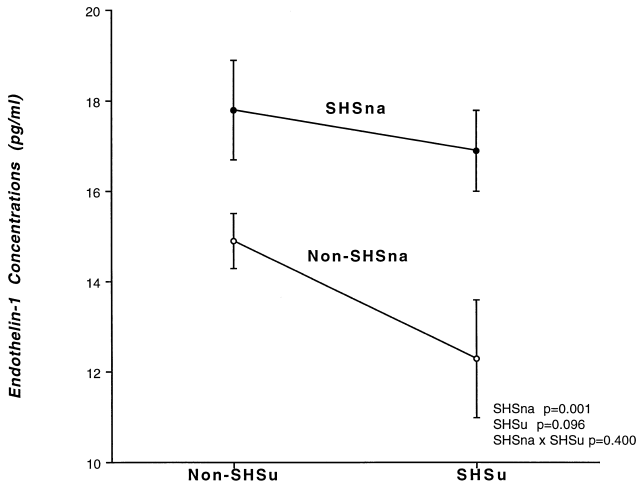


Figure 5. This profile plot shows endothelin-1 concentrations in the plasma of rats from the four groups. SHSna = rats exposed to SHS in the neonatal period; non-SHSna = rats not exposed to SHS in the neonatal period; SHSu = rats from mother rats exposed to SHS in utero for 3 weeks; non-SHSu = rats from mother rats not exposed to SHS in utero. Data are presented as mean value ± SEM. The p values were derived from two-way ANOVA.

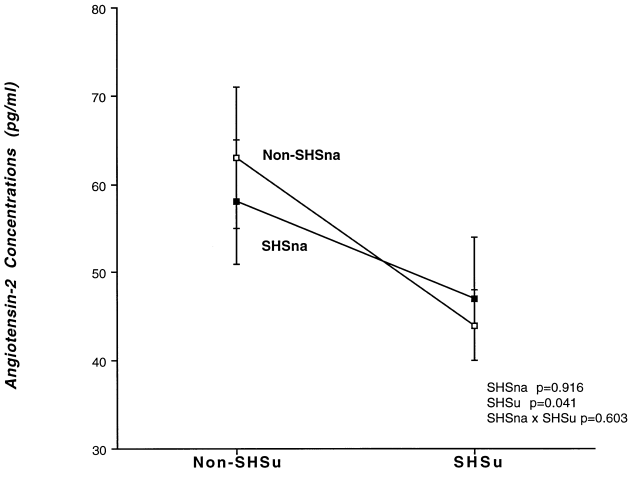
A poorer collateral circulation in rats exposed to SHS is one possibility.

Infarct size and gender. In previous infarct studies, we only used female Sprague-Dawley rats because male rats had a higher mortality due to arrhythmias during the occlusion and reperfusion period. The present study provides the first comparison of the cardiovascular effects of SHS exposure between male and female rats. We showed that the infarct size in male rats was significantly higher than that in female rats, regardless of SHS exposure. Male sex hormones, such as testosterone, reduced endothelium-dependent relaxation and augmented dysfunction associated with SHS (16). Female sex hormones, such as estrogen, may also have some protective effect on myocardial ischemia (29,30). A recent in vivo study from our group (31) showed that the female sex hormone, 17β-estradiol, attenuates endothelin-1–induced coronary vasoconstriction, possible through an effect on the endothelin-A receptor. It is possible that differences in infarct size between male and female rats is due to sex hormone-related differences in endothelin activity. However, as discussed earlier, the effect of SHS of increasing infarct size was clearer in female rats.

Endothelin-1, angiotensin-2 and SHS exposure. Endothelin-1 is a potent vasoconstrictor agent with a potential role in systemic hypertension and coronary artery disease (32). Smokers tended to have higher endothelin-1 plasma levels within 10 min after the onset of smoking (33). Long-term smoking is associated with an impaired endothelium-dependent vasodilator response to low dose endothelin-1, and short-term smoking enhances endothelin-1–induced vasoconstriction (34). In the present study, endothelin-1 levels in plasma in neonatal rats exposed to SHS were significantly higher than those in non-SHS rats. The higher levels of endothelin-1 may increase vasoconstriction and thus increase myocardial infarct size. The mechanism of nicotine-induced vasoconstriction may be independent of the release of endothelin or prostaglandin derivatives from endothelial cells (35). Other tobacco smoke components, such as carbon monoxide or tar, may be responsible for the increase in plasma endothelin-1 in smokers (33). This increase in the level of endothelin-1, a powerful vasoconstrictor and mitogen, may play an important part in the development of atherosclerosis arising from smoking (36). Angiotensin-2, as a vasoconstrictor, can accelerate the progression of vascular disease (37). In the present study, angiotensin-2 plasma levels of neonatal rats exposed to SHS in utero were significantly lower than those of rats not exposed to SHS. The mechanism underlying this finding is unclear. It is possible that SHS influences renal development, leading to lower levels of renin and thus angiotensin. Alternatively, the effect of SHS on the neonatal lung (23–25), or on other organs that express levels of angiotensin-converting enzyme activity, might account for our observation.

Clinical relevance. The rats were exposed to smoke from the burning tip of the cigarette in the exposure chamber. Therefore, they were exposed to a mixture of fresh and aged sidestream smoke, which is the same mixture received by people exposed to SHS. The exposure chamber we used had an interior volume (3.6 m^3) similar to that of an automobile sedan (3.7 m^3). If four passengers each smoked four cigarettes per hour, that microenvironment would be similar to the one in the present rat study. In our previous study, the average concentrations of air nicotine and carbon monoxide (1,103 $\mu g/m^3$ and 92 ppm) in the SHS exposure chamber were about twofold higher than those in human heavy smoking environments (50 to 500 $\mu g/m^3$ and 5 to 50 ppm) (12). These exposure levels are well below those that occur in active smokers. The duration of

Figure 6. This profile plot shows angiotension-2 concentrations in the plasma of rats from the four groups. SHSna = rats exposed to SHS in the neonatal period; non-SHSna = rats not exposed to SHS in the neonatal period; SHSu = rats from mother rats exposed to SHS in utero for 3 weeks; non-SHSu = rats from mother rats not exposed to SHS in utero. Data are presented as mean value ± SEM. The p values were derived from two-way ANOVA.



exposure to SHS in the present study was only 360 h spread over 12 weeks (6 h/day, 5 days/week). Compared with many chemical toxicology studies that involve doses several orders of magnitude above ambient levels, the levels of exposure of SHS we used were realistic.

The plasma nicotine concentrations in the present study were 39 ± 5 ng/ml. By comparison, rats receiving 4 mg/kg per day of nicotine, a total dose equivalent to ~1 pack of cigarettes per day, have plasma nicotine levels ~250 ng/ml (38,39), six times the levels we observed. Thus, the levels of nicotine we observed in our rats are comparable to what would be expected from a heavy level of passive smoking, as opposed to active smoking. The duration of SHS exposure, however, was short, even compared with a rat's lifetime (about 2 to 3 years) (40). Myocardial infarct size significantly increased with just 360 h of SHS exposure spread over 12 weeks. This is equivalent to 2% of the projected lifespan. Assuming a human lifespan of 75 years, an equivalent period of exposure to SHS would be 6 h per day for 6 years. Thus, these data may well have clinical relevance.

Conclusions. Exposure to SHS in pregnant rats significantly increased birth mortality and decreased neonatal body weight. Exposure to SHS in the neonatal to adolescent period and male gender increased myocardial infarct size in a young rat model of ischemia and reperfusion. These results are consistent with epidemiologic studies demonstrating that SHS increases the risk of death due to heart disease.

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