Second hand tobacco smoke, arterial stiffness and altered circadian blood pressure patterns are associated with lung inflammation and oxidative stress in rats.

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Running head: Tobacco smoke alters circadian blood pressure pattern

Key words: second hand tobacco smoke; arterial stiffness; nitric oxide; oxidative stress; blood pressure; circadian rhythm; endothelial dysfunction; inflammation.
Abstract

Chronic smoking and second hand tobacco smoke exposure are major risk factors for cardiovascular disease that are known to adversely alter the structural and mechanical properties of arteries. The objective of this study was to determine the effects of subchronic second hand tobacco smoke exposure on circadian blood pressure patterns, arterial stiffness, and possible sources of oxidative stress in conscious, unsedated radiotelemetry-implanted rats. Pulse wave dP/dt was used an indicator of arterial stiffness, and was compared to both structural (wall thickness) and functional (nitric oxide production and bioactivity, endothelin-1 levels) features of the arterial wall. In addition, histology of lung, heart, and liver were examined as well as pulmonary and hepatic detoxifying enzyme activity (cytochrome P450 – specifically CYP1A1).

Subchronic second hand tobacco smoke exposure altered the circadian pattern of heart rate and blood pressure, with a loss in the normal dipping pattern of blood pressure during sleep. Second hand tobacco smoke exposure also increased pulsewave dP/dt in the absence of any structural modifications in the arterial wall. Furthermore, although nitric oxide production and endothelin-1 levels were not altered by second hand tobacco smoke, there was increased inactivation of nitric oxide as indicated by peroxynitrite production. Increased lung neutrophils or pulmonary CYP1A1 may be responsible for the increase in oxidative stress in rats exposed to second hand tobacco smoke. In turn, this may be related to the observed failure of blood pressure to dip during periods of sleep and a possible increase in arterial stiffness.
1. Introduction

Substantial epidemiological evidence indicates that smoking is a major factor, if not the number one preventable risk factor for cardiovascular disease (21). Many of the traditional risk factors for cardiovascular disease, including smoking, adversely alter endothelial function as well as the structure and mechanical properties of the arterial wall (14). The endothelium releases nitric oxide (NO) which in turn causes vasodilation (11), but also releases powerful vasoconstrictors such as endothelin-1 that physiologically balance the effect of NO (40). Although much attention has been paid to alterations in NO release or activity in cardiovascular disease (11), elevated endothelin-1 release is also commonly reported in atherosclerosis and hypertension (40). Arterial stiffness is a powerful, independent predictor of cardiovascular risk (2) and is influenced by both structural and functional features of the arterial wall (17). Increasingly it is being recognized that second-hand tobacco smoke (also commonly referred to as environmental tobacco smoke or ETS) poses a similar risk for cardiovascular disease (1). However, it is not clear whether the same smoking-associated changes in endothelial function or arterial stiffness also occur with second hand tobacco smoke exposure.

It is a paradox that while smoking increases arterial stiffness, epidemiological studies have generally shown that the blood pressure of smokers is lower than that of nonsmokers (e.g. 16). A previous study from this laboratory on the acute effects of second hand tobacco smoke in rodents supports this observation (12), while another study from this laboratory showed that acute second hand tobacco smoke effects increased blood pressure in pigs (43). It has long been known that circadian rhythms are a characteristic feature of blood pressure regulation and hypertension (36). Ambulatory
blood pressure monitoring is increasingly being used in humans to evaluate the effects of lifestyle (e.g. smoking) on blood pressure. It provides greater accuracy than casual measurements and allows for the analysis of circadian rhythms in blood pressure (32). A previous study reported that stress and mainstream cigarette smoke in rats altered the daily rhythm characteristics of heart rate, body temperature, and locomotor activity (31). However, whether second hand tobacco smoke has a similar effect on blood pressure patterns is not known. Polycyclic aromatic hydrocarbons (PAHs), agonists at the aryl hydrocarbon receptor (AhR), are found in the tar particulate phase of cigarette smoke (19,33). This receptor is an orphan receptor that is known to be activated by dioxins, PAHs and many other environmental contaminants, but has an unknown function in normal physiology (28). It is however, part of the Per-ARNT-Sim (PAS) superfamily of proteins, many of which are involved in circadian clock signaling. Recent findings have led to the hypothesis that the AhR plays a role in circadian rhythms (48). Two recent studies have reported that subchronic exposure to AhR agonists, either dietary 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (23) or intranasal administration of the PAH, benzo-a-pyrene (13) caused significant increases in blood pressure. A striking finding of the latter study conducted in this laboratory (13) was that intranasal benzo-a-pyrene provoked a failure of blood pressure to dip during periods of sleep/inactivity in rats. The altered circadian blood pressure pattern elicited by intranasal benzo-a-pyrene was associated with pulmonary inflammation and increased oxidative stress-mediated inactivation of NO. Thus, a major goal of this study was to examine whether subchronic second hand tobacco smoke exposure would cause a similar alteration in the circadian
rhythm of blood pressure, then to explore if this is related to pulmonary oxidative stress, inflammation and AhR activation.

We hypothesize that similar to smoking, exposure to a high, but environmentally relevant level of second hand tobacco smoke will increase endothelin-1 (ET-1) and that oxidative stress arising from lung inflammation and/or AhR activation will reduce NO bioactivity, leading to increased arterial stiffness and blood pressure. We have previously demonstrated that pulse wave dP/dt collected from blood pressure telemetry-implanted rats can be used as an indicator of acute, active changes in arterial stiffness in rats (12). Therefore the objective of this study was to determine whether daily 1-hr second hand tobacco smoke exposure for 28 days would induce changes in arterial stiffness and circadian blood pressure patterns in rats. A second objective was to examine whether any changes were related to oxidative stress, inflammation, and/or cytochrome P450 1A1 activity (CYP1A1; an indicator of AhR activation) in the lung and liver. In addition, structural (wall thickness) and functional (NO production and bioavailability, ET-1) features of the arterial wall were examined.
2. Materials and Methods

2.1 Animals and surgery

All protocols were approved by the Animal Research Ethics Board at the University of Saskatchewan in accordance with the Canadian Council on Animal Care guidelines. Male Sprague Dawley rats (195-225g) were housed individually under standard conditions (12 hr light:12 hr dark) with food and water available ad libitum except during exposures. A sub-sample of rats from each treatment group (n=4 rats/group) were surgically implanted with blood pressure radiotelemetry devices as described previously (12). Briefly, a PA-C10 radiotelemetry blood pressure transmitter (Data Sciences International, St. Paul, USA) was implanted into the femoral artery and advanced toward the iliac artery with the transmitter body placed subcutaneously in the left flank. Rats were allowed to recover from surgery for at least 14 days before experiments.

2.2 Second Hand Tobacco Smoke (SHS) exposures

Second hand tobacco smoke (mainstream plus sidestream smoke) was generated with a single cigarette manual smoking machine from CH Technologies Inc (Westwood, USA) at a rate of 3 puff/min (57 ml/puff, 2-s duration). The second hand tobacco smoke was mixed with indoor air and pumped into a 89.5 L inhalation chamber. Pumps controlling inflow and outflow were both set at 6 L/min. Animals were entered into the exposure study in a staggered fashion (n=2 rats per day for each treatment). During exposures, rats were restrained in individual wire mesh tubes within the chamber and
exposed together (n=8) to second hand tobacco smoke from 3 regular-sized cigarettes (Canadian Classics, Rothmans, Benson & Hedges, Canada) during a one hour exposure period every day for 28 days. Sham-exposed rats (n=8) were restrained and placed in an identical clean exposure chamber under the same conditions except clean, unfiltered room air was pumped into the chambers for one hour every day for 28 days. Exposures were conducted between 8:00-10:00 AM every day (corresponding to the beginning of the ‘lights on’ or sleep period every day) except on days when weekly 24-hr reads were performed when exposures were performed after 10:00 AM. A one-month exposure period was chosen based on a previous report indicating that a similar exposure period was sufficient to induce myocardial remodeling in rats (Zornoff et al., 2006).

Exposure conditions were assessed in preliminary experiments by monitoring the total particulate concentrations in the chambers for sham and second hand tobacco smoke exposures without rats using a SKC constant airflow pump (Universal 224-PCXR4, Eighty Four, PA) fitted with pre-weighed mixed cellulose ester filters (0.8 μm, SKC Inc., Eighty Four, USA). The SKC pump sampled air at 2 L/min via a separate air sampling port than those used for air inflow/outflow. The regular outflow pump was adjusted to 4 L/min so that total outflow remained balanced with total inflow at 6 L/min. Air was sampled for 1 hour for sham conditions or for a shorter time (20 minutes) for second hand tobacco smoke exposure conditions to prevent cellulose filters from becoming saturated with the higher particulate conditions. For both exposure conditions, data was calculated and expressed as total particulates that would have been generated during a 1-hr continuous exposure. Carbon monoxide (CO) levels produced during exposures were also measured by placing a T40 Rattler CO monitor (Industrial Scientific, Corp., Oakdale,
USA) within the chamber without rats during preliminary experiments. Separate preliminary experiments to assess O₂/CO₂ air levels were conducted with 8 rats in the exposure chamber using a Criticare Poet IQ Multiparameter gas monitor (Criticare Systems, Inc., Waukesha, USA). CO₂ levels remained <1% and mean O₂ levels were 21% (n=3 determinations) during the sham exposures. The measured particulate concentrations (Table 1) during second hand tobacco smoke exposures were found to be within the maximum range reported in smoking homes in the USA (5), but lower than that reported for tobacco smoke levels in automobiles with the windows closed (20). Therefore, the second hand tobacco smoke exposure conditions in the current study are on the high end, but still relevant to what humans may encounter in the real world.

2.3 Plasma nitrate/nitrite, endothelin-1, cotinine, nitrotyrosine, and ethoxyresorufin-o-deethylase (EROD)

Blood samples were collected at 30 minutes after sham and second hand tobacco smoke exposures by immersing the tail in warm tap-water for 2 minutes, then inserting a butterfly catheter into the tail vein and allowing gravity to facilitate bleeding into a tube from the catheter. Plasma nitrate/nitrite (NOx) levels were measured using a commercially available enzyme-based kit (Nitric Oxide Quantitation Kit, Active Motif North America, Carlsbad, USA). Plasma ET-1 (R&D Systems, Inc, Minneapolis, USA), cotinine (Bio-Quant, Inc, San Diego, USA), and nitrotyrosine (Cell Sciences, Canton, USA) levels were quantitated using commercially available enzyme-linked immunosorbent assays. Rat liver and lung homogenates were analyzed for EROD activity (a CYP1A1-specific marker enzyme activity), as previously described (44). A positive
control sample (liver microsomes prepared from rainbow trout injected 2x with 10 mg/kg benzo-a-pyrene over 72 hrs) was analyzed in every sample series to ensure assay performance.

2.4 Endothelium-dependent vasorelaxation measurements

All chemicals were purchased from Sigma-Aldrich, St. Louis, USA unless specified otherwise. On day 29 of the experiment, rats (n=8 per treatment group) were anaesthetized with isoflurane and euthanized with pentobarbital sodium (0.44 ml/kg i.v.). Ring segments were rapidly excised from the aorta for tissue bath studies of endothelial-dependent relaxation, as previously described (45). Phenylephrine (10^{-7} M) was then added to each bath to pre-constrict the aortic rings. At the plateau in force, carbachol was added in cumulative concentrations from 10^{-9} M to 10^{-3} M. Vasorelaxation was expressed as a percentage of the plateau force to phenylephrine.

2.5 Histological Analysis

Whole heart, a portion of the lung (lower left lobe), thoracic aorta immediately after the arch, and the abdominal aorta with iliac branches were dissected from rats euthanized at Day 29 of treatment (n=8 per treatment group) and transferred into neutral buffered formalin. After 24 hours, samples were transferred to 70% ethanol and stored until histological analysis could be performed. Samples were paraffin-embedded and cross-sectioned (5 μm, serial sections), then stained with hematoxylin and eosin. Size analyses (wall thickness and luminal diameter) and histopathology were performed in heart and all arteries collected. However, none of these analyses showed any significant
effect of second hand tobacco smoke exposure (data not shown). For the lung, cross sections were scanned for 4 zones of high neutrophils, where the number of neutrophils was counted at 400x magnification. A single mean value per animal was used for all statistical comparisons.

2.6 Arterial stiffness, blood pressure and statistical analyses

Over the course of the 28 day exposure period, blood pressure and heart rate data was collected weekly for 20 minutes before daily exposures began (approximately 22 hours after the previous day’s exposure). Manual rather than continuous reads were performed daily in order to conserve battery life of the telemetry implant since implants were turned off between reads. Also, in order to obtain the circadian pattern of blood pressure and heart rate, data was collected once a week for a period of 24 consecutive hours (10 minutes of automatic sampling per rat per hour) starting within 2 hours of the end of the daily exposure and ending prior to the exposure periods the next day. Arterial pulsewave dP/dt has previously been validated by our laboratory to reflect active changes in arterial stiffness using radiotelemetry in rats (12). With arterial stiffening (whether through active or structural changes), pulse wave velocity throughout the arterial tree increases and the reflected wave tends to increase systolic pressure. Thus, the amplitude of the incident wave has been shown to increase in humans, along with the slope of the rise in arterial pressure over time (dP/dt) (30). Therefore, values for dP/dt were extracted from arterial pressure waveforms and averaged over 10 minute segments every hour using DSI Dataquest ART™ Analysis software (St. Paul, MN). In order to minimize effects of variation among individuals in baseline blood pressure values and determine
treatment effects, pre-exposure (baseline or day 0) blood pressure, heart rate, and dP/dt values were subtracted from post-exposure values from the same individual. The percent dip in blood pressure was calculated as the blood pressure, heart rate, or dP/dt value during the dark (mean value for 12-hr period) minus the value during the light period (mean value for 12-hr period) and multiplied by 100. The mean values during each portion of the light cycle were chosen to represent periods when the rats were expected to be awake/active versus sleeping/inactive, respectively. All data are expressed as mean ± standard error of the mean (SEM). Differences among groups were detected using one-way (single time point data), two-way (data with factors for week of exposure and treatment), or three-way (data with factors for time of day, week of exposure and treatment) analysis of variance (ANOVA) followed by Tukey’s posteriori tests as appropriate. However, all organ weights and tissue sizes were analyzed by analysis of covariance (ANCOVA) with body weight as a covariate.
3. Results

Second hand tobacco smoke exposure had higher total suspended particulates and carbon monoxide levels compared to sham exposure (Table 1). Plasma cotinine levels of tobacco smoke-exposed rats were also significantly higher compared to sham-exposed rats at 30 minutes after exposure. EROD activity was analyzed in liver and lung microsomes as an enzymatic marker for CYP1A1 induction and biomarker of exposure to the PAH component of second hand tobacco smoke. Lung microsomes of tobacco smoke-exposed rats showed higher EROD activity than those of sham-exposed rats while liver EROD activity was not significantly different between treatment groups (Table 1).

There were no significant differences in body, lung or liver weight of rats exposed to second hand tobacco smoke compared to sham after 28 days of exposure (data not shown).

To obtain the circadian pattern of blood pressure, data was collected once a week for a period of 24 consecutive hours and was expressed as a change from baseline (values obtained in the same individual rat prior to entering experiment subtracted from value obtained during experiment). Second hand tobacco smoke altered the circadian pattern of systolic pressure, diastolic pressure and heart rate (Figure 1; p<0.05 for treatment factor in separate 3-way ANOVAs for each end-point) as well as pulse pressure (data not shown). Systolic pressure, diastolic pressures and pulsewave dP/dt were higher in tobacco smoke-exposed compared to sham-exposed rats (Figure 1A, 1B and 1D) with this difference becoming significantly greater over time (p<0.05 for week factor in 3-way ANOVAs). In contrast, heart rate in tobacco smoke-exposed rats was significantly lower at several time points during the period where lights were out compared to sham-exposed
rats (Figure 1C). Paradoxically, heart rate tended to be higher in most time points during periods where lights were on in the same tobacco smoke-exposed compared to sham-exposed rats (Figure 1C). Blood pressure and $dP/dt$ of sham-exposed rats were slightly lower at week 4 than they were at baseline (a negative change from baseline; Table 2) while in the tobacco smoke group, blood pressure and $dP/dt$ had increased from the baseline values (a positive change from baseline; Table 2 and Figure 1).

The 24-hour circadian pattern shows that second hand tobacco smoke increased systolic and diastolic pressure to a greater extent during the period of light, a time of inactivity/sleep in rats (Figures 1A and 1B). More specifically, there was a significant reduction in the percent dip of systolic and diastolic pressures from the dark/active period to the light/inactive period in the tobacco smoke-exposed rats (Table 2), while the percent dip for heart rate and $dP/dt$ were not significantly affected by second hand tobacco smoke exposure (Table 2). When the data was averaged for the entire 24 hour period, the change in systolic and diastolic pressures from baseline were also significantly increased in tobacco smoke-exposed rat compared to sham-exposed rats (Table 2). In the tobacco smoke-exposed group, the 24 hour average blood pressure and heart rate at week 4 of the exposure were $124 \pm 1$ mmHg (systolic blood pressure), $87 \pm 2$ mmHg (diastolic blood pressure), $309 \pm 5$ bpm (heart rate). At 4 weeks of sham exposure, blood pressure and heart rate were $122 \pm 1$ mmHg (systolic), $84 \pm 2$ mmHg (diastolic), and $324 \pm 10$ bpm. It is important to note that while the data for systolic pressure, diastolic pressure and heart rate in the current experiment all exhibited significant circadian fluctuations ($p<0.05$ for time of day factor in 3-way ANOVAs; Figure 1), $dP/dt$ did not ($p=0.656$ for time of day factor; Figure 1D). However, second hand tobacco smoke exposure did cause a
significant increase in dP/dt that continued to increase further with each week of exposure (Figure 1D).

In order to better characterize the underlying chronic effects that may be developing, blood pressure data was recorded immediately before the daily exposure. This time was approximately 22 hours after the previous day’s exposure and thus the acute effects of the previous day’s second hand tobacco smoke exposure would be lowest, if not negligible. Second hand tobacco smoke significantly increased dP/dt by the end of week 1 in this period immediately before daily exposure, and this effect persisted throughout the remaining experiment (Figure 2). No significant differences in systolic pressure, diastolic pressure or heart rate were observed at these same times immediately prior to daily exposure (data not shown). Plasma nitrate/nitrite and ET-1 levels were not significantly different between the tobacco smoke- and sham-exposed rats (Figure 2 and Figure 3). However, plasma nitrotyrosine levels were significantly increased by second hand tobacco smoke exposure at day 28 (Figure 3). There was no significant difference in contractile response to 10\(^{-7}\) M phenylephrine in aorta isolated from rats after 4-weeks of second hand tobacco smoke exposure (0.20 ± 0.04 g tension/mg tissue; n=7) compared to sham exposure (0.22 ± 0.03 g tension/mg tissue; n=8). Furthermore, endothelium-dependent vasodilation to acetylcholine in the same isolated aorta was not significantly different between tobacco smoke- and sham-exposed rats (Figure 4).

Histological analysis of lung tissue showed an increased number of neutrophils in the lungs of rats exposed to second hand tobacco smoke after 28 days (Figure 5). In quantitative analyses, the number of lung neutrophils was significantly increased in tobacco smoke-exposed rats (5.4 ± 0.7 neutrophils/view, n=8) compared to sham-exposed
rats (3.0 ± 0.2 neutrophils/view, n=8 rats). No other differences in any other blood cell
type (e.g. no change in macrophage numbers) or pathological changes were noted in the
lungs (Figure 5), liver or arteries. Second hand tobacco smoke also had no significant
effect on heart weight, heart morphology or left ventricular wall thickness (data not
shown).
4. Discussion

The major finding of the current study is that second hand tobacco smoke exposure alters the circadian pattern of blood pressure and heart rate, significantly reducing the dipping pattern of blood pressure during sleep. The increase in pulse wave dP/dt observed with second hand tobacco smoke occurred early in the exposure period (by the end of week 1) and did not show a circadian pattern similar to blood pressure and heart rate. Furthermore, the increase in pulse wave dP/dt occurred in the absence of any structural alterations of the arterial wall which may instead be related to the observed reduction in NO bioactivity or other changes in functional regulation of arterial contractility.

Blood pressure in mammals is known to exhibit a circadian rhythm where blood pressure dips during the night and rises in the morning hours (27), a cycle which is inverted in nocturnal animals (25). In preliminary studies, we have observed an average 5% and 8% dip in systolic and diastolic pressures, respectively, in untreated, healthy Sprague Dawley rats using radiotelemetry. In humans, nondipping (<10% decrease in nocturnal blood pressure) and reverse-dipping (increased nocturnal blood pressure) blood pressure patterns are associated with increased cardiovascular mortality (32). Studies in humans have reported that heart rate (9) and daytime blood pressure (4,29) are increased in smokers, while nocturnal blood pressure dipping has been reported to be similar between smokers and nonsmokers (29). However, whether second hand tobacco smoke causes similar effects on blood pressure patterns has not been previously reported. In humans, elevated heart rate and blood pressure during the day has been attributed to the acute effects of nicotine from continuous smoking, with withdrawal occurring at night. In
previous studies from this laboratory, acute second hand tobacco smoke exposure was found to increase pig blood pressure (43), but had no acute effect on blood pressure in rats (12). Instead, subchronic tobacco smoke-exposed rats in the current study exhibited a non-dipping or reverse dipping blood pressure during sleep. Rats have previously been reported to exhibit increased locomotor activity after mainstream smoke exposure (31), but the current study found no significant change in activity with second hand tobacco smoke exposure (data not shown). Thus, the failure of blood pressure to dip in the tobacco smoke-exposed rats was not due to an acute hyperactivity response to nicotine and does not appear to be attributable to acute effects of nicotine.

The circadian clock influences both central and peripheral mechanisms of blood pressure regulation (36). NO has been implicated in the control of circadian variation in blood pressure in rats (47) and increased production of reactive oxygen species can disrupt timing of the circadian clock (18). In hypertensive individuals, a reversed or nondipping nocturnal blood pressure pattern is associated with increased arterial stiffness (24). In the current study, second hand tobacco smoke significantly increased pulse wave dP/dt which may indicate arterial stiffness increased. However, this conclusion requires corroboration with other tests of arterial stiffness in future experiments. Since there was no significant circadian pattern to dP/dt, the influence of second hand tobacco smoke on circadian blood pressure patterns and arterial stiffness may occur via different mechanisms.

Endothelium-derived nitric oxide (NO) and endothelin-1 (ET-1) function as mutual antagonists in the determination of vascular tone and several lines of evidence indicate that they play direct roles in the regulation of arterial stiffness (10,12,26). A
reduction in NO bioavailability is a key feature of endothelial dysfunction, and chronic smoking in humans is associated with impaired endothelium-dependent vasodilation in arteries exhibiting increased stiffness (34). In the current study, although plasma ET-1 and nitrate/nitrite levels were not significantly altered in tobacco smoke-exposed rats, there was an increase in peroxynitrite formation indicating reduced NO bioavailability. This increased oxidative stress-mediated inactivation of NO after 28-days second hand tobacco smoke exposure agrees well with a recent study reporting increased oxidative stress in mice exposed for 16- and 32-weeks to mainstream cigarette smoke (39). With increased oxidative stress and NO inactivation, one would expect to observed endothelial dysfunction. However, this was not evident in the current study in aorta isolated from rats exposed to second hand tobacco smoke for 28 days compared to sham-exposed rats. Instead endothelium-dependent relaxation in the current was intact in isolated aorta from tobacco smoke-exposed rats. Again, these findings agree well with the recent mouse study which found that isolated aorta did not exhibit impaired endothelium-dependent dilation to acetylcholine at 16-weeks exposure, but did by 32-weeks of tobacco smoke exposure (39).

Evidence from the same mouse study suggested that oxidative stress originated from activated mononuclear and multinuclear leukocytes, producing oxidative stress and endothelial impairment at 32 weeks exposure (39). There is increasing evidence that inflammatory reactions are involved in the pathophysiology of hypertension (37) as well as abnormalities in vascular function, including endothelial dysfunction and arterial stiffness in humans (15). Cigarette smoke exposure results in the attraction and activation of macrophages and neutrophils in the lung (38,42) and circulation (46), agreeing well
with the observed increase in pulmonary neutrophils in the current study. Not only can
these cells generate reactive oxygen species themselves (35), but they also release
proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-\(\alpha\)) (3,6) and
interleukin-6 that can travel via the blood to induce vascular oxidative stress (8) and
endothelial dysfunction in the systemic circulation (22). Thus, there is good evidence to
suggest that tobacco smoke-mediated increases in inflammation play a role in endothelial
dysfunction and arterial stiffness. In addition, another study suggested that a non-dipping
pattern of blood pressure was associated with higher C-reactive protein levels (41),
suggesting that inflammation may also play a role in the altered circadian blood pressure
patterns.

Alternatively, superoxide anion production may instead originate from a number
of other sources in cigarette smoke. While the gas-phase free radicals in cigarette smoke
are only stable for a short time, tar-phase radicals can generate additional reactive oxygen
species and are much more stable, having the potential to leave the pulmonary circulation
and reach the peripheral vasculature (38). Tar phase PAHs in cigarette smoke cause AhR
stimulation which in turn increase expression of genes such as CYP1A1 (7,13,33). PAHs
are also metabolically activated by CYP1A1 to form quinone structures that generate
superoxide anions (28). In the current study which examined effects of second hand
tobacco smoke and not direct smoking, the majority of cytochrome P450 substrates were
likely metabolized in the lung since CYP1A1 activity was increased in lung, but not liver
tissue. Further support for a role of CYP1A1 comes from two studies with AhR agonists.
First, blood pressure increased in rats after 7 days intranasal exposure to benzo-a-pyrene
(13). Second, oral exposure to TCDD in wild-type, but not CYP1A1 knockout mice
increased blood pressure after 15 days (23). Taken together, these results suggest that CYP1A1 activation by PAHs within cigarette smoke could have contributed to increasing blood pressure in the current study. It should be noted that if AhR agonists found within tobacco and other sources of smoke are verified in future studies to cause the changes in blood pressure, then implications for adverse cardiovascular effects extend much beyond tobacco smoke to air pollution and a variety of common environmental contaminants.

In summary, subchronic second hand tobacco smoke exposure in rats results in a loss or reversal in the normal dipping pattern of blood pressure during sleep and appears to increase arterial stiffness in the absence of any structural modifications to the arterial wall. Although smoking has been implicated as a cause of failure of blood pressure to dip during sleep, this is the first study to our knowledge that establishes a causal link between second hand tobacco smoke exposure and altered circadian blood pressure patterns. These findings were associated with increased neutrophil infiltration, increased CYP1A1 activity in the lung and a reduction in systemic NO bioactivity which may be causally related to arterial stiffness and overall increases in blood pressure even though endothelial dysfunction was not observed in the current study. However, which of these processes, if any, is responsible for the failure of blood pressure to dip is unclear and requires further investigation.

5. Grants

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**FIGURE LEGENDS**

**Figure 1.** Subchronic second hand tobacco smoke (also called environmental tobacco smoke or ETS) exposure alters the circadian pattern of A: systolic pressure, B: diastolic pressure, C: heart rate (beats per minute or bpm), and D: dP/dt of the arterial pressure pulse wave. Rats were exposed daily for one hour to sham (unlit cigarette; n=4; filled circles) or second hand tobacco smoke (3 cigarettes; n=4; open circles) for 28 days. Results from the end of week 1 and week 4 are shown. Data is expressed as a change in blood pressure, heart rate, or dP/dt from individual pre-exposure values and plotted as mean ± standard error of the mean (SEM). Results were analyzed by 3-way analysis of variance (ANOVA) with treatment, week, and time of day as factors. *p<0.05 versus sham-exposed group in Tukey’s posteriori test after 3-way ANOVA. There were no significant interactions between the factors in 3-way ANOVA analyses.

**Figure 2.** A: Pulse wave dP/dt 22 hours after the previous day’s tobacco smoke exposure and B: plasma nitrate/nitrite (NOx) levels in rats exposed daily for one hour to sham (unlit cigarette; filled circles) or second hand tobacco smoke (also called environmental tobacco smoke or ETS; 3 cigarettes; open circles) for 28 days. Resting dP/dt data (n=4 rats per treatment group) was collected over a 20 minute period before daily exposures and blood samples (n=8 per treatment group) were collected after exposure. Data is expressed as a change in dP/dt from pre-exposure values and plotted as mean ± standard error of the mean. *p<0.05 versus sham-exposed group in Tukey’s posteriori test after 1-way analysis of variance.
**Figure 3.** A: Plasma nitrate/nitrite (NOx), B: nitrotyrosine, and C: endothelin-1 levels in rats exposed daily for one hour to sham (unlit cigarette; n=8 rats; filled bars) or second hand tobacco smoke (also called environmental tobacco smoke or ETS; 3 cigarettes; n=8; hatched bars) after 28 days. Data are expressed as mean ± standard error of the mean. *p<0.05 versus sham-exposed group in 1-way analysis of variance.

**Figure 4.** *Ex-vivo* endothelium-dependent vasorelaxation in isolated aortic rings precontracted with $10^{-7}$ M phenylephrine from rats exposed daily to sham (unlit cigarette; n=8 rats; filled circles) or second hand tobacco smoke (also called environmental tobacco smoke or ETS; 3 cigarettes; n=8; open circles) for 28 days. No significant differences were found between treatment groups.

**Figure 5.** Representative photomicrographs showing increased neutrophils (arrows) in the lungs of rats exposed daily for one hour to A: sham (unlit cigarettes) compared to B: second hand tobacco smoke (3 cigarettes) after 28 days. Sections were stained with hematoxylin and eosin and neutrophils counted at 600x magnification.
Table 1  Second hand tobacco smoke exposure conditions, plasma cotinine concentrations, and liver and lung ethoxyresorufin-o-deethylase (EROD) activity.

Temperature and carbon monoxide (CO) were measured every 30 seconds for a total of one hour (sham) or 20 minutes (second hand tobacco smoke) in preliminary experiments in the absence of rats and are expressed as mean CO for a one hour exposure. Particulates were sampled continuously for one hour (sham) or 20 minutes (second hand tobacco smoke) and are expressed as total particulate matter for a one hour exposure period. Rats were then exposed daily for one hour to sham (unlit cigarette) or second hand tobacco smoke (3 cigarettes) for 28 days. Blood samples collected after exposure on day 28 were used for plasma cotinine quantitation (n=8 rats per group) and data are expressed as mean ± standard error of the mean (SEM). EROD activity was analyzed in liver and lung microsomes prepared from rats (n=8 per group) after 28 days of second hand tobacco smoke or sham exposure.

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<tr>
<td><strong>Plasma Cotinine (ng/ml)</strong></td>
<td>2.1 ± 0.4</td>
<td>51.6 ± 4.8**</td>
</tr>
<tr>
<td><strong>Liver EROD (fmol min⁻¹ mg⁻¹)</strong></td>
<td>0.46 ± 0.03</td>
<td>0.46 ± 0.04</td>
</tr>
<tr>
<td><strong>Lung EROD (fmol min⁻¹ mg⁻¹)</strong></td>
<td>0.06 ± 0.02</td>
<td>0.22 ± 0.04*</td>
</tr>
</tbody>
</table>

*p<0.01 and **p<0.0001 versus sham-exposed group 1-way analysis of variance
Table 2  Percent dip and average blood pressure, heart rate (beats per minute or bpm), and dP/dt of the arterial pressure pulse 4 weeks after second hand tobacco smoke exposure. Blood pressure telemetry-implanted rats were exposed daily for one hour to sham (unlit cigarette; n=4 rats) or second hand tobacco smoke (3 cigarettes; n=4) for 28 days. Blood pressure data collected during a 24 hour period were divided into light and dark periods and expressed as a percent dip (from dark/active to light/inactive period) or were averaged for the entire 24 hours and expressed as a change from individual baseline values. Data are shown as mean ± standard error of the mean.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Systolic Pressure</th>
<th>% Diastolic Pressure</th>
<th>% Heart Rate</th>
<th>% dP/dt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>6.5 ± 1.0</td>
<td>8.3 ± 2.1</td>
<td>17.1 ± 0.7</td>
<td>7.9 ± 2.2</td>
</tr>
<tr>
<td>Second hand tobacco smoke</td>
<td>-0.3 ± 1.6*</td>
<td>-0.4 ± 1.5*</td>
<td>13.2 ± 1.9</td>
<td>6.6 ± 3.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Δ Systolic Pressure (mmHg)</th>
<th>Δ Diastolic Pressure (mmHg)</th>
<th>Δ Heart Rate (bpm)</th>
<th>Δ dP/dt (mmHg/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>-2 ± 2</td>
<td>-3 ± 2</td>
<td>-75 ± 2</td>
<td>-457 ± 15</td>
</tr>
<tr>
<td>Second hand tobacco smoke</td>
<td>8 ± 2*</td>
<td>4 ± 1*</td>
<td>-71 ± 11</td>
<td>171 ± 216</td>
</tr>
</tbody>
</table>

*p<0.05 versus sham-exposed group in 1-way analysis of variance