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Third-hand Smoke: Impact on Hemostasis and Thrombogenesis

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Abstract: Cigarette smoking is a major risk factor for acute coronary thrombosis. In fact, both active/first-hand smoke and passive/second-hand smoke exposure are known to increase the risk of coronary thrombosis. Although recently a new risk has been identified and termed third-hand smoke (THS), which is the residual tobacco smoke contaminant that remains after a cigarette is extinguished, it remains to be determined whether it can also enhance the risk of thrombogenesis, much like first-hand smoke and second-hand smoke. Therefore, the present studies investigated the impact of THS exposure in the context of platelet biology and related disease states. It was found that THS-exposed mice exhibited an enhanced platelet aggregation and secretion responses as well as enhanced integrin GPIIb-IIIa activation. Furthermore, it was found that THS exposure shortens the tail bleeding time and the occlusion time in a model of thrombosis. Thus, our data demonstrate for the first time (at least in mice) that THS exposure increases the risk of thrombosis-based disease states, which is attributed, at least in part, to their hyperactive platelets.

Key Words: platelet, tobacco, third-hand smoke, thrombogenesis, hemostasis


INTRODUCTION

There is accumulating evidence that tobacco use causes multiple human diseases and is associated with a tremendous economic burden.1–3 In fact, cardiovascular disease (ie, thrombosis) is considered the main cause of death due to smoking.4,5

In terms of the detailed mechanism underlying its cardiovascular effects, there is ample evidence showing that smoking has the capacity to modulate platelet function. For example, platelets isolated from smokers exhibited an increased stimulated6–8 and spontaneous aggregation.9 Thus, it is thought that smoking-induced platelet-mediated thrombotic mechanisms may be involved in the pathophysiology of cardiovascular disease of smokers and is not just a result of a primary atherogenic effect.10

As more research has been performed, it is now evident that the deleterious effects of smoke are not limited to smokers [first-hand smoke (FHS)], rather they are shared with those who are in enclosed spaces with smokers. Thus, the harm that is imposed by smoke does not end with its users. In fact, second-hand smoke (SHS) is even now thought to be more toxic than smoke that is directly inhaled.11

Recently, the term “third-hand smoke” (THS) has been coined to describe the residual tobacco smoke contamination that remains after a cigarette is extinguished. Previous research has demonstrated that smoking in the home is linked to persistently high levels of tobacco toxins, long after active smoking has occurred.12–14 In fact, just a single day of smoking in an indoor setting exposes people to tobacco toxins within that setting in the future, potentially for days and even months. These toxins exist in various forms, including particulate matter deposited in a layer onto surfaces indoors or as dust or other particles in the air.14,15

Given the development of THS as a possible health concern and the fact that far less research has focused on the perceived harm from its exposure, investigating its negative consequences is clearly warranted/critical. It is noteworthy that evidence has underscored THS as an unappreciated danger to human health,16–20 particularly in the very vulnerable/sensitive populations of infants and children, as well as in adults and workers in environments where smoking is allowed.21 In this connection, it was found that relatively high levels of THS exist on various surfaces within the homes of smokers, even those that are located in bedrooms of their family members.14 Interestingly, THS residue remains as residents in these homes long after those who generated them, ie, smokers, have moved out,15 just as those associated with FHS and SHS. Regarding the means by which THS enters the body of a living being, it has been shown that inhalation, skin absorption, and ingestion are the major means for THS exposure.14 To this end, it was demonstrated that the exposure of nonsmokers to THS (and SHS) has the capacity to produce high toxicant blood levels.21,22 Surprisingly, however, a significant proportion of smokers and even nonsmokers disbelieve that THS exposure is harmful to human beings.20 Based on these considerations, there has been a growing interest in...
understanding the toxicity of THS exposure and whether it has any detrimental effects on human health. In this connection, a very recent study by Martins-Green et al.\(^2\) demonstrated that exposure to THS alone impairs wound healing, stimulates high levels of inflammatory cytokines, and increases lipid levels, which is a precursor for multiple diseases such as cardiovascular disease, among other negative effects.

Based on these aforementioned considerations, we investigated the impact of THS exposure in the context of platelet activation, hemostasis, and thrombogenesis. We observed that THS exposure increases the risk of thrombosis and enhances hemostasis in mice. Furthermore, evidence indicates that these effects derive, at least in part, from hyperactive platelets (ie, enhanced aggregation, glycoprotein IIb-IIIa activation and secretion responses) because of THS exposure. Collectively, our findings support the notion that THS exposure is detrimental to health and participates in thrombosis-based disease states.

### MATERIALS AND METHODS

#### Reagents and Materials

Adenosine diphosphate (ADP) was obtained from Sigma Aldrich (St. Louis, MO). U46619 was from Cayman Chemical (Ann Arbor, MI). Fluorescein isothiocyanate (FITC)-conjugated anti–P-selectin and JON/A antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA) and Emfret analytics (Würzburg, Germany), respectively. Stir bars and other disposables were from Chrono-Log Corporation (Havertown, PA). Other reagents were of analytical grade.

#### Animals

Animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Western University of Health Sciences. C57BL/6 mice were divided into normal vivarium/clean air (control) and THS groups. The THS group was exposed to THS from right after weaning to 24 weeks (6 months), whereas the control group was exposed to clean air for the same period.

#### THS Exposure

Common household fabrics were placed in smoking inhalation apparatus (Teague Enterprises, Woodland, CA) and subjected to SHS as described by Martins-Green et al.\(^2\) Briefly, each cage contained 10 g of curtain material (cotton), 10 g of upholstery (cotton and fiber), and two 16 in\(^2\) pieces of carpet (fiber) to maintain equal exposure levels across experimental groups. Two packs (20 cigarettes per pack) of 3R4F research cigarettes were smoked each day, 5 days per week, and smoke was routed to a mixing compartment and distributed between 2 exposure chambers containing 8 cages with the materials. We use the gravimetric method to determine the particulate concentrations. Whatman grade 40 quantitative cellulose filter papers are first weighed, then introduced into the filtering device, and after running the test for 15 minutes, the filter is weighed again to determine the particulate mass that has accumulated during this time. This procedure is repeated with 2 more filters, and the average of the 3 masses calculated gives the total particulate matter (TPM) values for each chamber. All cigarettes were smoked and stored in accordance with the Federal Trade Commission (FTC) smoking regimen. At the end of each week, cages were removed from the exposure chamber, bagged, and transported to the vivarium where mice were placed into the cages. For the next week, an identical set of cages and fabric was then prepared and exposed to smoke in the same way as described above. Using 2 sets of cages and material, each of which were exposed on alternating weeks, we ensured that mice inhabited cages containing fabric that had been exposed to both fresh and aged THS.

#### Murine Platelet Preparation

Clean air–exposed or THS-exposed (C57BL/6) mice were anesthetized, and blood was collected from the heart. Coagulation was inhibited by 3.8% wt/vol sodium citrate solution (1 part sodium citrate to 9 parts blood). Murine platelet-rich plasma was obtained by centrifugation (170g) at room temperature (22°C). Platelets were counted with an automated hematology analyzer and their count adjusted to 7 × 10\(^7\) platelets per milliliter in a total volume of 0.5 mL before each experiment.

#### In Vitro Platelet Aggregation

Clean air–exposed or THS-exposed platelets were activated with ADP (2.5–5 \(\mu\)M) or the thromboxane receptor agonist U46619 (0.25 \(\mu\)M). Platelet aggregation was measured by the turbidimetric method using model 490 aggregometer (Chrono-Log Corporation). Each experiment was repeated 4 times with blood pooled from at least 3 different groups of THS and clean air mice.

#### Flow Cytometric Analysis

Flow cytometric analysis was performed as we described before.\(^23\) In brief, platelets (2 × 10\(^9\)) from THS- or clean air–exposed mice were stimulated with ADP (2.5 \(\mu\)M) or U46619 (0.25 \(\mu\)M) for 3 minutes. Next, the reactions were stopped by fixing the platelets with 2% formaldehyde for 30 minutes at room temperature, and platelets were incubated with FITC-conjugated anti–P-selectin or JON/A antibodies at room temperature for 30 minutes in the dark. Finally, the platelets were diluted 2.5-fold with HEPES/Tyrode’s buffer (pH 7.4) before the fluorescent intensities were measured using a BD Accuri C6 flow cytometer. Results were analyzed using CFlow Plus (BD Biosciences, Franklin Lakes, NJ). Each experiment was repeated at least 3 times with blood pooled from at least 3 different groups of THS and clean air mice.

#### Tail Bleeding Assay

Clean air–exposed or THS-exposed mice were subjected to the tail transection technique as described previously.\(^23\)–\(^25\) Briefly, these mice were anesthetized and placed on a 37°C homeothermic blanket. Tail was transected 5 mm from the tip using a sterile scalpel. After transection, the tail was immediately immersed in saline (37°C, constant temperature), and the time to bleeding cessation was measured. Bleeding time of 10 minutes was considered as the cutoff time for the purpose of statistical analysis.
In Vivo Thrombosis Model

These studies were performed as described previously.23–25 Briefly, clean air–exposed or THS-exposed mice were anesthetized with ketamine (200 mg/kg). Then, the left carotid artery was exposed and cleaned, and baseline carotid artery blood flow was measured with Transonic Micro-Flowprobe (0.5 mm; Transonic Systems Inc., Ithaca, NY). After stabilization of blood flow, 7.5% ferric chloride (FeCl₃) was applied to a filter paper disc (1-mm diameter) that was immediately placed on top of the artery for 3 minutes. Blood flow was continuously monitored for 30 minutes or until blood flow reached stable occlusion (0 blood flow for 2 minutes). Data were recorded, and time to vessel occlusion was calculated as the difference in time between stable occlusion and removal of the filter paper (with FeCl₃). An occlusion time of 30 minutes was considered as the cutoff time for the purpose of statistical analysis.

Statistical Analysis

Analysis of the data was performed using GraphPad PRISM statistical software (San Diego, CA) and presented as mean ± SEM. The Mann–Whitney test was used for the evaluation of differences in mean occlusion and bleeding times. Analysis was also conducted using t test. Significance was accepted at $P < 0.05$ (2-tailed $P$ value), unless stated otherwise.

RESULTS

THS-exposed Platelets Exhibited Enhanced Platelet Aggregation

Nothing is known regarding THS effects on platelet function. Hence, platelets were collected from mice exposed to either THS or normal vivarium air as control, and platelet aggregation was studied. We observed for the first time that THS significantly enhanced platelet aggregation induced by ADP (2.5 μM) compared with the clean air–exposed controls (Fig. 1). The enhanced aggregation was evident regardless of the dose used (5 μM ADP; Fig. 1). Furthermore, a similar profile was found when platelets were stimulated with a thromboxane receptor agonist, namely U46619. Thus, platelet aggregation was found to be elevated in THS-exposed platelets in response to U46619 (0.25 μM) when compared with those exposed to clean air (Fig. 1). Together, these results suggest that the THS-exposed mice have hyperactive platelets.

THS-exposed Platelets Exhibited Enhanced Glycoprotein IIb-IIIa Activation

We next sought to investigate whether the enhanced aggregation response observed in the THS mice would be accompanied by a heightened integrin GPIIb-IIIa activation. Indeed, our flow cytometry analysis revealed that GPIIb-IIIa activation is enhanced in response to 2.5 μM ADP or 0.25 μM U46619 (Fig. 2). This finding is consistent with the enhanced aggregation response observed in the THS mice.

FIGURE 1. THS-exposed platelets exhibit enhanced platelet aggregation. THS-exposed and control platelets were stimulated with (A) ADP (2.5–5 μM) or (B) U46619 (0.25 μM) for 8 minutes. Each experiment was repeated 4 times, with blood pooled from at least 3 different groups of THS-exposed and clean air–exposed mice.

THS-exposed Platelets Exhibited Enhanced Platelet Secretion

In light of the enhanced platelet aggregation and GPIIb-IIIa activation observed in the THS platelets, we investigated the impact of THS on alpha granule secretion. Indeed, our results (Fig. 3) revealed that (2.5 μM) ADP-mediated and (0.25 μM) U46619-mediated secretion from the alpha granules was enhanced in the THS platelets compared with the clean air control. These data further support the notion that the THS-exposed mice have hyperactive platelets.

Effect of THS Exposure on Hemostasis

In light of the finding that THS enhances platelet aggregation, secretion, and GPIIb-IIIa activation and because cigarette smoking/FHS and SHS are known to exert numerous in vivo effects, we next investigated whether THS exposure produces in vivo effects in the context of platelet function. We addressed this issue by conducting the tail bleeding time assay to assess hemostasis in the THS-exposed mice. Indeed, our studies, using THS-exposed mice, revealed that they have significantly shortened bleeding times when
compared with their clean air–exposed controls (Fig. 4). This is the first evidence that THS exposure does in fact enhance hemostasis, which suggests a prothrombotic phenotype.

**Effect of THS Exposure on Thrombogenesis**

Given that the health consequences of THS exposure are a constant subject of debate, studies addressing this issue are clearly warranted. Additionally, platelet aggregation and secretion are major contributing factors in occlusive arterial thrombosis formation. Therefore, we determined whether THS exposure has effects on thrombogenesis. It was found that THS-exposed mice, when compared with the clean air controls, exhibited a shortened time for occlusion in a FeCl$_3$-induced carotid artery injury thrombosis model. In fact, complete occlusion of the vessel was found to be significantly shortened and to occur by 2.5 minutes after FeCl$_3$ treatment in THS-exposed animals, whereas the occlusion in air-exposed control animals took more than 28 minutes (Fig. 5). Taken together, the above data provide evidence that the THS-exposed mice exhibited increased risk of thrombosis, which is consistent with the enhanced aggregation, GPIIb-IIIa activation, and secretion, as well as shortened bleeding time phenotype we have observed.

**DISCUSSION**

Although exposure to SHS or “environmental” tobacco smoke is much less (1%) than that of a heavy smoker, its “excess” risk for cardiovascular disease is around 33% of that
Effect of Third-hand Smoke on Platelet Function

FIGURE 5. THS-exposed mice exhibit shortened occlusion time. Mice were anesthetized, and the FeCl₃-induced carotid artery injury model was performed as discussed in Methods. Each point represents the occlusion time of a single animal.

that exposure of non-smokers to THS has the capacity to produce high toxicant levels,14,21 and that exposure to THS does impose public health issues,15 such as impaired wound healing and increased lipid levels, among others.21 To this end, however, and despite the growing number of risks and negative health effects thought to be associated with THS exposure, whether exposure has specific consequences on platelets and platelet-dependent disease states remains to be determined. On this basis, in this study, we used a mouse model for THS exposure (developed by Martins-Green et al) that approximates that of children and others in environments contaminated by THS and presented the first line of evidence that exposure of mice to THS does indeed enhance their platelet aggregation and secretion responses and GPⅡb-Ⅲa activation. Second, we found that THS has the capacity to shorten their tail bleeding time (hemostasis response), indicating that its adverse health effects do manifest in vivo. Finally, we also documented for the first time that THS exposure increases the risk of thrombogenesis, using an animal model of arterial thrombosis. This latter finding is consistent with the enhanced platelet aggregation, secretion, and GPⅡb-Ⅲa activation phenotype observed in these mice. As for dose dependency, given the data suggesting a nonlinear dose–response relationship in the intensity of exposure to passive smoking/SHS,24 it is reasonable to propose a similar profile for THS exposure. These studies are the scope of a future investigation.

Collectively, these data demonstrate that THS-exposed mice have an enhanced hemostasis and thrombosis phenotype, which appears to be underlined, at least in part, by “hyperactive” platelets.

It is noteworthy that THS-exposed mice exhibit changes in liver metabolism that, in human, have important implications for development of coronary thrombosis, stroke, or type 2 diabetes.31,32 In terms of a real scenario for consequences for children of smoking parents, a recent study showed that SHS and its residue (THS) do harm non-smokers.27 Our studies in mice that are never exposed to smoke, but are exposed to residues of the smoke, strongly and clearly implicate tobacco smoke residues in thrombosis-based disease states. It follows that children in environment where smoking is, or has been allowed, are vulnerable/prone or at significant risk for suffering from long-term health issues.

CONCLUSIONS

Our studies show for the first time that THS exposure modulates platelet function, in vitro and in vivo. These findings also underscore the negative consequences of an underappreciated threat to human health. Future studies should provide mechanistic insight regarding hyperactivity of platelets in THS-exposed mice.

REFERENCES


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