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Enhanced platelet reactivity and thrombosis in *Apoe*^{-/-} mice exposed to cigarette smoke is attenuated by P2Y₁₂ antagonism

Anping Dong, MD, PhD,

VA Medical Center Lexington, Division of Cardiovascular Medicine, The Gill Heart Institute, University of Kentucky

Jessica Caicedo,

Division of Cardiovascular Medicine, The Gill Heart Institute, University of Kentucky

Sung Gu Han, PhD,

Graduate Center for Toxicology, University of Kentucky

Paul Mueller,

Division of Cardiovascular Medicine, The Gill Heart Institute, University of Kentucky

Sibu Saha, MD, MBA,

Division of Cardiothoracic Surgery, The Gill Heart Institute, University of Kentucky

Susan S. Smyth, MD, PhD, and

VA Medical Center, Lexington, Division of Cardiovascular Medicine, The Gill Heart Institute, University of Kentucky

C. Gary Gairola, PhD

Graduate Center for Toxicology, University of Kentucky

Anping Dong: adong2@uky.edu; Jessica Caicedo: jlcaic2@uky.edu; Sung Gu Han: sung.gu.han@uky.edu; Paul Mueller: pamuel3@uky.edu; Sibu Saha: ssaha2@uky.edu; Susan S. Smyth: SusanSmyth@uky.edu; C. Gary Gairola: cggair01@uky.edu

Abstract

Introduction—Smoking increases the risk of acute arterial thrombosis, including myocardial infarction, likely due to multi-factorial effects on the vasculature. Heightened platelet reactivity may be among the adverse effects of smoke exposure.

Methods—To examine the effects of smoke exposure on platelet function in an atherosclerotic environment, *Apoe*-deficient female mice, maintained on a Western diet, were exposed (4 hrs/d, 5d/wk) to sidestream cigarette smoke in a whole-body exposure chamber for 12 weeks. A separate group of wild type C57BL/6J mice were also exposed to smoke in an identical fashion.

Results—In comparison to control *Apoe*^{-/-} mice exposed to filtered ambient air, smoke-exposed *Apoe*^{-/-} mice displayed a 1.8 ± 0.3 fold enhanced ADP-induced fibrinogen binding *ex vivo* ($P < 0.001$) and had a shorter time to thrombotic occlusion following ferric chloride injury of the carotid artery (median time to thrombosis of 8 vs. 13 min; $P = 0.015$). Administration of the direct-acting P2Y₁₂ antagonist cangrelor blunted *ex vivo* fibrinogen binding and attenuated thrombosis (median time 20 min) in *Apoe*^{-/-} mice exposed to sidestream smoke. The effects of smoke exposure required a proatherosclerotic background, as wild-type C57BL/6J mice exposed to smoke displayed similar fibrinogen binding and thrombotic occlusion times as did control mice.

Conclusions—Our results demonstrate that exposure to smoke heightens platelet reactivity and thrombosis in *Apoe*^{−/−} mice and implicate signaling through platelet P2Y₁₂ receptor as a mediator of the adverse consequence of smoke exposure. These results may partially explain the recent observations that smokers derive greater clinical benefit from the P2Y₁₂ antagonist clopidogrel than do non-smokers.

Keywords

Apoe; cigarette smoking; thrombosis; P2Y₁₂

Exposure to cigarette smoke is a well-established risk factor for cardiovascular disease(1;2). Cigarette smoking may accelerate the progression of atherosclerosis(3), possibly through effects on inflammation and lipid oxidation(1). Studies in patients presenting with acute coronary syndrome have suggested that smokers may be more likely to present with platelet-rich thrombi(4) than their non-smoking counterparts. One possible explanation is that cigarette smoking heightens platelet activation and thereby increases the likelihood of platelet thrombosis at sites of vascular injury, such as plaque rupture or erosion. Platelets can be activated by a wide range of agonists, including those that work through G-protein-coupled receptors (e.g. thrombin, ADP, and epinephrine), and by adhesive protein – receptor interactions (e.g. collagen – glycoprotein VI and von Willebrand factor/shear – glycoprotein Ib). As a consequence of activation, platelets secrete granular contents, release thromboxane A₂, and aggregate to one another. Many studies report that cigarette smoking acutely increases plasma markers of platelet secretion(5;6), urinary thromboxane B₂ (an indicator of platelet thromboxane A₂ production)(7) and *ex vivo* platelet aggregation. In a study using laser-light scatter aggregometry, habitual smokers tested >10 hours after cigarette smoking had greater small spontaneous platelet aggregates and more ADP and epinephrine-induced medium and large aggregates than did nonsmokers(8). However, other investigators have found that platelets from smokers have less susceptibility to agonist-induced aggregation(9). Exposure to second hand smoke also substantially increases the risk of heart disease(10). Secondhand smoke exposure comes largely from sidestream smoke produced by the smoldering end of cigarettes. Sidestream smoke extracts increase activation of isolated platelets and enhance platelet susceptibility to shear-stress(11). A recent report from the Institute of Medicine indicated that smoke-free public policies are associated with reductions in the incidence of heart attacks. In several of the 11 studies reviewed, beneficial effects were observed within months of the institution of the smoking ban.

Despite these observations, a definitive link between exposure to cigarette smoke, platelet reactivity and heightened thrombosis in the setting of atherosclerosis remains to be established, in part due to a lack of experimental atherothrombosis models to study the effects of cigarette smoke. In dogs, exposure to cigarette smoke increased the spontaneous reductions in coronary blood flow in the Folts model of stenosed, but non-atherosclerotic, coronary arteries(12). We previously reported that exposure to sidestream smoke increases the severity of atherosclerosis in *Apoe*-deficient mice fed a Western diet without detectable histological and immunocytochemical changes in the lesion composition(13). We now use this model to confirm the effects of smoke exposure on thrombosis in an atherosclerotic milieu(14) and to evaluate the effects on markers of platelet reactivity and platelet-dependent thrombosis.

Materials and Methods

Animals

Female *Apoe*-deficient (*Apoe*^{−/−}) mice on the C57Bl/6J background and wild-type (WT) C57Bl/6J (8–9 weeks old) were purchased from the Jackson Laboratories (Bar Harbor, ME) and housed in HEPA filter equipped cages. Mice were fed a Western diet (21% anhydrous milk

fat, 34% sucrose, 0.2% cholesterol; Harlan Teklad 88137) and water *ad libitum*. To control for the effects of diet, one group of WT mice were maintained on standard rodent chow (Purina 5058). All studies conformed to the recommendations of “Guide for the Care and Use of Laboratory Animals” (Department of Health, Education, and Welfare publication number NIH 78-23, 1996) and were performed in accord with approval from the Institutional Animal Care and Use Committees.

Tobacco smoke exposure and monitoring

After one week of acclimation, animals were randomly divided into two equal groups: control and smoke-exposed. Exposure to sidestream cigarette smoke (SSCS) was performed as we have previously described (13). Mice were exposed to SSCS 4h/day, 5 days/week for 12 weeks. Control groups were maintained in filtered ambient air and otherwise handled in an identical fashion to the smoke-exposed group. To monitor smoke inhalation, plasma cotinine was measured by a commercial ELISA (Immunoanalysis, CA). Additionally, at the end of the experiments, lungs were harvested, and CYP1A1 protein levels in lung tissue microsome preparations were measured by immunoblot analysis(15). Results are presented as an average of CYP1A1 band densities normalized to β -actin.

Blood collection and platelet function analysis

Blood was collected from isoflurane-anesthetized mice into EDTA or 0.32% sodium-citrate as previously described(16). Plasma was prepared by centrifugation of EDTA-anticoagulated blood, and soluble P-selectin levels in plasma were determined by ELISA (R&D systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. For flow cytometry, whole blood was immediately diluted 1:40 in Tyrode’s buffer containing 1 mM $MgCl_2$ (17). ADP (4 μ M) or vehicle was added to aliquots of diluted blood in the presence of 64 μ g/ml FITC-anti-fibrinogen antibody (Dako) or 20 μ g/ml FITC-anti-P-selectin antibody (BD Science). After a 10 min incubation, samples were fixed with paraformaldehyde and analyzed on a FACScalibur flow cytometer (Becton-Dickinson). The mean fluorescence intensity (MFI) of platelets, identified by characteristic forward and side-scatter properties, was recorded.

Ferric chloride induced thrombosis

Carotid artery thrombosis was induced by the application of ferric chloride, and thrombotic occlusion was monitored by Doppler flow probe (0.5VB307; Transonic Systems Inc, Ithaca, NY) as we have previously described(18). To detect enhanced thrombosis, we modified the delivery of ferric chloride by applying two pieces of filter paper saturated with ferric chloride on opposite sides (one beneath and one above) of the carotid artery as described by Wang *et al.* (19). In preliminary experiments, we tested the effects of different concentrations of ferric chloride and found that an 8% solution ($FeCl_3 \cdot 6H_2O$ w/v; Sigma Chemical Co.) evoked variable thrombosis times in *Apoe*^{-/-} mice. This concentration was used in all experiments. The filter papers were applied for 3 min and then removed. Carotid blood flow was monitored for 20 min after application of the filter papers. In some experiments, mice were administered cangrelor (The Medicines Company) 100 μ g/kg $\times$ 2 doses 10 min apart, with the last dose given immediately prior to initiation of thrombosis.

Statistical analysis

For *in vitro* or *ex vivo* analysis, results are reported as mean $\pm$ SD. Differences between control and SSCS groups was analyzed by t-test. Based on the results of preliminary experiments, we calculated that 8 – 10 mice per group would be needed in the carotid artery thrombosis model to detect a 30% decrease in the time to thrombosis with smoke exposure. Differences between groups were analyzed by t-test, one-way analysis of variance, or rank sums as appropriate.

Statistical significance was set at $P < 0.05$. Statistics performed using the SigmaStat (SPSS) software package.

Results

We previously demonstrated that exposure of female *Apoe*-deficient mice to sidestream cigarette smoke (SSCS) promotes atherosclerotic lesion development without changing serum cholesterol concentration(13). In this study, we sought to determine if exposure to SSCS also increased platelet reactivity and thrombosis. *Apoe*- mice fed a Western diet were exposed to ambient air or SSCS for 12 weeks. At the end of the exposure, there was no difference in body weight or body composition score in the two groups of mice. Plasma cotinine levels in *Apoe*- mice exposed to SSCS were significantly higher than that of control *Apoe*- mice exposed to filtered ambient air (Table 1). Lung tissue microsomal CYP1A1 protein content in the SSCS-exposed group was also higher than in the control mice (Table 1). Together, these results confirm effective inhalation of tobacco smoke by animals under our exposure conditions. Although differences in metabolism between humans and mice make direct comparison difficult, the plasma cotinine levels in mice exposed to SSCS are roughly similar to those observed in humans exposed to at least moderate smoke.

We first examined the effects of smoke exposure on *ex vivo* markers of platelet function. In plasma collected from mice exposed to SSCS or filtered ambient air, no significant differences were observed in levels of soluble P-selectin, a protein that is expressed and shed from activated platelets and endothelial cells (Table 1). Similarly, in samples of whole blood collected from SSCS-exposed or control mice, no differences in platelet P-selectin expression were observed by flow cytometry (Figure 1A). Because others have reported that agonist-induced P-selectin expression is lower in hypercholesterolemic patients(20), we compared plasma P-selectin levels in mice on regular chow. Plasma P-selectin in mice on regular chow was 169 ± 26 and 208 ± 18 ng/ml ($P = 0.292$) with SSCS-exposure.

ADP-induced platelet fibrinogen binding was 1.8 ± 0.3 fold higher in whole blood collected from *Apoe*- mice exposed to SSCS than in samples from control mice (13 ± 3.9 versus 7.7 ± 1.2 MFI; $P < 0.001$; Figure 1B). *Apoe*- mice exposed to SSCS were treated with the direct acting P2Y₁₂ receptor antagonist cangrelor, and blood was collected for *ex vivo* analysis of fibrinogen binding. Cangrelor treatment reduced fibrinogen binding to levels observed in control *Apoe*- mice (Figure 1B), suggesting that at least some of the effects of smoke exposure may be through increased P2Y₁₂ signaling.

We sought to determine if the higher ADP-induced fibrinogen binding observed in *ex vivo* samples collected from *Apoe*- mice chronically exposed to smoke translated to alterations in platelet-dependent thrombosis. We used a ferric chloride injury model to elicit thrombosis in the carotid artery of anesthetized mice. To detect enhanced thrombosis, we examined the response to 8% ferric chloride, which in control *Apoe*- mice ($n = 11$) resulted in thrombotic occlusion in 8 of 11 (72%) mice within 20 min (Figure 2) with a median time to thrombosis of 12.85 min (mean $\pm$ SD: 14.1 ± 4 min). In *Apoe*- mice exposed to SSCS, thrombotic occlusion occurred in 8 of 9 (88.9%) with a median time to thrombosis of 7.6 min (mean $\pm$ SD: 9.2 ± 4 min), which is significantly different from that observed in *Apoe*- mice exposed to ambient air ($P = 0.015$; Figure 2).

Our *ex vivo* analysis suggested that smoke exposure increased ADP-induced fibrinogen binding and that this effect could be partially reversed by treatment of mice with the P2Y₁₂ antagonist cangrelor. To determine if P2Y₁₂ antagonism also alters thrombosis in mice exposed to SSCS, we examined the time to thrombosis in mice treated with the direct acting P2Y₁₂ antagonist cangrelor. We elected to use a direct-acting P2Y₁₂ antagonist to avoid any confounding issues

that may arise from the effect of smoke on the CYP-mediated metabolism of pro-drugs such as clopidogrel or prasugrel. Only one of five (20%) mice treated with cangrelor exhibited thrombotic occlusion within 20 min following application of ferric chloride (Figure 2), suggesting that P2Y₁₂ antagonism attenuates thrombosis in animals following smoke exposure.

Altogether, our results indicate that exposure of *Apoe*^{-/-} mice to sidestream smoke increases ADP-induced platelet fibrinogen binding and promotes a pro-thrombotic state. To determine if the atherosclerotic *Apoe*^{-/-} background were necessary for the effects of smoke exposure on platelet function and thrombosis, we repeated our experimental design using wild-type (WT) C57Bl/6J mice. In contrast to the effects observed in *Apoe*^{-/-} mice, WT mice exposed to SSCS did not display differences in thrombotic occlusion of the carotid artery following application of ferric chloride (Figure 3). In WT mice fed a Western diet and exposed to smoke, 4 of 8 (50%) had thrombotic occlusion of the vessel within 20 min of ferric chloride application. The median time to thrombosis in WT mice exposed to smoke was 10.4 min (25 – 75% CI, 8.33 – 20), as compared to 14.6 min (25 – 75% CI, 8.48 – 20; *P* = 0.677 by rank sum test) in control mice on Western diet (*n* = 8). Interestingly, the median time to thrombosis was not different in WT mice on normal rodent chow (13.9 min; 25 – 75% CI, 9.25 – 20; Figure 3). In agreement with these results, chronic smoke exposure did not alter ADP-induced fibrinogen binding to platelets in whole blood collected from WT mice fed Western diet. Treatment of smoke-exposed WT with cangrelor (*n* = 8) lowered ADP-induced fibrinogen binding to levels near those observed in the absence of ADP (Figure 4).

Discussion

In this study, we have found that exposure of *Apoe*-deficient mice to sidestream smoke significantly increased *ex vivo* ADP-induced fibrinogen binding to platelets in whole blood and shortened the time to thrombotic occlusion of the carotid artery following ferric chloride injury. Smoke exposure did not alter plasma levels of soluble P-selectin or platelet P-selectin expression. Interestingly, the effect of smoke on platelet reactivity required the *Apoe*-deficient background, as smoke exposure did not enhance thrombosis in C57Bl/6J mice. Several explanations could account for these findings. First, an interaction between smoke exposure and elevated plasma cholesterol and/or atherosclerosis could be required for the observed platelet effects. This could account for the reports of variable effects of smoking on platelet function in humans. Alternatively, smoke exposure may enhance platelet reactivity by promoting the development of atherosclerosis. Atherosclerosis and hyperlipidemia can increase platelet reactivity and propensity to thrombosis in mice(21;22). By promoting atherosclerosis, sidestream exposure may indirectly heighten platelet reactivity. Finally, it is possible that we were unable to detect a small effect of smoke exposure on thrombosis in wild-type mice. Nonetheless, our findings indicate an interactive effect of smoking and atherosclerosis on platelet function and thrombosis. One limitation to our study is the exclusive use of female mice. Sex differences in platelet function and thrombosis have been reported in humans and mice, which may relate to direct or in-direct hormonal effects on platelet function (reviewed in (23)). Thus, it is not clear if our findings would extend to male *Apoe*^{-/-} mice exposed to smoke.

Under the conditions studied, feeding wild-type C57Bl/6J mice a Western diet did not appreciably accelerate thrombosis. A previous report indicated that a diet rich in lard attenuates platelet function in C57Bl/6 mice (24), perhaps through elevations in blood glucose, which is also observed in mice fed Western diet. Other factors that promote arterial thrombosis, such as Factor V Leiden, lack effect in mice fed high-fat diet(25). Our results suggest that any effect of Western diet on thrombosis is minimal in the experimental model tested.

We observed that the direct acting P2Y₁₂ antagonist cangrelor lowered ADP-induced fibrinogen binding to platelets from SSCS exposed *Apoe*^{-/-} mice to levels observed in control *Apoe*^{-/-} mice. Cangrelor also extended the time of thrombotic occlusion in response to ferric chloride injury. Thus, platelet P2Y₁₂ receptor antagonism is effective in preventing thrombosis in *Apoe*^{-/-} mice exposed to smoke.

Our findings of the benefits of cangrelor are in agreement with several recent clinical studies suggesting that smokers derive greater clinical benefit from the P2Y₁₂ antagonist clopidogrel (26) or dual anti-platelet therapy with aspirin and clopidogrel (27) than do non-smokers. In *ex vivo* analysis, clopidogrel, but not aspirin, attenuated platelet aggregation in smokers (28). Other studies indicate that aspirin alone is not sufficient to block the effects of smoking on platelet function. For example, *ex vivo* platelet thrombus formation on porcine arterial media is heightened acutely by cigarette smoking in patients with coronary artery disease who were taking aspirin (29). Cigarette smokers also have higher levels of plasma markers of inflammation and platelet reactivity, even after consumption of aspirin (30). Interestingly, in the PURSUIT trial (Platelet Glycoprotein IIb/IIIa in Unstable Angina: Receptor Suppression Using Integrilin Therapy), inhibition of the platelet integrin α IIb β 3 (glycoprotein IIb/IIIa receptor) with eptifibatide did not improve 30 day outcomes in current smokers over non-smokers or former smokers (4). Thus, anti-platelet therapy with a P2Y₁₂ antagonist such as clopidogrel may have unique benefits in smokers. There are several possible explanations. First, clopidogrel is a pro-drug that undergoes hepatic metabolism that is essential for its anti-platelet effects. Smoking alters a number of CYP isoforms in a manner that may enhance the generation of the active clopidogrel metabolite (31). Indeed, smokers have a different pharmacokinetic profile of the inactive clopidogrel carboxylate metabolite following a 75 mg oral dose than do non-smokers (32). In addition, smoking inhibits the ability of platelets to degrade ADP (33), which in turn could enhance responsiveness.

While there are a number of mechanisms by which smoking may increase cardiovascular thrombotic events, including alterations in antithrombotic/prothrombotic/fibrinolytic factors and vascular dysfunction, our findings support the notion that chronic exposure to cigarette smoke in an atherogenic environment promotes platelet reactivity and increases the propensity to thrombosis. Both our data and recent clinical data indicate that P2Y₁₂ antagonism may uniquely target the pro-thrombotic effects of smoking.

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Figure 1. Flow cytometric analysis of platelet reactivity in whole blood from *Apoe*^{-/-} mice without and with exposure to sidestream smoke (SSCS)

Platelet surface expression of P-selectin (A) and 4 μ M ADP-induced fibrinogen binding to platelets (B) in blood from control mice (n= 8) and SSCS-exposed mice (n=8). In one group of *Apoe*^{-/-} mice exposed to SSCS, cangrelor was administered prior to blood collection. The mean fluorescent intensity for each condition was normalized to the value obtained for control, unstimulated platelets.

Figure 2. *Apoe*-deficient mice exposed to sidestream smoke (SSCS) have accelerated thrombosis after ferric chloride injury

Thrombosis was performed as described in methods. A. Time to thrombotic occlusion in *Apoe*^{-/-} mice on a Western diet (WD) without (n = 11) or with SSCS exposure (n = 9). One group of *Apoe*^{-/-} mice exposed to SSCS was treated with the P2Y₁₂ antagonist cangrelor before application of ferric chloride. B. The data are presented as the percent of mice with thrombotic occlusion at varying times following application of ferric chloride.

Figure 3. Exposure to sidestream smoke (SSCS) does not accelerate thrombosis after ferric chloride injury in wild-type (WT) C57BL/6J mice

Thrombosis was performed as described in Methods. A. Time to thrombotic occlusion in WT mice on a normal rodent chow (NC) or Western diet (WD) without ($n = 8$) or with SSCS exposure ($n = 9$). One group of WT on WD mice exposed to SSCS was treated with the P2Y₁₂ antagonist cangrelor ($n = 8$) before application of ferric chloride. B. The data are presented as the percent of mice with thrombotic occlusion at varying times following application of ferric chloride.

Fibrinogen binding to platelets in blood from C57Bl/6J mice

Figure 4. Flow cytometric analyses of platelet reactivity in whole blood from wild-type (WT) C57Bl/6 mice without and with exposure to sidestream smoke (SSCS)

4 μ M ADP-induced fibrinogen binding to platelets in WT mice and SSCS-exposed WT mice. In one group of WT mice exposed to SSCS, cangrelor was administered prior to blood collection. All mice were on Western diet for 12 weeks. The mean fluorescent intensity for each condition was normalized to the value obtained for control, unstimulated platelets..

Table 1

Effects of sidestream smoke exposure on lung CYP1A1 and plasma P-selectin and cotinine levels

	Control Apoe	Smoke-Exposed	
P-selectin (ng/ml)	170 $\pm$ 10	167 $\pm$ 11	P>0.05
CYP1A1/actin ratio	0.3 $\pm$ 0.09	1.4 $\pm$ 0.09	P <0.05
Cotinine (ng/ml)	Ud	144 $\pm$ 30	P<0.05

Plasma P-selectin is expressed as mean $\pm$ SEMLung CYP1A1 is expressed as mean $\pm$ SEM

ud = below detection level