Increased Serotonin Receptor Density and Platelet GPIIb/IIIa Activation Among Smokers

Jerome H. Markovitz, Lelland Tolbert, Suzan E. Winders

Abstract—This study sought to determine whether depressive symptoms and/or platelet serotonin receptor (5HT$_{2A}$) density are associated with increased platelet activation (PA) found among smokers. Flow cytometric detection of PA was used to study 36 smokers and 16 nonsmokers, aged 18 to 48 years. Subjects were tested at baseline and after either smoking 2 cigarettes (smokers) or a similar resting interval (nonsmokers). Assessment of PA included both platelet secretion and fibrinogen receptor (GPIIb/IIIa) binding. Platelet 5HT$_{2A}$ receptor binding and saturation were tested using [3H]LSD, and depressive symptoms were measured using the Beck Depression Inventory. Platelet 5HT$_{2A}$ receptor density was increased among smokers versus nonsmokers (82.7 ± 67.7 versus 40.0 ± 20.2 fmol/mg protein; $P<0.005$), and there was a dose-dependent relationship between receptor density and packs/d among smokers. Baseline wound-induced GPIIb/IIIa binding at 1 minute and GPIIb/IIIa binding in response to collagen stimulation in vitro was increased among smokers ($P<0.05$); there were no changes in PA among smokers after smoking, and platelet secretion was not elevated among smokers. Depressive symptoms were associated with 5HT$_{2A}$ receptor density among nonsmokers ($P<0.005$), but no such relationship was evident among smokers; PA was unrelated to 5HT$_{2A}$ receptor density in either group. The findings indicate that smoking is associated with increased platelet serotonin receptor density and with increased GPIIb/IIIa receptor binding, although these 2 factors are not related to each other or to depressive symptoms among smokers. Serotonergic dysfunction may be an important factor in the development of cardiovascular disease among smokers. (Arterioscler Thromb Vasc Biol. 1999;19:762-766.)

Key Words: smoking ■ platelet activation ■ thrombosis ■ serotonin receptors ■ depression

Cigarette smoking is a well-documented risk factor for cardiovascular disease. The specific mechanisms involved in this relationship, however, are not entirely clear. One proposed mechanism is an increased propensity toward thrombosis among smokers, including increased platelet activation (PA). The connection between smoking and platelet activation has been frequently studied. Although animal studies have shown increased PA in response to cigarette smoke exposure, the connection between PA and smoking has not been found in all studies in humans. Perhaps in part because of the variety of methods used to assess platelet function among smokers. Thus, despite compelling evidence from animal studies, the relationship between smoking and PA has not been consistently demonstrated in humans.

The mechanisms by which smoking increases PA are also not clearly established. Increases in plasma catecholamines are known to occur with smoking, and this has been the primary mechanism suggested. However, serotonergic dysfunction also plays a role in PA, and serotonergic dysfunction has been found in some studies of smokers. Interestingly, both adrenergic and serotonergic dysfunction have been associated with clinical depression, and depression is far more prevalent among smokers, suggesting a possible link between PA and depression among smokers. The increase in platelet serotonin receptor (5HT$_{2A}$) density consistently found in clinically depressed patients may have a direct impact on PA. The present investigation sought to determine the relationship between smoking and PA, using wound-induced flow cytometric detection of PA as previously measured in our laboratory. Platelet 5HT$_{2A}$ receptor binding and saturation, and depressive symptoms were also assessed as factors that might mediate between PA and smoking. The study examined PA both at baseline and after smoking (among smokers only). The major hypotheses for the study were (1) smokers have greater PA, higher platelet 5HT$_{2A}$ receptor density, and more depressive symptoms than nonsmokers; and (2) the relationships of depressive symptoms and/or serotonergic dysfunction with PA account in part for the increased PA seen among the smokers.

Methods

Subjects
A total of 52 subjects (36 smokers and 16 nonsmokers), aged 18 to 48 years participated. Subjects were recruited through local advertisements and leaflets circulated in the university community. Sub-
jects were otherwise healthy (ie, without a previous diagnosis of clinical cardiovascular disease, hypertension, diabetes, or other smoking-related diseases) and were not taking any medications that could interfere with platelet testing. All smokers reported smoking at least 1 pack per day; individuals smoking less than this amount were excluded from the study. Informed consent was obtained from all subjects, and the study was approved by the Institutional Review Board of the University of Alabama at Birmingham.

Design

All subjects were tested in the morning. Smokers were asked to refrain from smoking overnight and during the morning before testing. Subjects initially completed the Beck Depression Inventory, a well-characterized instrument for depressive symptoms.35 Smoking dose was assessed by self-reported packs per day. Then an 18-gauge intravenous catheter was inserted into a vein in the arm for blood sampling; subjects received a slow infuse of saline throughout the testing period, to avoid the use of heparin. All subjects then rested quietly for 25 minutes, after which initial blood testing was done. Blood was drawn from the catheter for baseline venous PA using a 2-syringe technique, and a modified bleeding time test was performed concurrently (see below). Nonsmokers then rested for an additional 10 minutes, while smoking subjects smoked 2 of their own brand of cigarettes during a 10- to 15-minute period. The blood collection and bleeding time test were then repeated, with additional blood taken for platelet serotonin receptor studies. The catheter was removed, and the session was complete.

Platelet Activation Measures

In addition to venous blood for resting and in vitro activation studies, a modified bleeding time procedure was performed as described previously,32–34 with samples taken at 1 and 2 minutes after incision for activation levels. After the samples were taken, the wound was bandaged. The bleeding time procedure was repeated after smoking or, for nonsmokers, an additional 10-minute rest period as described above. This method has been shown previously to be highly reproducible in our laboratory.32 Platelet function testing was performed as described previously.32,33 Whole venous blood, collected in 3.8% sodium citrate, was added to tubes containing Walsh’s buffer, a saturating solution of the anti-CD42b antibody S22-FITC (Gentak, Inc), and saturating solutions of 1 of the 2 antibodies for activation detection: AC1.2-PE (Becton Dickinson) for detection of P-selectin expression (an indicator of platelet secretion) and biotinylated anti-LIBS-1, specific for GPIIb/IIIa, specific for GPIIIb/IIIa (ligand binding), which was graciously provided by Dr. Mark Ginsberg. Negative and positive control tubes were prepared for each subject, and additional tubes were prepared using 100 μg/mL of equine collagen, type I (Chronolog, Co). In vitro testing was performed only from the initial venous sample. For the bleeding time samples, whole blood collected in heparinized capillary tubes was added immediately to similar tubes with 1 of the 2 activation antibodies. All tubes were mixed gently and incubated for 15 minutes. Five μL of a saturating solution of phycoerythrin-streptavidin (Southern Biotechnology Associates) was added to tubes containing biotinylated anti-LIBS-1, followed by a second incubation of 10 minutes. The reaction was stopped and the platelets fixed by the addition of 450 μL of 0.25% paraformaldehyde. Flow cytometric analysis and analysis was performed using a 10- to 15-minute period by a trained operator blinded to subject status. Acquisition was limited to include only particles with the characteristic properties of platelets, and samples were stained for the S22 antibody. Instead of using analytical markers as we have done previously,32–34 mean fluorescence intensity (MFI) was used to determine activation. Values of MFI for unstimulated venous blood (in the case of anti-LIBS-1) or the post-smoking GPIIb/IIIa measures, 2 smokers were eliminated from the 1-minute wound-induced secretion measure. For the baseline platelet secretion measures, 1 nonsmoker was eliminated from the post-smoking GPIIb/IIIa measures, 2 smokers were eliminated from the 1-minute wound-induced secretion measure, and 1 smoker was eliminated from the 2-minute wound-induced secretion measure. For the post-smoking GPIIb/IIIa measures, 2 smokers were eliminated from the 1-minute wound-induced activation measure, and 1 smoker was eliminated from the 2-minute wound-induced activation measure. Wilcoxon 2-sample tests, Student’s t tests, and χ^2 analysis were used to compare smokers and nonsmokers. Paired t tests were used to assess differences in PA after smoking. Linear regression was used to test relationships between PA levels and other factors including B_{max} and K_{d}.

Results

Characteristics of Smokers and Nonsmokers

Table 1 indicates that there were no substantial differences between groups in age, sex, or racial composition. As predicted, smokers had more depressive symptoms than the nonsmokers (P=0.01); 7 of the smokers had Beck Depression Inventory scores ≥10 (indicating possible clinical depression), whereas all nonsmokers had scores <10. Most of the smokers (29) reported smoking 1 pack/d, and the remaining 7 reported smoking 1.5 packs/d.
Platelet Serotonin Receptor Measures, Platelet Activation, and Smoking

Smokers had significantly higher \( B_{\text{max}} \) than nonsmokers, but there were no differences in \( K_d \) (Table 2). Furthermore, smokers who reported smoking 1.5 packs/d had higher \( B_{\text{max}} \) and \( K_d \) than smokers reporting 1 pack/d (82.7 (67.7) versus 71.6 (61.9) fmol/mg protein, \( P < 0.05 \); 0.75 (0.31) versus 0.69 (0.31) nmol/L, \( P < 0.05 \)), suggesting that the relationship was dose-dependent.

There were no differences in platelet secretion between smokers and nonsmokers before or after smoking. However, as shown in Table 3 and the Figure, GPIIb/IIIa receptor binding was significantly higher among smokers relative to nonsmokers for collagen activation in vitro (\( Z \) score = 2.4; \( P < 0.05 \)) and for wound-induced activation at 1 minute (\( Z \) score = 2.0; \( P < 0.05 \)). There were no changes in PA after smoking or resting in either group (\( P > 0.1 \); data not shown). As in previous studies, no racial or sex differences were seen in PA.

Smoking and Relationships Among Serotonin Receptor Density, Depressive Symptoms, and Platelet Activation

Among the nonsmokers, depressive symptoms were significantly correlated with \( B_{\text{max}} \) (\( r = 0.73, P < 0.005 \)). However, among smokers, there were no significant relationships between depressive symptoms and either \( B_{\text{max}} \) or PA (data not shown). There were also no significant relationships between depressive symptoms and PA among the nonsmokers (data not shown).

Discussion

The present results indicate that platelet serotonin receptor density and platelet GPIIb/IIIa receptor binding are elevated among smokers. Our initial hypothesis was that these factors would be related, and would also be covariant with depressive symptoms. Although there was evidence supporting this hypothesis among the nonsmokers, \( B_{\text{max}} \) and depressive symptoms were unrelated to PA among smokers, suggesting that other mechanisms of GPIIb/IIIa receptor binding (such as adrenergic activation) are operative among smokers.

TABLE 2. Platelet Serotonin Receptor Binding (\( B_{\text{max}} \)) and Saturation (\( K_d \)) in Smokers and Nonsmokers

<table>
<thead>
<tr>
<th>Variable</th>
<th>Smokers (n=38)</th>
<th>Nonsmokers (n=16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( B_{\text{max}} ), fmol/mg protein*</td>
<td>82.7 (67.7)</td>
<td>40.0 (20.2)</td>
</tr>
<tr>
<td>( K_d ), nmol/L</td>
<td>0.75 (0.31)</td>
<td>0.70 (0.40)</td>
</tr>
</tbody>
</table>

Values are mean (SD).

*\( P < 0.005 \).

This study is the first, to our knowledge, that has examined platelet serotonin receptor density among smokers, and this is the major new finding of the study. Receptor density was greater among smokers who reported smoking 1.5 packs/d usage than among smokers reporting 1 pack/d, indicating a dose-response relationship. Other studies have found greater serotonin metabolite excretion among smokers,21 and greater platelet serotonin content has also been found among smokers.22,23 Taken together, these studies indicate serotonergic dysfunction among smokers, which could be related to the increased incidence of coronary heart disease among smokers. Although the present study suggests that serotonergic mechanisms do not operate through increased PA among smokers, local vascular mechanisms may come into play, such as vasoconstriction in response to serotonin release,39–40 and smooth muscle cell proliferation.41 Such mechanisms could be the focus of future research in this area.

We also found increased GPIIb/IIIa receptor binding among smokers, although platelet secretion was not elevated. In addition, there were no changes in PA after smoking, in

TABLE 3. Resting Platelet Activation in Smokers and Nonsmokers

<table>
<thead>
<tr>
<th>Platelet Activation Measure</th>
<th>Smokers</th>
<th>Nonsmokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPIIb/IIIa receptor binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro—collagen*</td>
<td>8.4 (14.3)</td>
<td>−0.8 (3.7)</td>
</tr>
<tr>
<td>Wound-induced—1 minute*</td>
<td>34.0 (51.8)</td>
<td>13.7 (29.5)</td>
</tr>
<tr>
<td>Wound-induced—2 minutes</td>
<td>19.0 (27.5)</td>
<td>19.4 (31.2)</td>
</tr>
<tr>
<td>P-selectin expression (secretion)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>In vitro—collagen</td>
<td>80.2 (64.7)</td>
<td>72.0 (44.3)</td>
</tr>
<tr>
<td>Wound-induced—1 minute*</td>
<td>47.0 (28.6)</td>
<td>39.4 (22.9)</td>
</tr>
<tr>
<td>Wound-induced—2 minutes</td>
<td>50.0 (26.3)</td>
<td>55.9 (40.0)</td>
</tr>
</tbody>
</table>

Values are mean (SD) fluorescence intensity units (activation minus unstimulated venous sample).

*Smokers vs nonsmokers, \( P < 0.05 \) by Wilcoxon two-sample test.
agreement with some studies but not others; this finding, however, may be limited by the lack of assessment of smoking abstinence during the morning hours before the study (see below). The present study used flow cytometric detection of PA, which has been cited as the best available method of assessing platelet function; hence, the results may differ from earlier studies using other methods of assessing PA. In addition, there may have been other factors, such as differences in deprivation of smoking or other procedural details, that could account for results differing from earlier studies.

Platelet secretion was not related to smoking, consistent with our previous work in restenosis after coronary stenting. The general lack of a relationship between smoking and platelet secretion is not entirely unexpected, as adrenergic mechanisms, which are likely responsible for increased PA in smokers, may affect fibrinogen receptor binding without affecting platelet secretion. Taken together with our previous work, the present study indicates that not all measures of PA are the same, and that assessment of the activation of GPIIb/IIIa, the final common pathway to platelet aggregation, may generally prove to be more worthwhile than other measures.

Although the present findings indicate a strong relationship between subclinical depressive symptoms and Bmax in nonsmokers, they also indicate that smoking may be an important confounding variable in studies of platelet receptor density among patients with clinical depression. None of these studies reported the smoking habits of either the patients or the control subjects. Smokers have an increased incidence of major depression over their life span and the present study indicates that smokers have increased receptor density that is independent of depressive symptoms (although clinical depression could not be determined using the Beck Depression Inventory alone). Future work in this area would benefit from taking smoking into consideration.

The present study is limited by a lack of more objective measures of smoking, such as exhaled carbon monoxide, to assess morning abstinence from smoking and self-dosing behavior; hence our ability to assess the acute effects of smoking may have been diminished. In addition, alcohol intake (chronic and recent) was not measured, and alcohol is known to inhibit PA. Given the higher amount of alcohol consumption among smokers, this factor may have accounted for lower PA in some smokers in the present study, which may have attenuated the effect of smoking on PA. A number of outlying values (>3 standard deviations from the mean) were present and were eliminated from the analysis to avoid potential artifact effect, but most of these values (9 of 11) were found among the smokers; therefore, if any bias occurred in eliminating these values, it was toward the null hypothesis. Finally, although no subject had clinical cardiovascular disease, it is possible that subclinical disease, including endothelial dysfunction commonly found among smokers, may have played a role in the increased PA and platelet serotonin receptor density. Specifically, endothelial production of nitric oxide, a potent platelet inhibitor, may have been decreased among smokers. Hence, the increases in PA among smokers in this study may have been related to endothelial dysfunction caused by smoking, rather than the direct effect of smoking per se. However, smoking also diminishes release of nitric oxide from platelets. Further studies of smokers with overt or subclinical cardiovascular disease may be useful to further assess the mechanisms responsible for increased PA among smokers.

In summary, smoking is associated with increased platelet serotonin receptor density and with increased GPIIbIIIa receptor binding. The relationship between serotonin receptor density and smoking appears to be dose-related, but increased PA among smokers does not appear to be related to increased serotonin receptor density. Serotonergic dysfunction among smokers may be an important mediator linking cardiovascular disease to smoking, and further research in this area is indicated.

Acknowledgments
Supported by Grant 1K08HL02975-01 from the National Heart, Lung, and Blood Institute. The authors thank Dr. John Mann and Yung-Yu Huang for their generous technical advice and assistance in the studies of platelet serotonin receptor measures.

References
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doi: 10.1161/01.ATV.19.3.762
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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