Original Article

Smoking-Induced Alterations in Platelet Membrane Fluidity and Na⁺/K⁺-ATPase Activity in Chronic Cigarette Smokers

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Aim: Cigarette smoking is a recognized risk factor for cardiovascular diseases and has been implicated in the pathogenesis of atherosclerosis. Platelet adhesiveness and aggregation increases as a result of smoking. Cigarette smoking modifies haemostatic parameters via thrombosis with a consequently higher rate of cardiovascular events, but smoking-induced alterations of platelet membrane fluidity and other changes have not been studied.

Methods: Thirty experimental and control subjects (mean age 35 ± 8) were selected for the study. Experimental subjects had smoked 10 ± 2 cigarettes per day for 7–10 years. The plasma lipid profile, platelet carbonyls, sulfhydryl groups, Na⁺/K⁺-ATPase activity, fluidity using a fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH), total cholesterol and phospholipids as well individual phospholipids were determined.

Results: Increases in the platelet membrane cholesterol phospholipid (C/P) ratio, phosphotidylethanolamine, phosphotidylserine with decreased phosphotidylcholine, Na⁺/K⁺-ATPase activity, fluidity and no significant change in phosphotidylinositol and sphingomyelin, as well as increases in plasma total cholesterol, LDL-cholesterol, protein carbonyls with decreased HDL-cholesterol and sulfhydryl groups were observed in cigarette smokers. Platelet membrane total phospholipids were positively correlated with plasma LDL-cholesterol (r = 0.568) and VLDL-cholesterol (r = 0.614) in cigarette smokers.

Conclusions: Increased plasma LDL-cholesterol, VLDL-cholesterol and total cholesterol might have resulted in the increased C/P ratio and decreased platelet membrane fluidity of cigarette smokers.


Key words: Atherosclerosis, Cigarette smoking, Na⁺/K⁺-ATPase activity, Platelets, Fluidity

Introduction

A puff of cigarette smoke introduces 10¹⁸ free radicals, with chief constituents of nicotine, NOx, aldehydes, peroxides, benzene, and epoxides, into the human body¹-³. Free radicals have been reported to play an important role in the pathogenesis of many diseases, including atherosclerosis⁴-⁶. Cigarette smoke with its highly oxidizing gas and tar phases enters the lungs, putting oxidative stress on the entire organism⁷. High doses of reactive oxygen species (ROS) lead to the inability of the biological system to cope with their production and result in chemical modification of biological molecules, causing severe metabolic malfunctions and damage to biological macromolecules⁸. The proposed potential mechanisms by which smoking increases the risk of cardiovascular diseases (CVD) include hemostatic disturbances, lipid abnormalities and vascular endothelial dysfunction⁹. After exposure to cigarette smoke, the endothelium becomes activated and induces the extrinsic coagulation pathway, causing platelet activation and enhanced platelet aggregation leading to thrombin and fibrin formation¹⁰.
Various key physiological processes, characteristic events/effects associated with cigarette smoking are attributed to interactions of the chemical constituents of cigarette smoke with membrane constituents and alterations associated with the physicochemical properties of membranes. Earlier studies revealed that smoking increases the excretion of a thromboxane A2 metabolite in urine, which is of platelet membrane origin. Passive smoking induces short-lasting activation of platelet function and the prostaglandin system, followed by rapid recovery after 15 minutes. Aqueous cigarette smoke extract inhibited the platelet plasma membrane-bound enzyme nucleoside triphosphate diphosphohydrolase, which plays a significant role in the extensive platelet activation and vascular inflammation. The cellular and molecular mechanisms by which cigarette smoke causes these abnormalities in platelets have not been thoroughly investigated.

Platelet lipid composition plays an important role in platelet functions related to haemostasis and thrombosis, and changes in platelet lipid content have been observed in clinical situations in which platelet activation occurs. Maintenance of the appropriate membrane lipid composition and fluidity is critical for the proper functioning of integral membrane proteins, membrane-bound enzymes, receptors and ion channels. The Na+/K+ ATPase is an integral membrane-bound protein that plays a key role in cellular osmotic regulation through the maintenance of the transmembrane gradients of Na+ and K+. A growing body of evidence indicates that the fluidity of the lipid environment influences Na+/K+ ATPase activity, modulates transmembrane transport processes and is a determinant of the passive permeability characteristics of a given bilayer. Since the composition and properties of the bilayer lipid environment influence the functions of proteins embedded in membranes, such as protein site accessibility, structural arrangement of the platelet surface, and rapid cell response to appropriate stimuli, the present study aimed to assess alterations in platelet membrane cholesterol, phospholipid composition, fluidity, Na+/K+ ATPase activity, and the correlation of plasma lipoprotein alterations with platelet membrane fluidity in cigarette smokers.

Methods

Chemicals

Bovine serum albumin, 2-thiobarbituric acid, cholesterol, ouabain, adenosine triphosphate disodium salt (ATP-Na2) and the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents were obtained from Qualigens (Glaxo India Ltd, Mumbai, India).

Experimental Details

Thirty human male volunteers (mean age 35 ± 8) in each group were chosen for the study. The first group was designated as the control or normal group and had no past or present history of smoking. The second group was designated as the experimental group, smoking at least 10 ± 2 cigarettes/day for the past 7 to 10 years. The subjects were not alcoholics and were free from chronic diseases, illness, and use of any tranquillizers, drugs and anesthetics. All volunteers were well informed about the experiment and their written consent was obtained. The present study was approved by the institutional ethics committee.

Isolation of Plasma and Platelets

Samples were obtained after the subjects had fasted overnight and smoked their last cigarette 8 hours before the experimental procedure. Venous blood was drawn into heparin-treated evacuated tubes and centrifuged immediately at 1,500 g for 15 minutes at 4°C to separate plasma and red cells. Platelets were prepared by differential centrifugation using the method of Menashi et al. Briefly, the blood (18 mL) was centrifuged at 200 g for 10 min at room temperature and the platelet-enriched plasma was carefully removed. The remaining pellet was washed three times with acid citrate dextrose (ACD) buffer (36 mM citric acid, 5 mM KCl, 90 mM NaCl, 5 mM glucose, 10 mM EDTA, pH 6.8) and the washings were pooled with the original supernatant. The pooled suspension was centrifuged again at 200 g for 10 min to remove any residual erythrocytes. The platelets were then collected by centrifugation at 2,000 g for 20 min and the platelet pellet was washed three times with 10 mL phosphate-buffered saline (PBS: 120 mM NaCl, 15.3 mM Na2HPO4, 1.46 mM KH2PO4, 1.68 mM KCl, pH 6.8). The final pellet was resuspended in 700 μL PBS for platelet analysis.

Plasma and Platelet Biochemical Profile

Plasma total cholesterol, HDL-cholesterol, LDL-cholesterol and VLDL-cholesterol levels were determined using commercially available kits (Span Diagnostics, Surat, India). The concentration of platelet protein carbonyls was determined using the 2,4-dinitrophenylhydrazine (DNPH) assay according to the method of Reznick and Packer. The platelet sulfhydryl groups and lipid peroxidation were determined. The activity of Na+/K+ ATPase was mea-

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Table 1. General characteristics and plasma lipid profile of controls and cigarette smokers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Cigarette Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>35 ± 8</td>
<td>35 ± 8</td>
</tr>
<tr>
<td>BMI</td>
<td>&gt;23.5</td>
<td>&gt;24.2</td>
</tr>
<tr>
<td>Number of cigarettes/day</td>
<td>No</td>
<td>8–12</td>
</tr>
<tr>
<td>Platelet count (mean ± SD)</td>
<td>220 ± 60</td>
<td>232 ± 62</td>
</tr>
<tr>
<td>Hypertension</td>
<td>SBP &gt; 130 mmHg, and/or DBP &gt; 89 mmHg</td>
<td>SBP &gt; 130 mmHg, and/or DBP &gt; 89 mmHg</td>
</tr>
<tr>
<td>Blood sugar</td>
<td>80–120 mg/dL (Fasting levels)</td>
<td>80–120 mg/dL (Fasting levels)</td>
</tr>
<tr>
<td>Glucose in urine</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Protein in urine</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Smoking history</td>
<td>–</td>
<td>7–10 years</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>Do not drink</td>
<td>Do not drink</td>
</tr>
<tr>
<td>Chronic diseases</td>
<td>Not found</td>
<td>Not found</td>
</tr>
<tr>
<td>Total cholesterol †</td>
<td>24.44 ± 1.7</td>
<td>56.06 ± 2.95 *</td>
</tr>
<tr>
<td>LDL-Cholesterol ‡</td>
<td>95.91 ± 4.97</td>
<td>222.6 ± 15.43 *</td>
</tr>
<tr>
<td>VLDL-Cholesterol ‡</td>
<td>23.84 ± 1.22</td>
<td>27.66 ± 1.62 *</td>
</tr>
<tr>
<td>HDL-Cholesterol ‡</td>
<td>41.35 ± 2.51</td>
<td>23.41 ± 2.86 *</td>
</tr>
</tbody>
</table>

Values are the mean ± SD of 30 human volunteers in each group. * indicates significant difference from controls. p < 0.05 is significant between groups. † Values are expressed as mg/dL. ‡ Values are expressed as µg/mg protein. BMI, body mass index, SBP, systolic blood pressure; DBP, diastolic blood pressure.

Quantitative measurement of platelet membrane fluidity was performed by the fluorescence polarization technique with DPH (1,6 diphenyl 1,3,5 hexatriene) as the fluorescence probe. Platelet preparations (50 µg protein) were suspended in 50 mmol/L DPH solubilized in tetrahydrofuran, and incubated at 37°C for 30 min. Fluorescence polarization was determined using a Hitachi fluorescence spectrophotometer (Hitachi, Japan) equipped with rotating polarizing filters with samples held at 25°C. Samples were excited at 360 nm and emission intensity was read at 435 nm. Polarization (P) and fluorescence anisotropy (γ) were calculated using the equation: P = I_{VV} - I_{VH} G / I_{VV} + I_{VH} G. Where I_{VV} and I_{VH} are the intensities measured parallel and perpendicular to the vertical axis of the excitation beam, and G is the correction factor, I_{VH} / I_{VV}. γ is calculated using the formula γ = 2P/(3-P). Platelet protein concentration was estimated as described by Lowry et al. 32.

Statistical Analysis

Data were subjected to statistical analysis, and values are the mean ± SD of thirty subjects in each group. The data were normally distributed and Student’s t-test was performed to find significant differences between groups. P < 0.05 was considered significant. Correlations between variables were assessed with Pearson’s correlation coefficients (r).

Results

Plasma Lipid Profile

Table 1 shows the general characteristics of the
subjects as well as plasma total cholesterol and lipoprotein patterns. A significant increase \((p<0.05)\) in plasma total cholesterol, LDL-cholesterol, VLDL-cholesterol with a significant \((p<0.05)\) decrease in HDL-cholesterol in the cigarette smoker group is evident from the study.

**Platelet Lipid Peroxidation, Protein Carbonyl Content and Total Sulphydryl Groups**

Fig. 1 shows the extent of lipid peroxidation in the platelet membrane of controls and cigarette smokers. Platelet membrane lipid peroxidation was significantly \((p<0.05)\) higher in cigarette smokers than in controls. Fig. 2 shows the platelet protein carbonyl content and total sulfhydryl groups of controls and cigarette smokers. A significant increase \((p<0.05)\) in protein carbonyl content with a significant decrease \((p<0.05)\) in total sulfhydryl groups was observed in cigarette smokers when compared to controls.

**Platelet Membrane Fluidity Studies**

Steady state fluorescent anisotropic studies of the platelet membrane using DPH were carried out and the anisotropic \((\gamma)\) values are presented in Fig. 3. The fluorescent anisotropic \((\gamma)\) values of the platelet membrane of cigarette smokers were higher than controls in the present study.

**Platelet Membrane \(\text{Na}^+/\text{K}^+\)-ATPase Activity, C/P Ratio and Individual Phospholipids**

The results of the present study clearly showed a significant decrease in \(\text{Na}^+/\text{K}^+\)-ATPase activity in cigarette smokers compared to controls (Fig. 4). A significant increase \((p<0.05)\) in platelet membrane cholesterol and phospholipid contents with significant increase \((p<0.05)\) in the consequent C: P ratio was observed in cigarette smokers (Table 2). Fig. 5 shows that cigarette smoking altered the content of platelet...
individual phospholipid classes. A significant increase was observed in the content of phosphatidyl ethanolamine and phosphatidylserine with a decrease in phosphatidylcholine, whereas no significant change was observed in the content of phosphatidylinositol and sphingomyelin.

**Comparison of Platelet Membrane Total Phospholipids with Plasma LDL and VLDL-Cholesterol**

Fig. 6 shows correlation (r) analysis between (a) plasma LDL-cholesterol and platelet membrane phospholipids and (b) plasma VLDL-cholesterol and platelet membrane phospholipids in smokers. The results of the present study clearly indicate that platelet membrane total phospholipids were positively correlated with plasma LDL-cholesterol (r = 0.568, p < 0.001) and plasma VLDL-cholesterol (r = 0.614, p < 0.001) in cigarette smokers.

**Discussion**

The adverse health consequences of smoking have been largely attributed to the abundance of reactive oxygen species and reactive nitrogen species that readily react with various biomolecules. Reactive oxygen species damage polyunsaturated fatty acids, leading to lipid hydroperoxide formation. In the present study, an increase in lipid peroxidation, carbonyl groups and decrease in sulphydryl groups was observed in the platelets of cigarette smokers. A great diversity of aldehydes are formed when lipid hydroperoxides break down in biological systems which are able to modify proteins both in vivo and in vitro. Increased lipid peroxides might have led to the covalent modification of proteins in cigarette smokers. ROS are known to damage polyunsaturated fatty acids and the oxidizable amino acids of transmembrane proteins through lipid peroxides, and cigarette smokers are more vulnerable to plasma membrane damage by these compounds. Earlier reports have shown that treatment of platelets with ROS such as H₂O₂ activates platelets by inducing Ca²⁺ release from agonist-sensitive Ca²⁺ stores, which is mediated by sulphydryl group oxidation.

Na⁺/K⁺ ATPase is a marker of membrane function as it is an integral membrane protein, depending on the chemical-physical properties of the microenvironment for its activity. Moreover, in association with membrane fluidity, it represents an index of membrane functionality, being involved in the modulation of phospholipid and protein interactions. This study showed a decrease in platelet Na⁺/K⁺ ATPase activity in cigarette smokers compared to controls. DPH studies revealed that chronic cigarette smoking resulted in a considerable increase in anisotropy (γ) values, indicating decreased fluidity in the platelet membrane. Further, the platelet membrane of cigarette smokers has shown an increase in the content of phospholipid, cholesterol and C/P ratio, suggesting alterations in platelet membrane microviscosity in cigarette smokers which is a critical determinant of platelet aggregation and secretion.

In blood, circulating platelets are continuously exposed to LDL, VLDL and HDL-cholesterol. Cholesterol plays an essential role in maintaining membrane fluidity and architecture whereas LDL and VLDL constitute a major source of phospholipids to the platelet membrane. Cigarette smokers in the present study showed increased plasma cholesterol, LDL, and VLDL, with decreased HDL. There is

<table>
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<tr>
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<th>Controls</th>
<th>Cigarette Smokers</th>
</tr>
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<tbody>
<tr>
<td>Total cholesterol</td>
<td>84.3 ± 4.64</td>
<td>95.1 ± 6.74 *</td>
</tr>
<tr>
<td>Total phospholipids</td>
<td>108.5 ± 5.18</td>
<td>116.7 ± 4.38 *</td>
</tr>
<tr>
<td>C/P ratio</td>
<td>0.77</td>
<td>0.82 *</td>
</tr>
</tbody>
</table>

Values are the mean ± SD of 30 human volunteers in each group. *indicates significant difference from controls. p < 0.05 is significant among groups. Values are expressed as µg/mg protein.
increasing evidence that cholesterol and lipoproteins affect platelet functions especially oxidized LDL. Animal models have shown that increased platelet cholesterol is due to the uptake of circulating cholesterol by megakaryocytes, which is then incorporated into future platelets. Increased plasma and platelet cholesterol may lead to alterations in platelet membrane physiological processes in cigarette smokers since dysregulation of cholesterol synthesis and transport with hypercholesterolemia is associated with increased platelet activity and hyperaggregability. Increased plasma LDL, VLDL and cholesterol might have altered the platelet membrane composition, which could be one reason to augment the sensitivity of platelets to aggregating agents generated by cigarette smoking.

Phospholipids (PLs) are involved in a variety of cellular events. Analysis of individual phospholipids of platelet membranes of cigarette smokers showed increases in the content of PE, PS with a decrease in PC and no significant change in the content of PI and sphingomyelin when compared to controls. Changes in phospholipids may alter the properties of biological membranes, since their polarity and shape may differ significantly from the structures of their parent molecules and they may alter lipid-lipid and lipid-protein interactions as well as membrane protein functions. In platelets, phospholipids play an important role in signal transduction processes, and platelet activation stimulates the cleavage of fatty acids from phospholipids. In this study, increased platelet membrane total phospholipid content was positively correlated with increased plasma LDL and VLDL in cigarette smokers. Since circulating lipoproteins are the major source of phospholipids, and PC, PE, and sphingomyelin can be transferred from LDL to human platelets, the observed increase in PE and PS might be due to the transfer of these phospholipids from LDL and VLDL to platelets of cigarette smokers. Moreover, LDL is known to supply PE by a specific mechanism, and VLDL could supply all types of phospholipids to platelets without consideration of their nature. Also, platelets might have been activated by the incorporation of cholesterol, which further could have stimulated phospholipid transfer from VLDL to platelet membranes in cigarette smokers since it can bind to platelet receptor CD36. The mechanism of cigarette smoke-induced alterations in the functions of the platelet receptor CD36 need further in-depth study.

Cigarette smoking is a reprehensible habit that has spread all over the world, and is a major lifestyle factor influencing human health. Chronic cigarette smokers have a higher prevalence of common diseases such as atherosclerosis and chronic obstructive pulmonary diseases with significant impact. As humans are oronasal inhalers, the inhaled smoke passes through
parts of the respiratory as well as digestive tract to enter the circulation, affecting plasma, blood cells and tissues, and causing oxidative damage. In conclusion, platelet membrane lipid peroxidation, protein oxidation, and the abnormalities observed in the platelet membrane cholesterol and phospholipid composition of cigarette smokers might have changed the activity of Na\(^+/\)K\(^+\) ATPases. Increased circulating cholesterol, LDL, and VLDL would have led to the uptake of cholesterol and phospholipids by platelet membranes to decrease platelet membrane fluidity, and resulted in platelet activation and aggregation, which are both primary and secondary effects on the initiation and progression of atherosclerosis and on thrombotic events in cigarette smokers. Investigations focused on the molecular mechanisms of cigarette smoking inducing the transfer of oxidized phospholipids from LDL and VLDL to platelet membranes and the resulting alterations in platelet membrane proteins and lipids leading to atherogenesis may be warranted.

**Acknowledgements**

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