Research Article

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EFFECT OF CIGARETTE SMOKING ON THE PRODUCTION OF 20-HYDROXYEICOSATETRAENOIC ACID IN HUMAN PLATELET

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ABSTRACT

Smoking showed to effect on platelet aggregation and the response to anti-platelet drugs. The arachidonic acid metabolite 20-hydroxyeicosatetraenoic acid (20-HETE) increases platelet aggregation and blood pressure. In this study, we investigated the influence of cigarette smoking on plasma 20-HETE levels and protein expression of 20-HETE producing enzyme CYP4A11 in isolated platelets from smoker (N=6) and non-smoker (N=6) volunteers. The protein expression and 20-HETE levels were analyzed using immunublot and high-performance liquid chromatography with Mass spectrometry assays, respectively. The results showed that plasma 20-HETE level was significantly higher among smokers than non-smokers (t test, p-value<0.05). The protein expression of CYP4A11 was significantly higher (t test, p-value<0.05) in platelets isolated from smokers, which was correlated with in vitro higher 20-HETE production, than non-smoker platelets. We concluded from this study that cigarette smoking increased the level of platelet activator 20-HETE through increasing the protein expression of CYP4A11. These findings may increase our understanding of the molecular mechanism of smoking in platelet aggregation and in the variation of anti-platelet drug response induced by smoking.

Keywords: CYP4A11, platelets, induction, 20-HETE

INTRODUCTION

Platelets are anucleate cells that play a major role in hemostasis. The activity of human platelets is affected by human health status and in response to xenobiotic compounds, such as drugs and environmental chemicals.[1,2] It is found that cigarette smoking increased platelet activation.[3] The mechanism by which cigarette smoking increases platelet aggregation is still not fully covered. Smoking cigarette contains many compounds including nicotine and hydrocarbons.[4] These cigarette compounds were reported to induce some cytochrome (CYP) P450s in liver, lung and gastro-intestinal tract.[5, 6]

Liu et al.[7], pointed out that 20-hydroxyeicosatetraenoic acid (20-HETE) is a platelet activator and showed that elevated plasma levels of 20-HETE were associated with decreased bleeding time.[7] The 20-HETE is an arachidonic acid metabolite, which is synthesized mainly through CYP4A and CYP4F subfamilies.[8]

The expression and the activity of arachidonic acid metabolizing-CYPs had been identified in human platelets.[9] It is found that CYP4A11, which is the main CYP in 20-HETE synthesis, was highly expressed in human platelet.

As 20-HETE and its synthesizing system had been identified in human platelets, it is hypothesized in this study that exposure to cigarette smoking increases 20-HETE levels and induces CYP4A11 and 20-HETE production in human platelets.

MATERIALS AND METHODS

Chemicals and reagents

Primary antibodies for CYP4A11 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Skimmed milk was purchased from Sigma Aldrich (St. Louis, MO, USA). 20-HETE, and 20-HETE-d6 were purchased from Cyamin (Michigan, USA). All other chemicals and organic solvents for 20-HETE assays were of the highest grade available from commercial sources.

Preparation of platelets

Platelets were isolated and washed from 5ml human blood samples as described previously.[9] All of the volunteers (smokers n=6, non-smokers n=6) signed on informed consent and they were healthy, as judged by medical history, and were not taking any drug medication at the time of blood collection.[9] This study was approved by the Institutional Review Board (IRB) committee. [9]

Protein extraction and immunoblot analysis

Platelet protein extraction and western blot analysis were performed as described previously.[9] Twenty µg protein was loaded onto a 13% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to a nitrocellulose membrane in a buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% (v/v) methanol. The membrane was treated with skimmed milk blocking buffer. Polyclonal mouse anti-CYP4A11 IgG, and monoclonal mouse anti-GAPDH IgG were used as primary antibodies. Immunoreactive proteins were detected by the enhanced chemiluminescence method according to the manufacturer's instructions (GE Healthcare Bio-Sciences, Buckinghamshire, UK).

Determination of 20-HETE levels in human platelets and plasma

Washed human platelets were incubated with 20µM arachidonic acid for 30 mins as it is published previously.[9] The 20-HETE from the in vitro supernatant reaction solutions and human

plasma samples of smokers and non-smokers were extracted by ethyl acetate after reducing the pH of the media to 3−4 with 2N H_2SO_4. Then, the residues were reconstituted in ethanol for analysis using liquid chromatography–mass spectrometry system (LC/MS) (Applied Biosystems, Foster City, CA); as published previously.[10]

Statistical analysis

All values were represented by the mean ± standard deviation (SD) of triplicate reactions. Statistical significance was analyzed by a two-tailed Student's *t*-test where the differences, as compared to control groups, were indicated as $P < 0.05$.

RESULTS

Pixel analyses of western blot bands showed that CYP4A11 protein expression was significantly higher (*t* test, p-value < 0.05) in platelet samples from smokers than non-smoker (Figure 1).

Figure 1

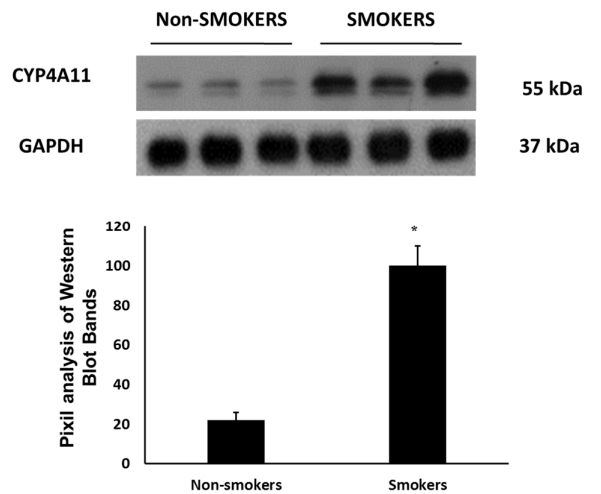

Figure1: Induction of CYP4A11 expression in smoker human platelets

The analysis of CYP4A11 expression was done using western blot. As it is shown, CYP4A11 protein expression was significantly (p value < 0.05) higher in smoker than non-smoker platelets. The GAPDH was used as a control of the variation in protein expression among different samples.

This induction of CYP4A11 protein in smoker platelets was correlated with higher capacity of platelets to synthesis 20-HETE. As shown on Figure 2, in vitro incubation of isolated smoker platelets with 20μM arachidonic acid showed higher significant (p-value < 0.05) capacity for 20-HETE production, almost 3-fold, in comparison with non-smoker platelets.

Figure 2

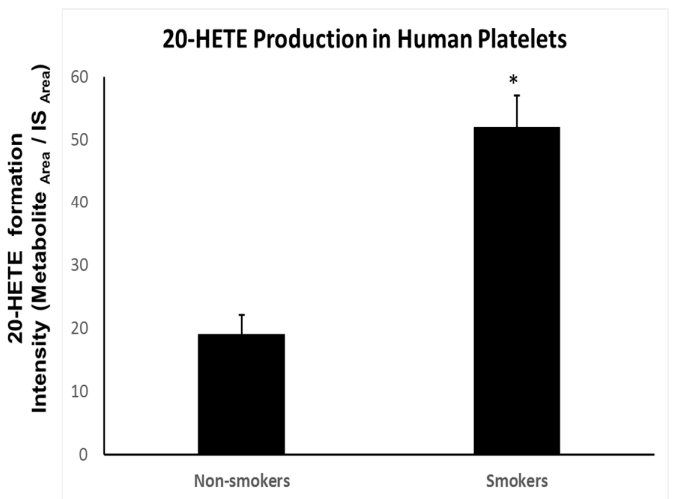

Figure 2. The 20-HETE production among smoker and non-smoker platelets

The levels of 20-HETE production was measured using LC/MS after incubation the washed platelets with 20μM arachidonic acid. The 20-HETE-d6 was used as internal standard. It is shown that 20-HETE production was significantly higher (p value < 0.05) among smoker than non-smoker platelets.

Clinically, the plasma levels of 20-HETE in smokers were almost double (p value < 0.05) the levels in non-smoker plasma samples, as they were analyzed by LC/MS method.

Figure 3

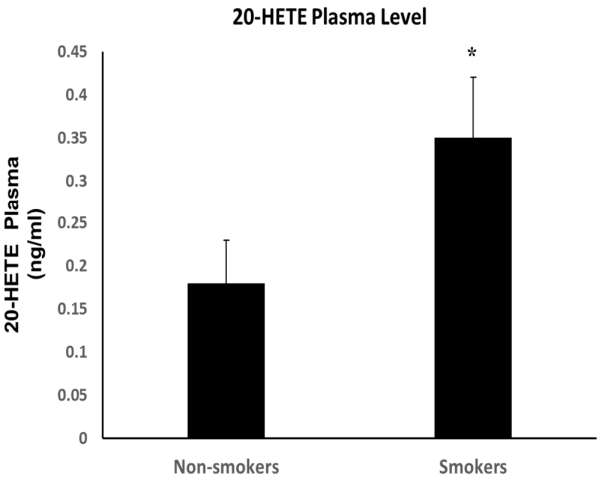

Figure 3. The plasma levels of 20-HETE among smoker and non-smoker volunteers

The quantification of plasma 20-HETE was done using LC/MS method. The plasma 20-HETE levels in smokers were 2fold higher in comparison to non-smoker volunteers.

DISCUSSION

Cigarette smoking is well known to affect harmfully on the cardiovascular system and blood hemostasis.[11] Until now, the mechanism by which cigarette smoking accelerates platelet aggregation is not fully understood. In this study, it is found that plasma 20-HETE in smokers was almost 2fold higher in comparison with non-smoker volunteers, which was associated with increased protein expression of CYP4A11 and in vitro 20-HETE production in smoker platelets. The results of this study may add a step toward understanding the mechanism of the influence of cigarette smoking on platelet aggregation through enhancing of platelet 20-HETE synthesis.

The CYP4A11 was found to be the main CYP in human platelets which synthesizes 20-HETE.[9] In addition, CYP4A11 expression was reported to be increased in endothelial dysfunction, in hypertensive animal models and in doxorubicin induced cardiotoxity.[12, 13] Furthermore, genetic polymorphism on *CYP4A11* gene was associated with cardiovascular diseases such as hypertension.[14] The increased CYP4A11expression in the cardiovascular toxicity was accompanied with increased 20-HETE levels.[12] In this study, it is found that smokers had higher platelet CYP4A11protein expression which may play a role in the cardiovascular complications induced by smoking.

The 20-HETE is peroxisome proliferator activator-alpha receptor agonist (PPAR-∝).[15] The binding sites for PPAR-∝ were identified on the promoter region of *CYP4A11* gene and it is reported to affect the expression of CYP4A11.[16] It might indicate that elevated 20-HETE induced by smoking play a role in the induction of CYP4A11 expression.

The protein content of the anucleated platelets comes mainly from megakaryocyte in the bone marrow.[17] Induced CYP4A11in platelet may occur in megakaryocyte in the bone marrow, or there are other molecular mechanisms of CYP4A11 induction in platelets, which need further investigations.

The 20-HETE is produced through CYP450 metabolism of arachidonic acid. It is a hypertensive chemical and increases platelet aggregation.[7] Elevated plasma 20-HETE levels was observed among human cardiovascular patients.[18] In this study, the results showed that 20-HETE levels were higher in clinical plasma and platelet samples from smokers. Platelets are not the only cells which produce 20-HETE. It is reported that human endothelial cells and leukocytes, liver and kidney have the capacity to produce 20-HETE.[12, 19] Therefore, the increased 20-HETE levels found in the plasma of smokers may also come from different 20-HETE synthesizing cells and tissues. However, increased production of 20-HETE in smoker platelets might contribute, at least in part, in the elevated plasma 20-HETE levels. Further studies are needed to investigate the effect of cigarette smoking on 20-HETE production among other tissues including the liver and kidney.

Cigarette smoking has been reported to affect on the response of ant platelet drugs. It is demonstrated that cigarette smoking decreased the response of aspirin (acetylsalicylic acid) in patients with ischemic heart disease.[20] It is also found that clopidogrel, which is platelet ADP receptor antagonist, was more effective than aspirin in inhibiting platelet aggregation among smokers.[21] Interestingly, 20-HETE was reported to activate platelet aggregation through ADP dependent mechanism.[7] This might indicate that elevated 20-HETE in smoker platelets plays, at least in part, a role in alteration of antiplatelet drug response. It is

recommended to investigate the effect of 20-HETE synthesis inhibitors on the response of antiplatelet drugs.

CONCLUSION

In conclusion, the present study showed that cigarette smoking increased the 20-HETE levels in human platelet and plasma which was correlated with increased protein expression of CYP4A11 in platelets. This study may increase our understanding of the influence of cigarette smoking on platelet activity, antiplatelet response and in the cardiovascular diseases.

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