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CIGARETTE SMOKING STIMULATES LIPOXYGENASE BUT NOT CYCLOOXYGENASE PATHWAY IN PLATELETS

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Summary: Male rats were exposed to freshly generated cigarette smoke once daily for 4 to 8 weeks. Inhalation of smoke was verified by elevated level of carboxyhemoglobin. Arachidonate metabolism through lipoxygenase and cyclooxygenase pathways in platelets was determined. Cigarette smoking increased 12-lipoxygenase activity significantly without affecting the cyclooxygenase pathway. In view of platelet-leukocyte interactions and potent chemotactic activity of 12-HETE for aortic smooth muscle cell migration, increased 12-lipoxygenase activity may predispose individuals to atherosclerosis, thromboembolism and emphysema commonly found in smokers.

Cigarette smoking is known to be a major risk factor for the development of a variety of cardiovascular diseases including myocardial infarction, thromboembolism and atherosclerosis (1). The underlying mechanisms by which cigarette smoking contributes to these disorders are yet to be defined. Platelets have been implicated in a wide variety of physiological and pathological processes (2). Their role in the primary arrest of bleeding is well established. However, the properties of adhesion, aggregation, and secretion that make the platelets useful in homeostasis also permit them to be deposited as thrombi in blood vessels and on heart valves. In addition, products that are stored or synthesized and released during platelet activation may mediate a variety of biological processes such as vascular permeability, smooth muscle proliferation, atherogenesis and immune reactions.

Among the products synthesized by platelets that show intimate relevance to vascular homeostasis are metabolites of arachidonic acid. Two separate pathways are known for the metabolism of arachidonic acid in platelets. The cyclooxygenase pathway is initiated by the synthesis of prostaglandin endoperoxides catalyzed by fatty acid cyclooxygenase followed by

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Abbreviations: 12-HETE, 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid; 12-HPETE, 12(S)-hydroperoxy-5,8,10,14-eicosatetraenoic acid; LTB_4, leukotriene B_4; TXA_2, thromboxane A_2; TXB_2, thromboxane B_2.

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the production of vasoconstrictive and pro-aggregatory TXA_2 which is readily hydrolyzed to stable TXB_2 (3), whereas lipoxygenase pathway is preceded by the synthesis of 12-HPETE catalyzed by 12-lipoxygenase and then the formation of 12-HETE (4). Both 12-HPETE and 12-HETE were previously known to be chemotactic and chemokinetic for leukocytes (5). Very recently, these lipoxygenase products were found to be extremely potent agents to induce migration of aortic smooth muscle cells (6). Their possible role in atherogenesis is strongly implicated. We are interested if cigarette smoking may alter the metabolism of either one of the two pathways or both in platelets. In the present study, we report that cigarette smoking stimulates specifically 12-lipoxygenase but not cyclooxygenase pathway in rat platelets.

MATERIALS AND METHODS

Materials: Arachidonic acid, glutathione (GSH) and thrombin (from human plasma, 3,000 NIH units per mg protein) were purchased from Sigma Chemical Co., St. Louis, MO. TXB_2 was obtained from the Upjohn Company, Kalamazoo, MI. 12-HETE was synthesized from arachidonic acid by human platelet suspension in the presence of indomethacin and GSH as described by McGuire et al. (7). Soluble calf skin collagen was supplied by Worthington Biochemical Corp., Freehold, NJ. [1-^{14}C]Arachidonic acid was purchased from Amersham, Arlington Heights, IL.

Smoke inhalation: Rats were divided into two groups, shams and smoke exposed, following a 7-day acclimatization period. Smoked animals were exposed to fresh whole smoke for 10 minutes daily, 7 times a week for 4 and 8 weeks. Smoke exposure was accomplished by placing unanesthetized rats in a flexible restrainer allowing the nose to protrude into a moving column of air spiked every minute with fresh smoke generated from a University of Kentucky 2R1 reference cigarette. The bodies of the animals were never exposed to smoke. Sham animals were handled exactly as the smoked animals, but the moving air column contained only room air passed through the smoke generating equipment. Separate restrainers and smoke generators were used for the smoke and sham groups to prevent exposure of the shams to smoke materials deposited on the equipment. Beginning the first week of smoke exposure, 0.2 ml of blood was sampled randomly from the retro-orbital sinus of animals in each group immediately after smoke exposure and the % carboxyhemoglobin determined using a CO-oximeter (IL282, Instrumentation Laboratory Incorported, Lexington, MA.).

Preparation of platelets: Twenty-four hours after the last smoke exposure animals were anesthetized by light ether and blood (about 9 ml) was drawn from the abdominal aorta into a 10 ml syringe containing 1 ml of 3.8% sodium citrate. Citrated blood was first centrifuged at 200 xg for 10 min to obtain platelet rich plasma which is further centrifuged at 1000 xg for 10 min to precipitate platelets. Platelets are then suspended in Ca^{++} and Mg^{++} free phosphate buffered saline containing 5.5 mM glucose. Cell numbers were determined by Coulter counter.

Preparation of cytosolic fraction from platelets: Cytosolic fraction from platelet was prepared as described previously (8). Briefly, platelet pellets were suspended in 0.05 M Tris-HCl, pH 7.5, and freeze-thawed three times. The suspension was centrifuged at 105,000 xg for 60 min. The supernatant designated as cytosolic fraction was used for 12-lipoxygenase assay.

Assay of thromboxane biosynthesis in platelets: One ml of platelet suspension (6.5×10^8) incubated at 37°C was challenged with arachidonic acid

(3 μg/ml) or thrombin (1 U/ml) or collagen (100 μg/ml) for 2, 10 or 20 min respectively. The reaction mixture was acidified with 1 N HCl and then neutralized with 1 M Tris base before an aliquot was diluted for radioimmunoassay of TXB_2.

Assay of 12-lipoxygenase: The assay was done as described previously with some modifications (8). The reaction mixture contained: [1-^{14}C]arachidonic acid (0.05 μCi, 0.82 nmol); GSH, 1 mM; and cytosolic fraction in a final volume of 1 ml of 0.05 M Tris-HCl, pH 7.5. The reaction was performed at 37°C for 2 min and terminated by acidification to pH 3.0. The reaction mixture was extracted with 5 ml of ethylacetate. The organic phase was evaporated to dryness under a stream of nitrogen. The residue taken up in ethanol was applied to a silica gel G plate. The plate was developed in a solvent system of petroleum ether/ethyl ether/acetic acid (50:50:1). The substrate and products were localized by autoradiography, and scraped separately into scintillation vials and the radioactivity was determined by liquid scintillation counting.

Radioimmunoassay of TXB_2: Radioimmunoassay was carried out essentially as described previously by us (9).

Protein Determinations: The method of Lowry et al. (10) was employed using bovine serum albumin as a standard.

RESULTS

Body weights and carboxyhemoglobin content after 4 and 8 weeks of cigarette smoke exposure are shown in Table I. Smoked rats appeared to gain less weight than shams during the whole period of smoke exposure. Evidence that the smoke group actually inhaled cigarette smoke is demonstrated by the significant increase in percent carboxyhemoglobin over shams immediately after a smoke exposure session.

When 12-lipoxygenase was assayed in the presence of GSH, smoke group showed a significant increase in activity as compared to shams following 4 and 8 weeks of cigarette smoke exposure as shown in Fig. 1. However, when platelets were incubated with exogenous arachidonate, production of TXB_2 as measured by radioimmunoassay was not significantly different between sham and smoke groups as shown in Table II. Furthermore, when plate-

TABLE I. Body Weight and Carboxyhemoglobin Content After 4 and 8 Weeks of Smoke Exposure

Group	0 Week	4 Weeks		8 Weeks	
	Wt (gm)[a]	Wt (gm)	COHb (%)[b]	Wt (gm)	COHb (%)
Sham	290 ± 2	314 ± 4	1.1	347 ± 9	0.5
Smoke	293 ± 2	299 ± 2	6.2	322 ± 6	8.5

[a]Values for body weight represent the mean ± S.E.M., n = 8.

[b]Values for COHb represent the mean % from two to three animals of that group.

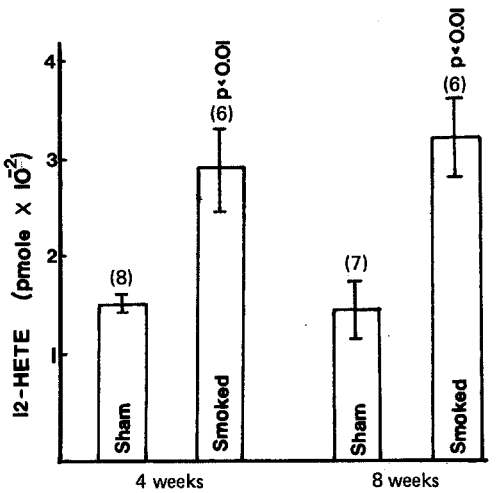

Fig. 1. Effect of cigarette smoke exposure on platelet 12-lipoxygenase activity.

Rats were exposed to cigarette smoke for 4 and 8 weeks. Cytosolic fraction of platelets was assayed for 12-lipoxygenase activity as described in Materials and Methods.

lets were stimulated either with thrombin or collagen to release endogenous arachidonate, subsequent synthesis of TXB_2 indicated no significant difference between the two groups as shown in the same Table.

DISCUSSION

Previously we reported that cytosolic fraction of platelets was able to metabolize arachidonic acid to several polar products in addition to 12-HPETE and 12-HETE (8). The more polar products and 12-HPETE were con-

TABLE II. Effect of Cigarette Smoke on Thromboxane Synthesis in Platelets

Length of Smoke Exposure	Stimulators	TXB_2 (pmole/6.5 x 10^8 platelets)		p-value
		Sham	Smoked	
4 Weeks	Arachidonate, 3 µg/ml	406 ± 38	436 ± 35	NS
	Thrombin, 1 U/ml	65 ± 12	55 ± 9	NS
	2 U/ml	135 ± 16	157 ± 18	NS
	Collagen, 100 µg/ml	41 ± 4	39 ± 4	NS
8 Weeks	Arachidonate, 3 µg/ml	1070 ± 51	1177 ± 50	NS
	Thrombin, 1 U/ml	159 ± 12	176 ± 6	NS
	2 U/ml	252 ± 9	289 ± 11	NS
	Collagen, 100 µg/ml	133 ± 35	111 ± 12	NS

[a] Each value is the mean ± S.E.M., n =8.

[b] Incubations of platelets with arachidonic acid, thrombin and collagen were performed for 2, 10 and 20 min, respectively.

[c] NS = not significant.

verged into a single 12-HETE product when assayed in the presence of GSH. This finding indicates that 12-HPETE can be effectively reduced to 12-HETE by a GSH-dependent peroxidase if supplemented with GSH before it is converted to other polar products. Therefore, 12-lipoxygenase was routinely assayed in the presence of GSH to simplify the system. The increase of 12-lipoxygenase activity found in smoked group was not solely due to a possible increase in GSH dependent peroxidase activity which could more effectively remove the product, 12-HPETE, as it was formed. In fact, when conversion of arachidonate by cytosolic fraction of platelets from smoked group was carried out in the absence of GSH, formation of 12-HPETE, 12-HETE and more polar products was generally increased (data not shown) indicating that it was indeed 12-lipoxygenase being stimulated.

Mechanism that leads to increased 12-lipoxygenase activity by cigarette smoking is presently unknown. Increased 12-HETE synthesis has been described in psoriasis (11). We have reported that estradiol treatment in rats stimulates 12-lipoxygenase activity in platelets (12) and cyclooxygenase activity in aortic smooth muscle cells (13). Increased cyclooxygenase activity was shown to be due to induction of the enzyme protein (14). Whether cigarette smoking induces synthesis of 12-lipoxygenase remains to be determined.

Conversion of arachidonate to TXB_2 through cyclooxygenase pathway by platelets did not show any difference between sham and smoked groups suggesting that neither cyclooxygenase nor thromboxane synthetase activities were altered by smoking. Similar conclusion can be reached using thrombin or collagen as an agonist to induce endogenous release of arachidonate and subsequent thromboxane synthesis. Furthermore, results from thrombin or collagen stimulation also suggest that receptor responsiveness to either agonists were not altered by smoking. Insignificant change of thromboxane synthesis in platelets has been reported for human smokers (15) and animals exposed to smoke (16). Therefore, a slightly increased plasma level of TXB_2 in human smokers reported by Mehta and Mehta (15) may be derived from sources other than platelets. Lubawy *et al.* (16) recently demonstrated that lung microsomes from smoke exposed rats did synthesize more TXB_2 than those from shams. It is possible that cyclooxygenase pathway in lung cells notably fibroblasts which have very active system synthesizing thromboxane (17) may be stimulated following cigarette smoke exposure. In view of the pro-aggregatory and vasoconstrictive nature of thromboxane, the consequence of increased levels of thromboxane may predispose individuals to cardiovascular disorders as proposed by Moncada and Vane (18).

The significance of stimulation of platelet 12-lipoxygenase by cigarette smoking can be several fold. Firstly, 12-HETE is an extremely potent agent to stimulate aortic smooth muscle cell migration (6). Migration of

these cells from media to intima is considered to be the first event following endothelial injury and platelet adhesion. Upon reaching the intima, they proliferate, incorporate lipids, biosynthesize and release connective tissue proteins. Repetition of these sequalae causes the formation of atheromatous plaques (19). Significant stimulation of platelet 12-lipoxygenase pathway following cigarette smoke exposure may predispose individuals to atherosclerosis commonly found in smokers. Secondly, 12-HPETE, the immediate product of 12-lipoxygenase, modulates neutrophil functions (20). This substance is more potent than 12-HETE in evoking neutrophil chemotactic and chemokinetic responses, enhancing the expression of C_{3b} receptors on neutrophils and augmenting significantly the release of lysosomal enzyme from neutrophils initiated by chemotactic fragments of C_5. Very recently, Maclouf et al. (21) reported that 12-HPETE stimulates leukotriene synthesis in leukocytes. Modulation of neutrophil functions by 12-HPETE reported previously may in part be mediated by leukotrienes notably LTB_4 produced. The stimulatory effect of platelet lipoxygenase product on the synthesis of leukotrienes in neutrophils supports the concept of the possible involvement of platelet-leukocyte interactions in some pathophysiological states. In this regard, one may speculate that abnormal homeostasis may partially account for the pathogenesis of smoking associated disorders such as emphysema and coronary heart disease. Thirdly, 12-HPETE like other hydroperoxy fatty acids is a potent inhibitor of prostacyclin synthetase. Increased synthesis of this product following smoking may distort thromboxane/prostacyclin balanced action in blood vessels and may contribute to the development of thromboembolism and atherosclerosis commonly found in smokers.

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