Effect of a Cigarette Smoke Extract on the Metabolism of the Proinflammatory Autacoid, Platelet-Activating Factor

Shuichi Miyaura, Hideshi Eguchi, and John M. Johnston

Cigarette smoking is associated with an increased incidence of a number of diseases. Minimal information is available at the molecular level concerning the mechanism of action of cigarette smoke. Platelet-activating factor (PAF) is one of the most potent proinflammatory agents described. PAF concentration may be regulated by the degradation of PAF as catalyzed by the plasma enzyme, PAF acetylhydrolase (PAF-AH). This enzyme is associated with the lipoprotein fraction. The exposure of low density lipoprotein to a cigarette smoke extract (CSE) has been shown to alter the charge of low density lipoprotein and its uptake by macrophages. The activity of PAF-AH in the lipoprotein fraction has been assayed after exposure to CSE. The activity of PAF-AH was inhibited by the CSE in a dose-dependent manner. The inhibition of PAF-AH by the CSE was not altered by superoxide dismutase or catalase addition. Sulphydryl compounds prevented and reversed the inhibition of PAF-AH caused by CSE. The inhibitor present in CSE was not nicotine, its major metabolite, (−)-cotinine, or several compounds known to be present in the extract. The charge alteration reaction(s) and PAF-AH inhibition appear to be localized at different sites of the lipoprotein molecule. The observed inhibition may account for the increase in the plasma PAF concentration that is known to occur in smokers. The increase of PAF may contribute to the increased incidence of cardiovascular and lung diseases known to be present in smokers. (Circulation Research 1992;70:341–347)

Cigarette smoking is a major risk factor in the development of a number of diseases, including cardiovascular diseases.1 The precise biochemical mechanisms by which cigarette smoke causes the various adverse effects are largely unknown. It has been suggested that platelet activation is one of the mechanisms by which the injury associated with the vascular diseases is initiated and/or propagated.2,3 1-O-Alkyl-2-acetyl-sn-glycero-3-phosphocholine (platelet-activating factor [PAF]) is one of the more potent proinflammatory agents known.4 PAF activity was first observed in the middle 1960s as a factor associated with histamine release from leukocytes.5–7 Subsequently, the name was coined to describe a factor released from stimulated basophils.8 The structure was elucidated by Demo-

poulos et al9 and Benveniste et al10 in relation to its proinflammatory action and by Blank et al,11 who identified PAF as a powerful hypotensive polar lipid of renal origin. PAF has recently been implicated in the pathophysiology of a number of human diseases, including asthma, endotoxic shock, ischemic diseases, diabetes mellitus, and hypertension (for reviews see References 4, 12, and 13). In addition, it is increasingly apparent that PAF has a fundamental role in a number of physiological processes, in particular those associated with reproductive biology.14,15 Recently, the PAF receptor from guinea pig lung has been cloned by Honda et al.16 It is interesting that the first lipid mediator receptor to be cloned was that of PAF and was of lung origin.

The relation of PAF to cigarette smoking was first suggested by the observation of Imaizumi,17 who reported that a higher plasma PAF concentration was found in smokers compared with nonsmokers. Previously, Yokode et al,18 in studies related to accumulation of low density lipoprotein (LDL) cholesterol esters by macrophages, reported that the treatment of LDL with an aqueous extract of cigarette smoke markedly stimulated its uptake. Farr and colleagues19,20 had previously reported that an enzyme was present in the LDL-enriched fraction of

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Received April 2, 1991; accepted October 16, 1991.
human plasma that inactivated PAF by cleavage of the acetyl residue in the sn-2 position. The enzyme was further characterized by Blank et al., who termed the enzyme PAF-acetylhydrolase (PAF-AH, EC 3.1.1.48). These investigators also differentiated a plasma form of the enzyme from an intracellular isoyme. Subsequently, it has been reported that the plasma PAF-AH activity was increased in the spontaneously hypertensive rat, in untreated hypertensive white males, in the stressed lizard, in atherosclerotic patients, and in patients after an ischemic stroke and was decreased in asthmatic children. We reported that the PAF-AH activity was decreased in maternal plasma during the latter stages of pregnancy in the rabbit and human and was rapidly increased in the newborn rabbit. It has been suggested that the enzyme plays an important role in regulating the concentration of PAF in plasma and various tissues. Stafforini et al. have purified and further characterized the plasma enzyme. The role of this enzyme in macrophage development has recently been described, and it has been reported that the enzyme secreted by macrophages is the plasma type. The potential role of PAF-AH in a number of human diseases, especially with relation to cardiovascular problems, is discussed in an excellent review.

Based on these studies, the present investigation was undertaken. In this investigation we have examined the effect of a cigarette smoke extract (CSE) on the activity of human plasma PAF-AH in an attempt to explain how cigarette smoke increases the PAF concentration in the plasma and the relation of these findings to the known inflammatory and cardiovascular responses associated with cigarette smoking.

Materials and Methods

Chemicals

1-O-Hexadecyl-2-[3H]acyetyl-sn-glycero-3-phosphocholine ([3H]PAF, 10 Ci/mmol, 1 Ci=37 GBq) was purchased from New England Nuclear, Boston, Mass. Nonradiolabeled PAF was obtained from Avanti Polar Lipids, Pelham, Ala. Bovine serum albumin (fatty acid poor), superoxide dismutase (SOD, 3,000 units/0.9 mg protein), catalase (58,000 units/mg protein), reduced glutathione (GSH), 2-mercaptopethanol, DL-dithiothreitol (DTT), L-cysteine, L-ascorbic acid, p-hydroxymercuribenzoic acid (p-MBA), N-ethylmaleimide (NEM), iodoacetamide, and 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co., St. Louis, Mo. In our initial studies, cigarettes were purchased from commercial sources. Cigarettes without filters were used unless stated otherwise. Standard filtered (1R4F) and nonfiltered (2R1) cigarettes were also obtained from the University of Kentucky Research Foundation, Lexington, Ky.

Preparation of CSE

CSE was prepared in a manner similar to that described by Yokode et al. Briefly, smoke from one cigarette was passed through 1 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl by aspiration at constant vacuum that was slightly below atmospheric pressure. Four to 5 minutes was required to consume one cigarette. The CSE was filtered through a membrane filter (0.45 µm) (Gelman Sciences Inc., Ann Arbor, Mich.) and then adjusted to pH 7.4 with NaOH (1N).

Treatment of Human Plasma With CSE

Human plasma was obtained from blood that was drawn in EDTA-containing tubes and centrifuged at 2,500g for 15 minutes. One hundred microliters of plasma was added to 1.0 ml phosphate buffer (pH 7.4, 10 mM) containing NaCl (0.15 M) and various quantities of the CSE prepared from one cigarette. The CSE and control tubes (without CSE) were incubated at 37°C for various time periods, and PAF-AH activity was determined by the assay described below.

PAF-AH Activity Assay

The activity of PAF-AH in plasma was assayed according to the method of Miwa et al. with minor modifications. The assay mixture contained Tris-HCl (30 mM, pH 7.5), [3H]PAF (50 µM, 4.0 µCi/µmol) in bovine serum albumin (0.13% final concentration), and various concentrations of plasma in a total volume of 0.5 ml. The assay mixtures and controls (without enzyme) were incubated for 20 minutes at 37°C. The reaction was terminated by addition of 0.5 ml trichloroacetic acid (14%), and the tubes were centrifuged at 3,000g for 5 minutes at 4°C. A 0.1-ml aliquot of the supernatant fraction was mixed with 5 ml scintillation cocktail (Budget-Solve, Research Products International Corp., Mt. Prospect, Ill.), and the trichloroacetic acid soluble [3H]acetate released from [3H]PAF was assayed by liquid scintillation spectroscopy. A standard plasma of known activity was assayed with each group of samples, and all values were corrected in accordance with this standard. All assays of each experiment were performed in triplicate. All experiments were carried out at least twice and in most cases five times, with the exception of the effect of sulfhydryl (SH) enzyme inhibitors on the PAF-AH activity of the LDL and high density lipoprotein fractions shown in Table 1. The results presented in each figure are mean±SD of a repre-

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<th>TABLE 1. Effect of Sulfhydryl Enzyme Inhibitors on Platelet-Activating Factor Acetylhydrolase Activity</th>
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<td>Residual activity (%)</td>
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Values are mean±SD and are expressed as the residual activity compared with control (without inhibitors). p-MBA, p-hydroxymercuribenzoic acid; NEM, N-ethylmaleimide; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid); LDL, low density lipoprotein; HDL, high density lipoprotein. Either whole plasma or a mixture of LDL plus HDL was incubated at 37°C for 4 hours in the presence of various sulfhydryl enzyme inhibitors at the concentration of 10 mM.
 grafics showing effects of cigarette smoke extract (CS) on platelet-activating factor-acetylhydrolase activity in human plasma. Panel A: Whole plasma (100 µl) was incubated at 37°C with (●) or without (○) 1,000 µl CS. Platelet-activating factor-acetylhydrolase activity was assayed at various time periods. Panel B: Whole plasma (100 µl) was incubated at 37°C for 3 (○) or 6 (●) hours with various amounts of CS. Values were expressed as the percent residual activity (mean ± SD) compared with the original activity before treatment. Significant (p<0.01) inhibition was observed at concentrations of the CS as low as 100 µl.

Results

Effect of CSE on PAF-AH Activity in Human Plasma

Incubation of human plasma with CSE resulted in marked inhibition of plasma PAF-AH activity (Figure 1A). As shown in the figure, PAF-AH activity was inhibited by addition of 1,000 µl CSE to values < 5% of the original activity. On the other hand, 98% of the activity remained after a 24-hour incubation at 37°C in the absence of CSE. Thus, the plasma PAF-AH is quite stable under these conditions. Figure 1B illustrates the effect of various concentrations of CSE. As can be seen, 250 µl CSE inhibited ~50% of PAF-AH activity when incubated at 37°C for 3 hours. The extract turned to a darker yellow color upon storage, and the inhibitory activity was decreased by 50% when stored at room temperature for 4 days (data not shown). Therefore, the CSE was prepared freshly before each experiment.

Three different brands of cigarettes with filters were examined. Approximately a 50% inhibition in plasma PAF-AH activity was observed when 1,000 µl of the CSE prepared from filtered cigarettes was used (data not shown). A 90% inhibition was obtained when the nonfiltered cigarettes were used. We have recently compared these findings with the inhibition observed for both filtered (IR4F) and nonfiltered (2R1) research cigarettes (University of Kentucky Research Foundation) and obtained similar results as we observed with the commercial cigarettes. When the CSE was treated with charcoal (50 mg charcoal per cigarette), its inhibitory effects were decreased by 80% (Figure 2).

Properties of Inhibition of PAF-AH Activity With CSE

It has been reported that several active oxygen species, such as superoxide anion or hydrogen peroxide, are produced in cigarette smoke and that these species are active in modifying various lipoproteins.18,33 It has been demonstrated that the superoxide anion reacts with LDL to form a more negatively charged species.18 This modified LDL is now readily taken up by macrophages by the scavenger pathway to form foam cells. As previously discussed, PAF-AH is known to be present in the plasma lipoprotein fraction. Therefore, the possible protective effect of SOD and catalase on the activity of plasma PAF-AH in the presence of CSE was examined (Figure 3). No change in the inhibition of PAF-AH activity by CSE was observed in the presence of various concentrations of the enzymes. Likewise, EDTA had no effect on the...
inhibition of PAF-AH by CSE, suggesting that redox-active metal ions such as Cu²⁺ or Fe³⁺, which are known to cause the peroxidation of lipoproteins,¹⁸ are not involved in the inhibition. In contrast, the incubation in the presence of increasing concentrations of GSH resulted in the protection of PAF-AH activity against the inhibition caused by CSE (Figure 3).

To further clarify the beneficial effect of GSH on the inhibition of PAF-AH activity by CSE, we examined other SH-containing compounds, including 2-mercaptoethanol, DTT, and l-cysteine. All these –SH-containing compounds protected against the inhibition caused by the CSE on PAF-AH activity (Figure 4). We also examined whether or not other chemicals that are capable of undergoing a redox reaction, such as ascorbate, protected PAF-AH activity in the presence of the CSE. Ascorbate, when added at a concentration of 5–20 mM, did not protect PAF-AH from inhibition by CSE.

In consideration of the effect of the –SH-containing compounds, we examined the possibility that PAF-AH contains active –SH groups. Human plasma was incubated at 37°C for up to 4 hours in the presence of a number of –SH reactive chemicals including p-MBA, NEM, iodoacetamide, or DTNB at concentrations up to 10 mM. As shown in Table 1, in no instance was there a significant inhibition of PAF-AH. Similar results were obtained when the purified lipoprotein fraction from human plasma was used as the enzyme source.

Reversibility of Inhibition of PAF-AH Activity With CSE

Figure 5 illustrates the inhibition of PAF-AH activity by CSE and its partial reversibility by the addition of DTT. Incubations were carried out for up to 4 or 8 hours with 200 µl CSE. A 55% inhibition of PAF-AH activity was observed (solid bar). When the enzyme was further incubated for 4 hours in the presence of 10 mM DTT, only a 35% inhibition was found (hatched bar). When the DTT was added simultaneously with the CSE, only a 10–15% inhibition was observed. It was also found that DTT alone...
did not activate PAF-AH activity (data not shown). Based on these observations, it is suggested that the activity of PAF-AH is partially reactivated by the addition of DTT (open bar versus hatched bar) and is protected by the addition of DTT at the beginning of the incubation (crosshatched bar).

Effect of Constituents of Cigarette Smoke

The chemical composition of cigarette smoke has been reported.34 We have incubated various classes of compounds known to be present in both the gaseous and particulate fractions with the PAF-AH present in plasma in an attempt to identify the chemical nature of the inhibitor. The amount of the various compounds used was 20 times that reported to be present in one cigarette. The compounds incubated were nicotine (438 mM), cotinine (438 mM), propylene glycol (200 mM), palmitic acid (240 mM), catechol (100 mM), hydroquinone (240 mM), furfural (230 mM), indole (5.7 mM), acetaldehyde (910 mM), acrolein (55 mM), acetonitrile (152 mM), hydrazine (42 μM), and the heavy metals nickel (75 μM) and cadmium (40 μM). None of the compounds used resulted in an inhibition even at these extremely high concentrations.

Discussion

In the reported experiments we have demonstrated the presence of inhibitor(s) of PAF-AH activity in a CSE. This finding may explain the increase of PAF, a potent proinflammatory agent, in the plasma of smokers.17 It has become apparent that the PAF concentration is regulated not only by its biosynthesis but also by degradation. The latter reaction is catalyzed by PAF-AH in which the biologically active PAF is converted into the inactive lysoPAF. Two isozymes of PAF-AH are known; one is an intracellular enzyme and the other is the plasma enzyme. Farr and colleagues19,20 were the first to characterize the plasma enzyme and demonstrated that the plasma form of the enzyme was associated with the lipoprotein fraction and was Ca2+ independent and that the preferred substrate for the enzyme contained a short acyl group in the sn-2 position. The different properties and molecular weights of the two isozymes of PAF-AH have been reported.22 The presence of PAF-AH in the LDL fraction prompted us to examine the role of a CSE on the activity of this enzyme, since the inhibition has also been demonstrated when purified lipoprotein fractions were used. Yokode et al18 demonstrated that treatment of LDL with a CSE alters the lipoprotein and results in a more anionic species. The more negatively charged species can then be taken up by macrophages by the scavenger pathway. Thus, macrophages are transformed into foam cells at an increased rate. This group of investigators also suggested that the modification of the LDL by cigarette smoke was, at least in part, mediated by a superoxide anion. Protection against conversion of LDL into a more anionic species was demonstrated by the presence of SOD.18

In contrast, the inhibition of PAF-AH by the CSE is not altered by the presence of SOD or catalase (Figure 3). Therefore, the inhibition by the CSE of the PAF-AH activity and the alteration of the charge of LDL occur at the same site and by different mechanisms. Most probably, the inhibition of PAF-AH activity and the modification of the charge of the LDL fraction is caused by a different component of the CSE. However, a relation between the oxidation of LDL and PAF-AH activity has recently been documented by the observation that oxidized species of phosphatidylcholines are substrates for PAF-AH.35,36 The presence of such oxidized phospholipids in plasma has also been described.37 The peroxidation of LDL has also been reported to be stimulated by Cu2+.38 The activity of PAF-AH, however, was not altered by the presence of heavy metal chelators such as EDTA (Figure 3). A variety of SH-containing compounds including GSH, mercaptoethanol, DTT, and cysteine protected the PAF-AH activity from inhibition by the CSE. The GSH concentration in plasma is \( -1 \mu M \),39 which is orders of magnitude lower in concentration than that used in the protective and reversal studies reported in this article. The biochemical mechanism by which SH compounds protect and reverse the inhibition of PAF-AH by the CSE may be of minimal importance. We are presently investigating the nature of the inhibition by focusing on the determination of the component in cigarette smoke responsible for the inhibition. There are almost 3,000 components present in cigarette smoke.34 We have used a representative compound from most of the known groups of compounds present in the particulate and gaseous phases of cigarette smoke to evaluate their effect on PAF-AH activity. The amount of each compound incubated with 10 μl serum was 20 times that found in one nonfiltered cigarette. None of the tested compounds inhibited PAF-AH.

The observed inhibition of PAF-AH by a CSE would explain the reported increase in the plasma PAF concentration of smokers.17 Since PAF is known to be one of the most potent proinflammatory agents described,12,13 its increase may play an important role in the increased incidence of certain cardiovascular diseases in smokers.40 In addition, the inhibition of PAF-AH caused by smoking may also contribute to the development of the emphysema associated with smoking. The lower blood pressure found in smokers versus nonsmokers might be explained by the increase in the hypotensive PAF.41

It is established that women on oral contraceptives are at increased risk of thrombosis.42 We have previously demonstrated that the activity of plasma PAF-AH is decreased by estrogens.43 The changes in PAF-AH activity by CSE may also explain why the combination of estrogen intake and smoking leads to at least an additive risk of a thrombotic episode.42 Although the precise chemical nature of the inhibitor in CSE is yet to be defined, the relation of PAF-AH to cigarette smoking provides a direct bio-
chemical link to many of the effects of cigarette smoke on various pathological processes. The determination of the chemical nature and the mechanism of action of the compound in CSE that inhibits PAF-AH activity would be of potential significance.

Acknowledgments

The authors appreciate the editorial assistance of Dr. Rene Frenkel and Dolly Tutton in the preparation of this manuscript.

References


KEY WORDS • Cigarette smoke extract • platelet-activating factor • lipoproteins • hypertension
Effect of a cigarette smoke extract on the metabolism of the proinflammatory autacoid, platelet-activating factor.
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Circ Res. 1992;70:341-347
doi: 10.1161/01.RES.70.2.341

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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