Cigarette Smoke Extract Inhibits Platelet Aggregation by Suppressing Cyclooxygenase Activity

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Introduction

Cigarette smoking has been recognized as a risk factor for various diseases including cancers, cardiovascular diseases, and respiratory diseases. In the development of cardiovascular diseases, platelets are known to play an important role by both aggregating themselves and releasing various bioactive substances such as growth factors, lysophospholipids, and chemokines. Accordingly, a large number of studies have been performed to investigate the effect of cigarette smoke on platelet function.

Abstract

The results of studies that were performed to determine whether cigarette smoking affects platelet function have been controversial, and the effects of nicotine- and tar-free cigarette smoke extract (CSE) on platelet function remain to be determined. The aim of this study was to determine the effect of CSE on platelet aggregation and to clarify the mechanism by which CSE affects platelet function. CSE inhibited murine platelet aggregation induced by 9,11-dideoxy-9α,11α-methanoepoxy-prosta-5Z,13E-dien-1-oic acid (U-46619), a thromboxane (TX) A2 receptor agonist, and that induced by collagen with respective IC50 values of 1.05 ± 0.14% and 1.34 ± 0.19%. A similar inhibitory action of CSE was also observed in human platelets. CSE inhibited arachidonic acid–induced TXA2 production in murine platelets with an IC50 value of 7.32 ± 2.00%. Accordingly, the inhibitory effect of CSE on collagen-induced aggregation was significantly blunted in platelets lacking the TXA2 receptor compared with the inhibitory effect in control platelets. In contrast, the antiplatelet effects of CSE in platelets lacking each inhibitory prostanoid receptor, prostaglandin (PG) I2 receptor and PGE2 receptor subtypes EP2 and EP4, were not significantly different from the effects in respective control platelets. Among the enzymes responsible for TXA2 production in platelets, the activity of cyclooxygenase (COX)-1 was inhibited by CSE with an IC50 value of 1.07 ± 0.15% in an uncompetitive manner. In contrast, the activity of TX synthase was enhanced by CSE. The results indicate that CSE inhibits COX-1 activity and thereby decreases TXA2 production in platelets, leading to inhibition of platelet aggregation.

Keywords

► cigarette smoke extract
► platelets
► thromboxane A2
► cyclooxygenase

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platelets. Cigarette smoking potentiates platelet aggregation induced by various stimulants,\(^7,8\) and platelet activity was increased by both the mainstream and sidestream of cigarette smoke.\(^9\) Furthermore, activated platelets from smokers showed spontaneous aggregation\(^10\) and contributed to the degree of platelet aggregation in response to collagen was lower in habitual smokers than in nonsmokers.\(^13\) In addition, it was shown that platelet aggregation induced by various stimulants and platelet hemostatic capacity were decreased or unchanged in smokers compared with those in nonsmokers.\(^14\) Thus, the effect of cigarette smoking on platelet function remains to be determined.

Nicotine is the representative bioactive agent contained in cigarette smoke, and the effect of nicotine on platelets has been investigated in some studies. Nicotine potentiates thrombin- or adenosine diphosphate (ADP)-induced platelet aggregation.\(^7,15\) In addition to nicotine, at least 4,800 chemical constituents have been identified in cigarette smoke,\(^16,17\) and some of them have been shown to affect platelet function,\(^18–20\) possibly being one of the reasons why the effect of cigarette smoking on platelets is complicated. Cigarette smoke is a complex aerosol consisting of a particulate phase and a gas phase. Among the constituents of the particulate phase, tar is de...
human platelets were approved by the Asahikawa Medical University Research Ethics Committee.

**Platelet Aggregation Study**

Platelet aggregation was examined by a previously reported method using an aggregometer (PAT-4A, Nihon Kohden, Tokyo, Japan) with a slight modification. Briefly, PRP (200 μL) being stirred at 37°C in a cuvette was preincubated for 5 minutes and then CSE was added to the PRP 1 minute before the addition of U-46619 (a TP agonist), collagen, or ADP. Unstimulated PRP and PPP were set to show 0 and 100% of light transmission, respectively, and a peak of light transmission represents platelet aggregation. U-46619 was added at a concentration to induce murine platelet aggregation of 40 to 55% (2.5 μg/mL) and 45 to 55% (3.5–4.5 μM), respectively. When using PRP prepared from TP−/− mice, the concentration of collagen was increased (2.5–3.5 μg/mL) to induce a degree of aggregation similar to that in PRP prepared from WT mice. For human platelets, U-46619 and collagen were added at concentrations to induce aggregation of 45 to 50% (0.5–0.8 μM) and 45 to 55% (0.5–1.0 μg/mL), respectively.

**Measurement of TXB₂ Content**

Washed platelets (100 μL) were preincubated in the absence of calcium and fibrinogen for 5 minutes at 37°C, and then CSE was added 5 minutes before the addition of 1 μM arachidonic acid or 10 nM PGH₂. After further incubation for 5 minutes at 37°C, the reaction was terminated by the addition of ice-cold 1 N HCl (25 μL), and the platelet suspension was centrifuged at 20,400 g for 10 minutes at 4°C. The supernatant was neutralized with a one-fifth volume of 1 M Tris (pH 10.4), and the content of TXB₂ was measured by using a TXB₂ enzyme immunoassay Kit (Cayman Chemical).

**Measurement of cAMP Production**

Washed platelets (100 μL) were preincubated in the presence of 1 mM IBMX, an inhibitor of phosphodiesterase, for 10 minutes at 37°C, and then CSE was added. After further incubation for 10 minutes at 37°C, the reaction was terminated by the addition of 30% trichloroacetic acid (25 μL), and the platelets were disrupted by sonication. The solution was centrifuged at 20,400 g for 10 minutes at 4°C. The content of cAMP in the supernatant was determined by using a radioimmunoassay kit (Yamasaki Shouyou, Chiba, Japan) after trichloroacetic acid had been extracted three times with water-saturated diethyl ether.

**Measurement of COX Activity**

COX-1 and COX-2 activities were measured using a COX Fluorescent Inhibitor Screening Assay Kit (Cayman Chemical). Briefly, CSE was added to a solution containing ovine COX-1 or human COX-2, and the mixture was incubated for 5 minutes at room temperature. To initiate the reaction, arachidonic acid was added to the mixture, and then the sample was further incubated at room temperature. After 2-minute incubation, the fluorescence was measured with an excitation wavelength of 535 nm and an emission wavelength of 590 nm using a microplate reader (Synergy H1; BioTek, Winooski, Vermont, United States). To determine the manner of the inhibitory action of CSE on COX-1, we examined the effect of CSE (3%) on the Michaelis constant ($K_m$) and the maximum velocity ($V_{max}$) of COX-1 within the Michaelis–Menten kinetics.

**Data Analysis**

All data except those shown in -Fig. 1A are expressed as means ± standard error of the mean (SEM). Statistical comparisons of data were made by repeated two-way analysis of variance. A p-value of < 0.05 was considered statistically significant.

**Results**

CSE Inhibits U-46619- or Collagen-Induced Platelet Aggregation

To determine whether cigarette smoke components other than nicotine and tar affect platelet aggregation, we examined the effect of nicotine- and tar-free CSE on aggregation in platelets prepared from WT mice (WT platelets). At a concentration of 3%, CSE alone did not induce platelet aggregation or platelet shape change (-Fig. 1A, left). Next, we examined whether CSE affects U-46619-induced platelet aggregation. CSE inhibited U-46619-induced platelet aggregation in a concentration-dependent manner with an $IC_{50}$ value of 1.05 ± 0.19% (-Fig. 1A [right], B). CSE also inhibited collagen-induced platelet aggregation in a concentration-dependent manner with an $IC_{50}$ value of 1.34 ± 0.19% (-Fig. 1C). On the other hand, the inhibitory effect of CSE on ADP-induced platelet aggregation was weak, and its inhibition was only 19.1 ± 6.3% of control aggregation at a maximum concentration (-Fig. 1D). CSE also inhibited U-46619- and collagen-induced aggregation of human platelets with potencies similar to those in murine platelets; the $IC_{50}$ values were 2.80 ± 0.37% and 2.65 ± 0.26%, respectively (-Fig. 1E, F).

CSE Inhibits TXA₂ Production in Platelets, and Its Inhibitory Action on Collagen-Induced Aggregation Is Attenuated in Platelets Lacking the TXA₂ Receptor

TXA₂ is known as a potent stimulator of platelets, and it is produced when platelets are activated. Therefore, it works as a central regulator of platelet activation in a positive feedback manner. To determine whether CSE affects TXA₂ production in platelets, we examined the effect of CSE on arachidonic acid-induced TXA₂ production. For assessment of the degree of TXA₂ production, we measured the content of TXB₂, a stable TXA₂ metabolite. To exclude TXA₂ production associated with platelet aggregation, we performed the experiment in a buffer without calcium and fibrinogen, which enable aggregation. Under this condition, CSE inhibited TXA₂ production in a concentration-dependent manner with an $IC_{50}$ value of 7.32 ± 2.00% (-Fig. 2A). To confirm that the reduced production of TXA₂ contributes to the inhibitory action of CSE, we examined the effect of CSE on collagen-induced aggregation in platelets lacking the TXA₂ receptor TP.
Since TP-mediated signaling plays a role in collagen-induced platelet aggregation, the concentration of collagen was increased to induce a degree of aggregation in TP−/− platelets similar to that in WT platelets. In TP−/− platelets, the inhibitory effect of CSE on collagen-induced aggregation was attenuated significantly compared with that in WT platelets; the IC₅₀ values were 3.51 ± 0.13% and 0.92 ± 0.15%, respectively (Fig. 2B). These results indicate that TXA₂-mediated signaling contributes to the inhibitory action of CSE on platelet aggregation.

Prostanoid Receptors Other Than TP Do Not Participate in the Inhibitory Action of CSE on Platelet Aggregation, and CSE Does Not Affect Intraplatelet cAMP Concentration

It is well known that PGI₂ plays a central role opposing TXA₂ in the regulation of platelet function; stimulation of the PGI₂ receptor IP leads to inhibition of platelet aggregation. In addition, we found in a previous study that selective agonists for PGE₂ receptor subtypes EP₂ and EP₄ potently inhibit platelet aggregation. To determine whether these prostanoid receptors contribute to the inhibitory action of CSE on platelet aggregation, we examined the effect of CSE on U-46619-induced aggregation of platelets lacking EP₂ (EP₂−/− platelets), EP₄ (EP₄−/− platelets), or IP (IP−/− platelets). U-46619 induced similar degrees of aggregation in these platelets as well as WT platelets and platelets prepared from F2-WT mice (F2-WT platelets). In both EP₂−/− and IP−/− platelets, the inhibitory effects of CSE on U-46619-induced aggregation were not
significantly different from the inhibitory effect in WT platelets. The IC50 values were 1.35 ± 0.14%, 1.46 ± 0.16%, and 1.14 ± 0.10%, respectively (Fig. 3A). In EP2−/− platelets, the inhibitory effect of CSE was also not significantly different from that in F2-WT platelets. The IC50 values were 1.31 ± 0.15% and 1.42 ± 0.18%, respectively (Fig. 3B). These results indicate that these prostanoid receptors do not participate in the inhibitory action of CSE.

To confirm that CSE does not affect the signaling of the inhibitory prostanoid receptors, IP, EP2, and EP4, we examined whether CSE affects the intraplatelet concentration of cAMP, a second messenger of the inhibitory prostanoid receptors. CSE had no significant effect on the cAMP concentration in washed platelets prepared from WT mice (Fig. 3C), indicating that the signaling of the inhibitory prostanoid receptors does not participate in the inhibitory action of CSE.

**CSE Inhibits Both COX-1 and COX-2 Activities**

Since TXA2 synthesis was suppressed by CSE (Fig. 2A), we next examined whether CSE affects the activity of COX, a rate-limiting enzyme in the biosynthesis of prostanoids. COX has two isoforms, constitutive (COX-1) and inducible (COX-2) isoforms. CSE inhibited both COX-1 and COX-2 activities in a concentration-dependent manner, with respective IC50 values of 1.07 ± 0.15% and 0.80 ± 0.12% (Fig. 4A). To determine further the pattern of the inhibitory action of CSE, we analyzed the kinetics of the inhibitory action on COX-1, which is responsible for TXA2 synthesis in platelets. It has been shown that COX-2 is expressed in only a few platelets prepared from healthy donors.30 CSE decreased Km of COX-1 from 3.37 ± 0.40 to 1.21 ± 0.41 μM and also decreased Vmax from 139.1 ± 6.8 to 44.6 ± 4.6 arbitrary fluorescence units (Fig. 4B), indicating that the pattern of COX-1 inhibition by CSE is uncompetitive.

**CSE Enhances TX Synthase Activity in Platelets**

TX synthase works downstream of COX, converting PGH2 to TXA2. We finally examined whether CSE affects the activity of TX synthase. To assess TX synthase activity, we added PGH2 to the platelet suspension and examined the increase in the content of the stable TXA2 metabolite TXB2 in the medium. In contrast to the effect on COXs, CSE enhanced
platelet aggregation differ from the results of a previous study showing that filtered gas phase extract inhibited ADP-induced aggregation in human and rabbit platelets.\textsuperscript{37} Although the reason of this difference in the effects of CSE on ADP-induced platelet aggregation remains to be clarified, the reason may be the species difference.

We next intended to clarify the mechanism by which CSE inhibits platelet aggregation. We showed that CSE inhibited arachidonic acid–induced TXA\textsubscript{2} production and that the inhibitory effect of CSE on collagen-induced aggregation was suppressed significantly in TP\textsubscript{\textsuperscript{-/-}} platelets compared with the inhibitory effect in WT platelets (\textbullet{}Fig. 2\textbullet{}). These results indicate that the inhibitory effect of CSE on platelet aggregation is derived from reduced TXA\textsubscript{2} production. In EP\textsubscript{2\textsuperscript{-/-}}, EP\textsubscript{4\textsuperscript{-/-}}, or IP\textsubscript{\textsuperscript{-/-}} platelets, the inhibitory effect of CSE on U-46619-induced aggregation was not significantly different from that in respective control platelets (\textbullet{}Fig. 3A, B\textbullet{}). Furthermore, CSE did not increase the content of cAMP in platelets (\textbullet{}Fig. 3C\textbullet{}). These results indicate that receptors coupling to G\textsubscript{\textalpha{}} expressed in platelets, including inhibitory prostanoid receptors, do not participate in the inhibitory effect of CSE on platelet aggregation.

COX-1 is an enzyme that is responsible for TXA\textsubscript{2} production in platelets. CSE inhibited COX-1 activity in a concentration–dependent manner, and the pattern of inhibition was uncompetitive (\textbullet{}Fig. 4\textbullet{}). In this study, we measured the peroxidase activity of COX, namely, the activity for conversion of PGG\textsubscript{2} to PGH\textsubscript{2}, the direct precursor of TXA\textsubscript{2}. Our results indicated that CSE binds to the COX-1–PGG\textsubscript{2} complex and stabilizes it, resulting in an uncompetitive inhibition of PGH\textsubscript{2} production. In contrast to the inhibitory action on COX-1 activity, CSE enhanced TX synthase activity in a concentration–dependent manner (\textbullet{}Fig. 5\textbullet{}). These results suggest that the inhibitory action of CSE on COX-1, an upstream enzyme of TX synthase in the arachidonic acid cascade, overcame the stimulatory action of CSE on TX synthase, leading to reduced TXA\textsubscript{2} production in platelets. Like the action of CSE, it has been shown that catechol inhibited arachidonic acid–induced platelet aggregation and that the inhibition of COX activity and TXA\textsubscript{2} production by catechol played a role in its antiplatelet effect.\textsuperscript{20} Because catechol is included in the particulate phase of cigarette smoke,\textsuperscript{16,21} it does not participate in the inhibitory effect of CSE, gas phase constituents of cigarette smoke. Further study is needed to determine what constituent(s) of CSE is responsible for the inhibitory effect of CSE on platelet aggregation.

Notably, CSE also inhibited the activity of COX-2 (\textbullet{}Fig. 4A\textbullet{}), a well-known player in inflammatory responses. On the other hand, it has been shown that CSE induces COX-2 expression in dendritic cells\textsuperscript{38} and in tracheal smooth muscle cells.\textsuperscript{39} Therefore, it is an interesting issue whether cigarette smoking increases or decreases COX-2-dependent prostanoid production under various inflammatory conditions in vivo.

This is the first report showing that CSE, the gas phase constituents of cigarette smoke, inhibits platelet aggregation and that its antiplatelet effect is derived from the inhibition of platelet COX-1 activity and resultant reduction in TXA\textsubscript{2} production.
Conflict of Interest
None declared.

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