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Participation of epoxygenase activation in saikogenin D-induced inhibition of prostaglandin E_2 synthesis

Yasufumi Toriniwa, Xiaochuan Lv, Yasuo Kodama, Yasushi Ohizumi, Makoto Yoshida and Norimichi Nakahata

Abstract

We examined the effect of saikogenin D on arachidonic acid metabolism in C6 rat glioma cells to clarify its anti-inflammatory mechanism. Incubation of C6 cells with saikogenin D for 20 min resulted in the inhibition of prostaglandin E_2 production and the accumulation of an arachidonic acid metabolite that was found to be 11,12-dihydroxyeicosatrienoic acid, a metabolite of 11,12-epoxyeicosatrienoic acid. C6 cells expressed rat epoxygenase mRNAs, CYP1A1, CYP2B1 and CYP2J3, which converted arachidonic acid to epoxyeicosatrienoic acids. 11,12-Epoxyeicosatrienoic acid inhibited A23187-induced prostaglandin E_2 production and SKF-525A, an inhibitor of epoxygenase, attenuated the saikogenin D-induced inhibition of prostaglandin E_2 production in C6 cells. Furthermore, 11,12-epoxyeicosatrienoic acid and 11,12-dihydroxyeicosatrienoic acid, but not saikogenin D, inhibited the activity of cyclooxygenase in a cell-free condition. These data suggest that saikogenin D activates epoxygenases that rapidly convert arachidonic acid to epoxyeicosanoids and dihydroxyeicosatrienoic acids, and then the metabolites secondarily inhibit prostaglandin E_2 production.

Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, Japan

Yasufumi Toriniwa, Xiaochuan Lv, Makoto Yoshida, Norimichi Nakahata

Department of Pharmaceutical Molecular Biology, Graduate School of Pharmaceutical Sciences, Tohoku University, Japan

Yasuo Kodama, Yasushi Ohizumi

21st century COE program "CRESCENDO", Graduate School of Pharmaceutical Sciences, Tohoku University, Japan

Makoto Yoshida, Norimichi Nakahata

Correspondence: M. Yoshida, Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aoba, Aramaki, Aoba-ku, Sendai 980-8578, Japan. E-mail: myoshida@mail.pharm.tohoku.ac.jp

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Introduction

An increasing number of studies have revealed the pivotal role of inflammation and glial activation in the pathogenesis of neurodegenerative diseases (Yermakova & O'Banion 2000). It has been proposed that nonsteroidal anti-inflammatory drugs are effective in preventing neural disorders, such as Alzheimer's disease, in part by reducing the toxic inflammatory response in the brain, although a mechanism of action other than inhibition of cyclooxygenase (COX) activity may be involved (Hull et al 2002). One possible key group of substances for brain anti-inflammation is the epoxyeicosatrienoic acids (EETs), which are oxidatively metabolized from arachidonic acid by multiple microsomal cytochrome P450 (CYP) enzymes referred to as epoxygenases (Capdevila et al 2002). Several cytochrome P450 enzymes, including members of the CYP1A, CYP2B, CYP2C and CYP2J subfamilies, have been reported to produce EET (Zeldin 2001). EETs have been known as an endothelial-derived hyperpolarization factor causing relaxation of vascular smooth muscle (Fisslthaler et al 1999; Earley et al 2003). Recently, EETs have been reported to inhibit prostaglandin E_2 (PGE_2) synthesis stimulated with lipopolysaccharide in rat monocytes (Kozak et al 2003). Furthermore, it has been shown that several EETs decrease the cytokine-induced expression of endothelial cell adhesion molecules by the inhibition of a pro-inflammatory transcriptional factor, NF-κB, through inhibiting IκB degradation (Node et al 1999). Although the intervention of EETs in brain inflammation is scarcely elucidated, in contrast to the roles in peripheral cardiovascular and inflammatory systems, the expression of cytochrome P450 enzyme is considered to have a relationship with brain inflammation (Nicholson & Renton 2002).

Recently, we have shown that saikogenin D, an active metabolite of saikosaponin d, a constituent of the root of *Bupleurum falcatum* that is used in oral Kampo medicines (Chinese herbal medicines) for the treatment of acute and chronic inflammatory diseases, has a strong inhibitory effect on PGE_2 production in C6 rat glioma cells (Kodama et al 2003). Interestingly, saikogenin D did not affect the conversion of arachidonic acid to

PGE_2 in microsomal preparations and arachidonic acid liberation from intact C6 cells, suggesting that saikogenin D has a new anti-inflammatory profile distinct from the direct inhibition of COX or phospholipase A_2 activity. In this study, we examined the mechanism of saikogenin-D-induced inhibition of PGE_2 synthesis concerned with epoxygenase activation and EET production, to clarify the anti-inflammatory property of saikogenin D.

Materials and Methods

Materials

Saikogenin D was hydrolysed from saikosaponin d (Wako Pure Chemical, Tokyo, Japan) with 1 N sulfuric acid and purified by ODS column chromatography. The chloroform solution of crude mixture of saikogenin D was applied to the ODS column and eluted by the solvent with an exponential gradient of chloroform–methanol (100:0 to 80:20 v/v). No contamination of saikosaponin d in the sample was confirmed by thin-layer chromatography separation. C6 rat glioma cells (JCRB9096) were obtained from Health Science Research Resources Bank (Osaka, Japan). A23187 was purchased from Sigma-Aldrich Japan (Tokyo, Japan). SKF-525A (proadifen) and recombinant COX-1 and COX-2 were purchased from Cayman Chemical (Ann Arbor, MI). Other chemicals and drugs were of reagent grade or of the highest quality available.

Female Wistar rats (SLC, Shizuoka, Japan) for liver sampling were kept in cages under conditions of constant temperature (21–24°C) with a 12-h light–dark cycle and allowed free access to a normal diet and water. All procedures for handling animals were approved by the Animal Experimentation Committee of Tohoku University Graduate School of Pharmaceutical Sciences.

Cell culture

C6 rat glioma cells were grown in F-10 medium containing 10% horse serum, 5% fetal bovine serum, 50 $\mu g\,mL^{-1}$ streptomycin and 50 $U\,mL^{-1}$ penicillin. Cells were grown in a 37°C humidified incubator under an atmosphere of 5% CO_2 in air.

Measurement of PGE_2 production

C6 rat glioma cells were cultivated in 12-well plates. Two days after seeding, the cells were washed twice with Eagle's minimum essential medium buffered with 20 mM HEPES, pH 7.35. After drug treatment, the medium was retrieved and indometacin (50 μM) and EDTA (5 mM) were added to terminate the COX reaction. PGE_2 was extracted from the medium twice with ethyl acetate under acidic conditions (pH 4.0). The sample was dried under nitrogen, and dissolved in 0.5 mL of 10 mM Tris HCl buffer. PGE_2 was measured by radioimmunoassay, as described previously (Nakahata et al 1996; Kodama et al 2003).

Analysis of arachidonic acid metabolites

Arachidonic acid metabolites were detected by the method described previously (Nakatani et al 2002) with slight modifications. C6 cells were seeded into 6-well plates. Two days after seeding, the medium was changed to F-10 medium containing 5% fetal bovine serum and 0.5 $\mu Ci\,mL^{-1}$ of [^{14}C]-arachidonic acid, and the cells were incubated for 24 h. The cells were washed twice with Eagle's modified essential medium–HEPES containing 5% albumin (pH 7.35). After pre-incubation with saikogenin D or indometacin for 10 min at 37°C, the cells were incubated with or without 10 μM A23187 for 10 min. The medium was transferred to a tube and indometacin (50 μM) and EDTA (5 mM) were added to terminate COX reaction. The metabolites were extracted twice with ethyl acetate under acidic conditions (pH 4.0). The sample was dried under nitrogen, dissolved in chloroform (100 μL) and applied to a thin-layer chromatography plate (LK6DF, Whatman). The developer used was the upper phase of an ethyl acetate–isooctane–acetic acid–water (110:50:20:100 v/v) mixture. Metabolites of [^{14}C]-arachidonic acid were visualized as radioluminogram with a Molecular Imager (GS363; Bio-Rad, Tokyo, Japan).

Reverse transcription–polymerase chain reaction (RT-PCR)

C6 cells were used two days after seeding into 6-well plates. Rat liver was obtained from adult female Wistar rats anaesthetized with sodium pentobarbital (50 $mg\,kg^{-1}$, i.p.). Total RNA samples were prepared from C6 cells and rat liver using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. They were reverse-transcribed into cDNA using the ReverTra Ace (Invitrogen, Tokyo, Japan), $oligo_{15}$ (dT) primer, 20 units RNase inhibitor and 10 mM dNTP. The sequences of primers for PCR were as follows: 5′-TTC ATT CCT ATC CTC CGT TAC-3′ (forward primer for CYP1A1), 5′-TCC TGT GGG TCT CTG CTG TGC-3′ (reverse primer for CYP1A1) (1010 bp) (Geng & Strobel 1995); 5′-TCA TCG ACA CTT ACC TTC TGC-3′ (forward primer for CYP2B1), 5′-GTG TAT GGC ATT TTA CTG CGG-3′ (reverse primer for CYP2B1) (255 bp) (Ibach et al 1998); 5′-TCA TCG ACA CTT ACC TTC TGC-3′ (forward primer for CYP2B2), 5′-AGT GTA TGG CAT TTT GGT ACG A-3′ (reverse primer for CYP2B2) (280 bp) (Ibach et al 1998); and 5′-CCT GGA TTT TGC TAA CAT TC-3′ (forward primer for CYP2J3), 5′-CTA AGC TCT TCT TTC CTA GT-3′ (reverse primer for CYP2J3) (227 bp) (Yamasaki et al 2004). Each reverse-transcribed cDNA was amplified in a PCR buffer containing 25 $U\,mL^{-1}$ Taq polymerase, 0.5 μM primers and 250 μM dNTP mixtures. PCR amplifications were performed in PTC-200 (Bio-Rad, Tokyo, Japan) with an initial denaturation step at 95°C for 10 min followed by 35 cycles of 95°C for 30 s, 55°C for 30 s and 72°C

for 30 s. A final extension step was carried out at 72°C for 5 min. After 1.5% agarose gel electrophoresis, PCR products were stained with ethidium bromide and detected by a UV detector (FAS III mini, TOYOBO, Osaka, Japan).

For relative quantification of gene expression, real-time PCR assay was performed using the DNA engine Opticone system (MJ Reseach, Waltham, MA, USA). PCRs were performed using 10 μL SYBR Premix Ex Taq (Takara Bio Inc., Otsu, Japan), and 0.4 M of each primer in a 20-μL reaction. Thermocycler conditions comprised an initial denaturation step at 95°C for 10 s followed by 35 cycles of 95°C for 10 s, 55°C for 20 s and 72°C for 30 s. A 1-step melting-curve analysis was also performed at the end of the run to ensure the crossover values obtained were due to the amplification of a specific product. The β-actin gene was used as an endogenous control, to normalize for differences in the amount of total RNA in each sample.

Assay of the enzymatic activity of COX-1 and COX-2

The activity of COX-1 and COX-2 was determined according to the procedure described previously (Nakatani et al 2002). Recombinant COX-1 or COX-2 enzyme protein (2.0 U each) was dissolved in 150 μL of 50 mM Tris-buffer containing 2 μM haematin and 5 mM tryptophan. The mixture was pre-incubated with or without drugs for 10 min at 37°C and further incubated with 20 μM arachidonic acid for 10 min at 37°C. Then, the reaction was terminated by the addition of 20 μL of 1 N HCl. PGE_2 was extracted and determined by radioimmunoassay as described above. COX activity was estimated from the level of PGE_2.

Statistical analysis

Values are expressed as mean ± s.e.m. Two-way analysis of variance and Scheffé's test for multiple comparisons were used to evaluate the effect of SKF-525A. All other data were analysed by one-way or two-way analysis of variance followed by Dunnett's test for multiple comparisons. Results with $P < 0.05$ were considered statistically significant.

Results

Effect of saikogenin D on PGE_2 production

The application of 10 μM A23187, a Ca^{2+} ionophore, for 10 min induced PGE_2 production in C6 rat glioma cells (Figure 1). While the pretreatment with saikogenin D at a concentration of 20 μM for 10 min did not affect basal PGE_2 release from C6 cells, it inhibited PGE_2 release induced by A23187 as previously shown by us (Kodama et al 2003). Indometacin pretreatment at a concentration of 10 μM also inhibited A23187-induced PGE_2 release.

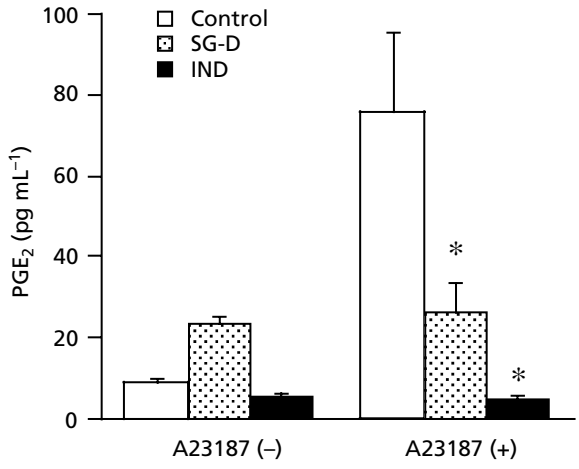

Figure 1 Effects of saikogenin D (SG-D) and indometacin (IND) on A23187-induced prostaglandin E_2 (PGE_2) production in C6 rat glioma cells. After pre-incubation with 20 μM saikogenin D or 10 μM indometacin for 10 min, the cells were incubated with or without 10 μM A23187 for 10 min. Each column represents mean ± s.e.m. of three independent experiments performed in triplicate. *$P < 0.05$ vs control.

Effect of saikogenin D on arachidonic acid metabolism

The application of 10 μM A23187 induced [^{14}C]-arachidonic acid liberation and [^{14}C]-PGE_2 production in C6 rat glioma cells labelled with [^{14}C]-arachidonic acid (Figure 2E). Saikogenin D (50 μM) and indometacin (30 μM) inhibited PGE_2 production induced by A23187 (Figures 2F and 2G). Interestingly, saikogenin D accumulated a metabolite of [^{14}C]-arachidonic acid distinct from PGE_2 or arachidonic acid (Figure 2C). This metabolite of arachidonic acid corresponded to 11,12-dihydroxyeicosatrienoic acid (11,12-DHET) based on the rate of flow value. Furthermore, saikogenin D facilitated A23187-induced DHET production (Figure 2F). Indometacin, however, did not affect A23187-induced DHET production (Figure 2G). While DHET is a metabolite of EET that is biosynthesized from arachidonic acid by epoxygenase (Sacerdoti et al 2003), no apparent peak corresponding to 11,12-EET was detected in any radioluminogram. These data prompted us to examine the possibility that saikogenin D facilitated epoxide production and the epoxide inhibited COX activity. To test this hypothesis, we investigated the involvement of epoxyeicosanoids in saikogenin-D-induced inhibition of PGE_2 production.

Epoxygenase expression in C6 rat glioma cells

First, we investigated whether cytochrome P450 enzymes were expressed in C6 cells using an RT-PCR method (Figure 3). The mRNAs of CYP1A1, CYP2B1 and CYP2J3, which are known to produce epoxyeicosanoids from arachidonic acid (Zeldin 2001), were expressed in C6 cells (Figure 3A). However, CYP2B2 was not detected in C6 cells. All mRNAs of examined cytochrome P450s were

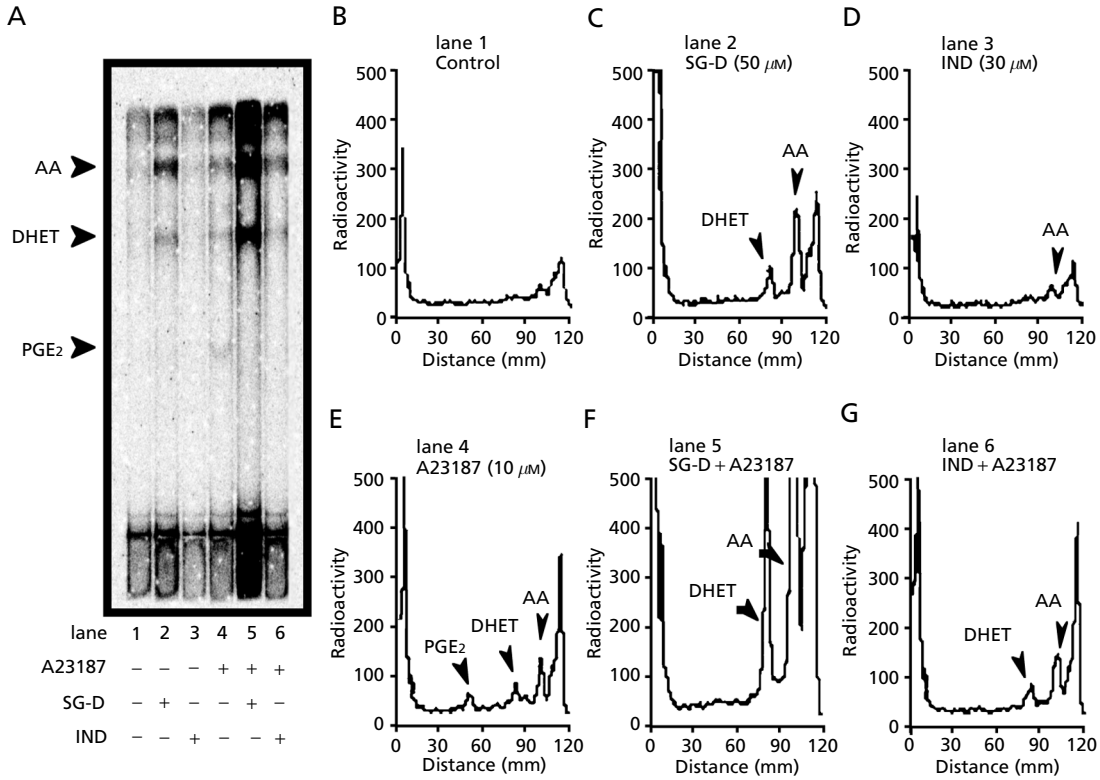

Figure 2 Effect of saikogenin D (SG-D) on arachidonic acid (AA) metabolites released into extracellular medium from C6 rat glioma cells. [^{14}C]-Arachidonic acid-labelled cells were incubated with 50 μM saikogenin D or 30 μM indometacin (IND) for 10 min, then incubated with or without 10 μM A23187 for an additional 10 min. Released arachidonic acid metabolites into the medium were separated by thin-layer chromatography. The bands of [^{14}C]-arachidonic acid metabolites on chromatogram (A) were detected by a molecular imager and the relative density of each lane was determined (B–G). The peaks of prostaglandin E_2 (PGE_2), arachidonic acid and 11,12-dihydroxyeicosatrienoic acid (DHET) were assigned from the rate of flow value using authentic standards. A representative result is shown, and data represent similar results in three separate experiments.

detected in rat liver. No contamination of genomic DNA was confirmed by the separate experiment that showed no bands in the sample without reverse transcription (data not shown). The incubation of C6 cells with 20 or 50 μM saikogenin D for 20 min did not affect the mRNA expression of CYP1A1, CYP2B1 or CYP2J3 (Figure 3B).

Effect of 11,12-EET on PGE_2 production stimulated by A23187

Because DHETs are metabolites of EETs, we hypothesized that saikogenin D facilitated EET production, which attenuated the generation of PGE_2. Thus, the direct effect of EET on PGE_2 production was examined in C6 cells. 11,12-EET inhibited A23187-induced release of PGE_2 from C6 cells in a concentration-dependent manner (Figure 4). However, it did not affect PGE_2 production in unstimulated cells.

Effect of SKF-525A on saikogenin-D-induced inhibition of PGE_2 production

To clarify the involvement of epoxides in saikogenin-D-induced inhibition of PGE_2 production, we investigated the effect of SKF-525A, an epoxygenase inhibitor, on saikogenin-D-induced inhibition of PGE_2 production. Without SKF-525A treatment, saikogenin D almost completely inhibited the A23187-induced PGE_2 production (Figure 5). SKF-525A attenuated saikogenin-D-induced inhibition of PGE_2 production in a concentration-dependent manner. SKF-525A itself did not affect the basal or A23187-induced PGE_2 production.

Direct effect of 11,12-EET and 11,12-DHET on COX activity

We examined whether saikogenin D, EET and DHET affected the conversion of arachidonic acid to PGE_2 by recombinant COX enzymes in a cell-free condition. As shown in Figure 6, indometacin, but not saikogenin D, inhibited PGE_2 production by recombinant COX-1 or COX-2. 11,12-EET and 11,12-DHET slightly reduced COX-1 activity but the difference was not statistically significant (Figure 6C). In contrast, COX-2 activity was significantly reduced by 11,12-EET and 11,12-DHET (Figure 6D). The results suggest that the inhibitory effect of saikogenin D on PGE_2 production is not

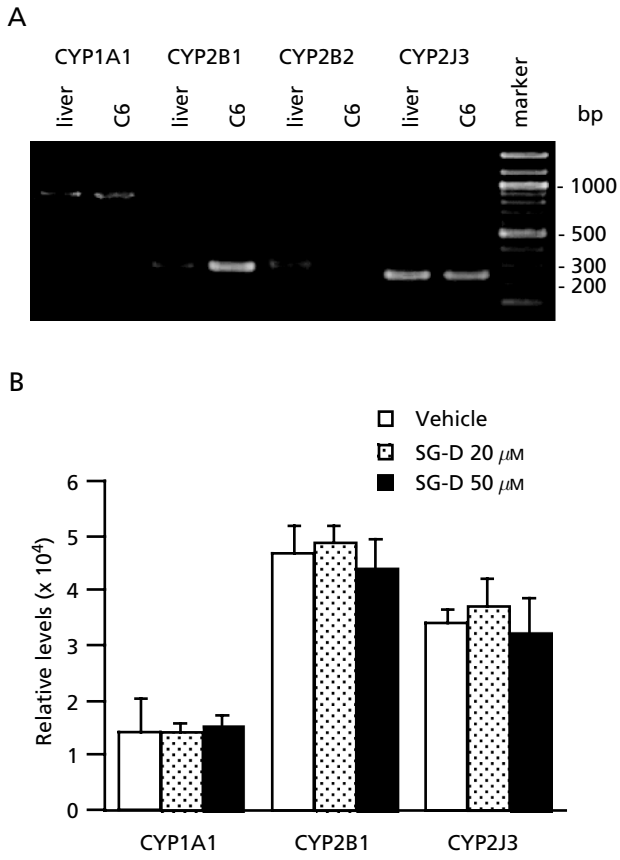

Figure 3 Expression of epoxygenase mRNA in C6 rat glioma cells. A. Reverse transcription–polymerase chain reaction was performed to examine the expressions of CYP1A1, CYP2B1, CYP2B2 and CYP2J3 mRNA in C6 cells, as described in Materials and Methods. B. Real-time PCR assay was performed in C6 cells incubated with 20 or 50 μM saikogenin D (SG-D) or vehicle for 20 min. The data are presented relative to β-actin. Each column represents mean ± s.e.m. of four independent experiments.

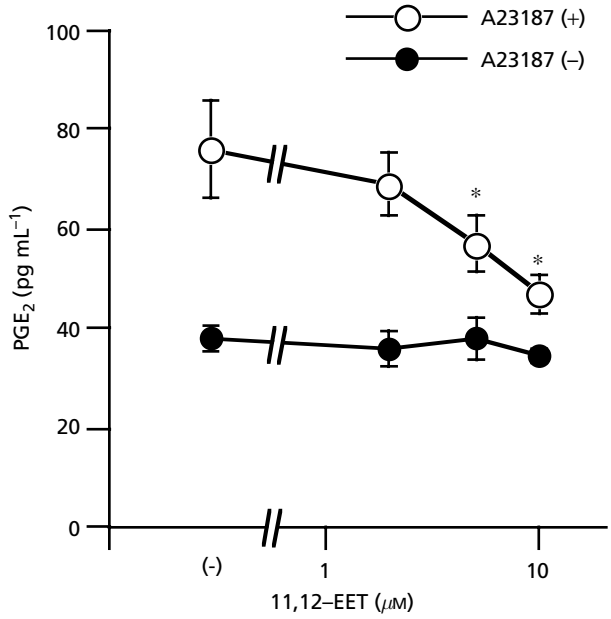

Figure 4 Effect of 11,12-epoxyeicosatrienoic acid (11,12-EET) on prostaglandin E_2 (PGE_2) production in C6 rat glioma cells. After pre-incubation with 11,12-EET for 10 min, the cells were incubated with (open circles) or without (closed circles) 10 μM A23187 for 10 min. Each point represents mean ± s.e.m. of three independent experiments performed in triplicate. *$P < 0.05$ vs A23187 alone.

attributed to the direct inhibition of COX activity, whereas 11,12-EET and 11,12-DHET inhibit activity of COX-2 directly.

Discussion

Our data revealed that saikogenin D rapidly activated the epoxygenase cascade of arachidonic acid metabolism, and the accumulated epoxyeicosanoids secondarily inhibited PGE_2 production. This is the first report to show the inhibition of PGE_2 production by an epoxygenase-activating drug in glial cells. Recent studies have suggested the important role of the epoxygenase pathway and epoxyeicosanoids in the pathophysiology of inflammation (Kozak et al 1998; Campbell 2000). Kozak et al (2003) have reported that 11,12-EET inhibited lipopolysaccharide-induced PGE_2 production, and SKF-525A, an epoxygenase inhibitor, potentiated PGE_2 production in rat monocytes. Node et al (1999) have reported that EETs inhibited the induction of cell adhesion molecules in endothelial cells stimulated with tumour necrosis factor-α, interleukin-1α or lipopolysaccharide. However, there are few reports of the anti-inflammatory effect of epoxides in glial cells that are known to play an important role in brain inflammation. Our data showed that 11,12-EET and 11,12-DHET inhibited COX-2 activity in a cell-free condition, and 11,12-EET inhibited PGE_2 production in C6 rat glioma cells. Furthermore, cytochrome P450 enzymes having epoxygenase activity (Zeldin 2001) were expressed in C6 cells, and SKF-525A attenuated saikogenin D-induced inhibition of PGE_2 production. These results suggest that the epoxygenase pathway exists in C6 cells, and epoxides functionally affect PGE_2 production. In addition to C6 cells, it has been shown that epoxygenases are expressed in primary cultures of rat hippocampal astrocytes (Alkayed et al 1996). It is therefore possible that epoxygenases in glial cells play an important role in brain inflammation.

It is noteworthy that brief application (20 min) of saikogenin D to C6 cells can activate the epoxygenase pathway of arachidonic acid metabolism. Well-known activators of epoxygenase, phenobarbital or β-naphthoflavone, require at least several hours to induce activity because their effects are accompanied by appropriate expression of enzyme proteins. In contrast, saikogenin D activated the epoxygenase pathway without any induction of CYP enzyme expression. One possible mechanism for the epoxygenase activation is COX inhibition, which prevents arachidonic acid conversion to PGH_2. However, our data in Figure 2D showed that indometacin did not increase the peak of an arachidonic acid metabolite,

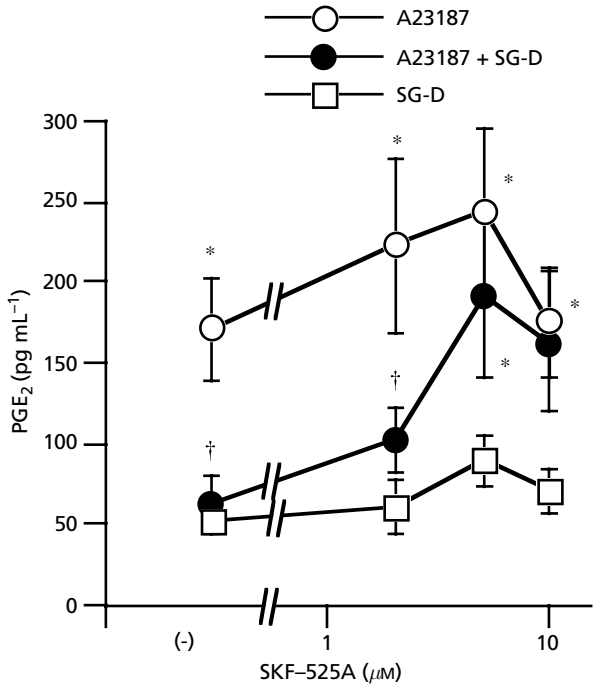

Figure 5 Effect of SKF-525A on saikogenin D (SG-D)-induced inhibition of prostaglandin E_2 (PGE_2) production in C6 rat glioma cells. After pre-incubation with SKF-525A for 10 min, the cells were incubated with (closed circles, open squares) or without (open circles) 20 μM saikogenin D for 10 min. Then, the cells were further incubated with (open and closed circles) or without (open squares) 10 μM A23187 for 10 min. Each point represents mean ± s.e.m. of three independent experiments performed in triplicate. *$P < 0.05$ vs corresponding values of a group incubated without A23187; †$P < 0.05$ vs corresponding values of a group incubated without saikogenin D.

11,12-DHET. In contrast to indometacin, saikogenin D activated epoxygenase without direct inhibition of COX activity. Alkayed et al (1997) have reported that brief application (30 min) of glutamate to cultured rat astrocytes activated the epoxygenase pathway of arachidonic acid metabolism through an increase in arachidonic acid liberation. Because 50 μM sakogenin D releases arachidonic acid (Figure 2C) through an increase in the intracellular Ca^{2+} concentration, as previously shown by our laboratory (Kodama et al 2003), this concentration of saikogenin D may affect the arachidonic acid metabolism and epoxyeicosanoid accumulation. However, 2–10 μM saikogenin D also inhibits A23187-induced PGE_2 production without changes in the intracellular Ca^{2+} concentration and arachidonic acid release (Kodama et al 2003), indicating that saikogenin D activates the epoxygenase pathway without changes in phospholipase A_2 activity. Although its precise mechanism for activation is still unclear, saikogenin D could be a useful drug for rapid activation of the epoxygenase pathway.

There are four isomers in EETs, 5,6-, 8,9-, 11,12- and 14,15-EET, whose biological efficacies are different from each other (Zeldin 2001). It has been reported that among EETs, 11,12-EET was the most effective in tumour necrosis factor-α-induced expression of vascular cell adhesion molecule-1 in human endothelial cells (Node et al 1999). Furthermore, 11,12-EET, but not 14,15-EET, inhibited tumour necrosis factor-α-induced NF-κB promoter activation (Node et al 1999). While 11,12-EET inhibited A23187 induced-PGE_2 production in this study, it has been shown that 14,15-EET had the highest efficacy in reducing PGE_2 production among the four isomers of EETs in vascular smooth muscle cells (Fang et al 1998). In human platelets, 14,15-EET has been reported to inhibit arachidonic-acid-induced platelet aggregation more effectively than 11,12-EET (Fitzpatrick et al 1986). Based on these reports, it is speculated that the isomeric efficacy of EETs are different among tissues or cells. In this study, we showed that A23187 and saikogenin D facilitated the production of an arachidonic acid metabolite that was assumed to be 11,12-DHET from the rate of flow value in thin-layer chromatograms (Figures 2C and 2E). The combination of A23187 and saikogenin D facilitated the production of 11,12-DHET from C6 cells (Figure 2F). Although the peak for 11,12-EET, a precursor of 11,12-DHET, was not found in our chromatograms, the increase in intracellular Ca^{2+} concentration and the application of saikogenin D can be thought to facilitate arachidonic acid metabolism to epoxyeicosanoids, especially 11,12-EET and 11,12-DHET. Furthermore, the incubation with 11,12-EET inhibited PGE_2 production in C6 rat glioma cells, suggesting an important role of 11,12-EET in the regulation of PGE_2 level in C6 cells. However, we cannot exclude the possibility that other isomers of EETs and DHETs contribute to the inhibitory effect of saikogenin D on PGE_2 production.

Many cytochrome P450 enzymes, such as CYP1A, CYP2B, CYP2C, CYP2D, CYP2J and CYP2N subfamilies, are known to act as epoxygenases (Zeldin 2001). Human CYP2J2, corresponding to rat CYP2J3, was identified as a potential source of EETs in human endothelial cells (Node et al 1999). Furthermore, CYP2J2 is highly expressed in human heart, and is one of the enzymes responsible for epoxidation of endogenous arachidonic acid pools (Wu et al 1996). CYP1As and CYP2Bs have been identified in rat brain and glial cells (Strobel et al 1995). CYP1A activity in rat astrocytes is affected by lipopolysaccharide, suggesting a possible role of CYP1A in brain inflammation (Nicholson & Renton 2002). CYP2B mRNA is reported to be strongly expressed in young cultured astrocytes (Ibach et al 1998). In this study, we showed that CYP2J3, CYP1A1 and CYP2B1 mRNAs were expressed in C6 rat glioma cells, but it is still unclear which cytochrome P450 isozyme mainly produces epoxyeicosanoids in C6 rat glioma cells. Further investigations are needed to elucidate the more detailed mechanism of epoxyeicosanoid accumulation induced by saikogenin D.

Conclusions

This study showed that brief incubation of C6 cells with saikogenin D resulted in the inhibition of PGE_2 production. An epoxygenase inhibitor cancelled this saikogenin-D-induced inhibition of PGE_2 production. Saikogenin D augmented the production of the arachidonic acid metabolite 11,12-DHET. Although saikogenin D did not affect COX activity directly, 11,12-EET, a precursor of 11,12-DHET, inhibited PGE_2

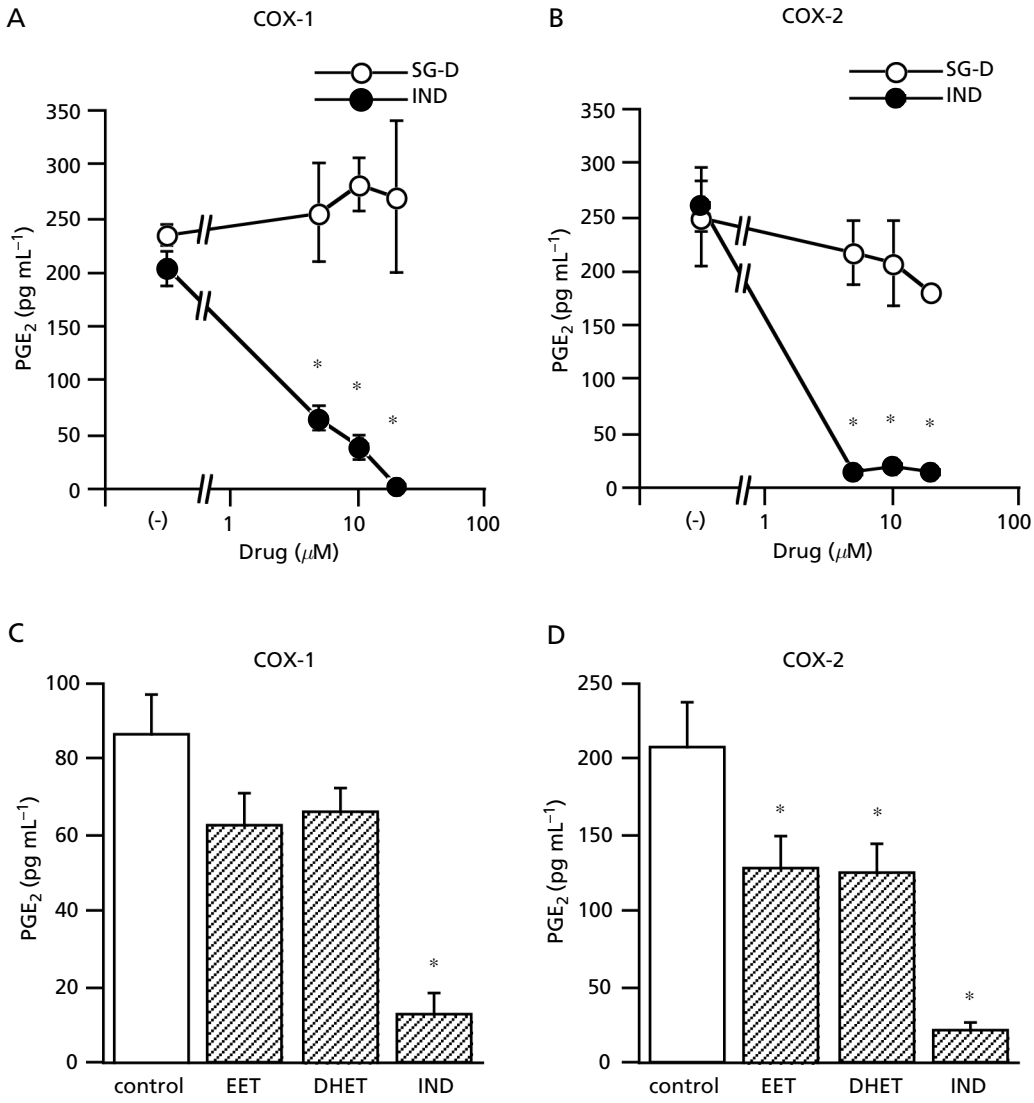

Figure 6 A, B. Effect of saikogenin D (SG-D) and indometacin (IND) on cyclooxygenase (COX) activity. The reaction mixture with COX-1 (A) or COX-2 (B) was pre-incubated with indicated concentrations of saikogenin D (open circles) or indometacin (closed circles) for 10 min, and further incubated with arachidonic acid for 10 min. C, D. Effect of 11,12-epoxyeicosatrienoic acid (EET) and 11,12-dihydroxyeicosatrienoic acid (DHET) on COX activity. The reaction mixture with COX-1 (C) or COX-2 (D) was pre-incubated with 10 μM 11,12-EET, 10 μM 11,12-DHET or 20 μM indometacin, and incubated with arachidonic acid for 10 min. Each value represents mean ± s.e.m. of three independent experiments performed in triplicate. *$P < 0.05$ vs control.

production both in C6 cells and in a cell-free condition. These data suggest that saikogenin D inhibits PGE_2 production through the accumulation of epoxyeicosanoids that inhibit COX activity. Saikogenin D is a unique and valuable drug for rapid modification of the epoxygenase pathway.

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