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An anti-platelet agent, OPC-29030, inhibits translocation of 12-lipoxygenase and 12-hydroxyeicosatetraenoic acid production in human platelets

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1 In human platelets, arachidonic acid is mainly metabolized by the two enzyme systems; cyclo-oxygenase and 12-lipoxygenase. Cyclo-oxygenase produces prostaglandin H_2 which is further converted to thromboxane B_2. 12-Lipoxygenase synthesizes 12(S)-hydroperoxyeicosatetraenoic acid which is reduced to 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE).

2 An anti-platelet compound, OPC-29030, dose-dependently inhibited 12(S)-HETE production with an IC_{50} of 0.06 ± 0.01 μM, but not synthesis of thromboxane B_2 in human platelets. Although the compound suppressed 12(S)-HETE production in human platelets, cytosolic 12-lipoxygenase activity was not inhibited up to 10 μM. Essentially identical data were obtained with a 12-lipoxygenase of human erythroleukaemia cells which had megakaryocyte/platelet-like properties.

3 OPC-29030 also suppressed production of 5(S)-HETE, a 5-lipoxygenase product, in rat basophilic leukaemia cells without inhibiting enzyme activity. It has been shown that 5-lipoxygenase binds to membrane 5-lipoxygenase-activating protein (FLAP) to produce 5(S)-HETE, and thus FLAP inhibitor suppresses cellular 5(S)-HETE production.

4 A FLAP inhibitor, L-655,238, suppressed platelet 12(S)-HETE production, but had no effect on the 12-lipoxygenase activity.

5 Western blot analysis showed that platelet 12-lipoxygenase translocated from cytosol to membranes upon thrombin stimulation, and OPC-29030 suppressed this process in a dose-dependent manner.

6 These results suggest that the 12-lipoxygenase of human platelets binds to FLAP or a similar protein, and OPC-29030 suppresses 12(S)-HETE production by inhibiting a certain step of the 12-lipoxygenase translocation.

Keywords: OPC-29030; 12(S)-HETE; 5(S)-HETE; 12-lipoxygenase; 5-lipoxygenase; FLAP; platelet; translocation

Abbreviations: FLAP, five-lipoxygenase activating protein; HEL, human erythroleukaemia; HETE, hydroxyeicosatetraenoic acid; HPETE, hydroperoxyeicosatetraenoic acid; α-MEM, α-modified Eagle's minimum essential medium; NDGA, nordihydroguaiaretic acid; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; RBL, rat basophilic leukaemia; TXB_2, thromboxane B_2

Introduction

A newly developed synthetic compound, OPC-29030, has inhibitory effects on platelet adhesion (Igawa *et al*., submitted) as well as thrombus formation under rheological conditions (Katoh *et al*., 1998). Potential mechanisms of action of this compound have been explored, and the compound was shown to inhibit production of 12(S)-hydroxy-5,8,10,14-eicosatetraenoic acid (12(S)-HETE) by platelets (Katoh *et al*., 1998; Uno *et al*., 1995). 12(S)-HETE is a reduced derivative of 12(S)-hydroperoxyeicosatetraenoic acid (HPETE) produced by a 12-lipoxygenase from arachidonic acid. 12(S)-HETE was shown to play roles in the regulation of platelet functions such as aggregation (Sekiya *et al*., 1989; 1990; 1991) and P-selectin expression (Ozeki *et al*., 1998). The mechanism of intracellular activation of platelet 12-lipoxygenase has not been fully elucidated, although Ca^{2+} induced translocation of the enzyme from cytosol to membranes was previously reported (Baba *et al*., 1989). 5-Lipoxygenase is another member of lipoxygenase family, which is involved in hypersensitivity and inflammatory diseases (Samuelsson, 1983). The enzyme primarily produces 5-hydroperoxyeicosatetraenoic acid, which is either reduced to 5(S)-HETE or converted to leukotrienes. The 5-lipoxygenase was shown to translocate from cytosol to membranes in leukocytes (Brock *et al*., 1995; Ford-Hutchinson, 1991; Vickers, 1995). In this process, the enzyme binds to membrane 5-lipoxygenase activating protein (FLAP). Thus, compounds that interact with FLAP such as MK-886 (Miller *et al*., 1990) and L-655,238 (Evans *et al*., 1991) were shown to suppress production of 5(S)-HETE and leukotrienes. The present study is to investigate the precise mechanisms underlying inhibitory effects of OPC-29030 on platelet functions. We found that OPC-29030 suppressed the platelet 12(S)-HETE production without inhibiting cytosolic 12-lipoxygenase activity. Moreover, our data using a FLAP inhibitor suggested that the platelet 12-lipoxygenase translocation was mediated by FLAP or a similar protein, and OPC-29030 inhibited this translocation process.

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Methods

Materials

OPC-29030 (Lot No. 97F89M) was obtained from the Second Tokushima Factory of Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan), a FLAP inhibitor, L-655,238 (REV 5901, *para isomer*) and nordihydroguaiaretic acid (NDGA) from Biomol Research Laboratories (Campus, PA, U.S.A.), thrombin from Motida Pharmaceutical Company (Tokyo, Japan), collagen from Arzneimittel Gmbh. (Munchen, Germany), [1-^{14}C]-arachidonic acid from Amersham (Buckinghamshire, U.K.), arachidonic acid and indomethacin from Sigma (St. Louis, MO, U.S.A.), N,N-dimethylformamide from Wako Pure Chemical (Osaka, Japan), α-modified Eagle's minimum essential medium (α-MEM) from Gibco BRL (New York, NY, U.S.A.), foetal bovine serum from Dainippon Pharmaceutical (Osaka, Japan), thin layer chromatography plate from Merck (Darmstadt, Germany), bond elute C18 columns from Varian (Harbor City, CA, U.S.A.), polyvinylidene difluoride (PVDF) membranes from Bio-Rad (Hercules, CA, U.S.A.), anti-12-lipoxygenase antibody (human platelets) from Alexis (San Diego, CA, U.S.A.), horseradish peroxidase-conjugated goat anti-rabbit IgG from Immunotech (Marseille, France), ECL Western blotting detection reagents from Amersham (Buckinghamshire, U.K.) and Titer Zyme® enzyme immunoassay kits for 12(S)-HETE and thromboxane B_2 (TXB_2) from PerSeptive Diagnostics (Cambridge, MA, U.S.A.). Human erythroleukaemia (HEL) cells were kindly provided by Dr Eto of Ajinomoto Company (Tokyo, Japan) and Dr S. Narumiya of Kyoto University. Rat basophilic leukaemia (RBL-2H3) cells were provided by National Institute of Health Science (Tokyo, Japan).

Cell culture

HEL cells were cultured in RPMI 1640 medium containing 10% heat-inactivated foetal bovine serum, 100 units ml^{-1} penicillin G and 100 μg ml^{-1} streptomycin sulphate, and RBL-2H3 cells in α-MEM supplemented with 10% heat-inactivated foetal bovine serum and 100 μg ml^{-1} streptomycin sulphate. The pH was adjusted to 7.4 with 7.5% sodium bicarbonate solution. The cells were cultured at 37°C in a humidified incubator with 5% CO_2 in air, and passed every 3–4 days.

Measurement of 12(S)-HETE and TXB_2 in human platelets and HEL cells

Blood was collected from healthy volunteers into a plastic syringe containing EDTA at a final concentration of 0.1%. Platelet-rich plasma was obtained by centrifuging the blood at $150 \times g$ for 10 min, and further centrifuged at $2500 \times g$ for 3 min. Platelet pellet was suspended in 50 mM HEPES-Tyrode buffer at pH 7.4 containing 1 mM $MgCl_2$ to give a density of 3×10^8 cells ml^{-1}. The platelet suspension (200 μl) was preincubated at 37°C for 2 min with the test drugs dissolved in N,N-dimethylformamide (final concentration of 0.1%), and then stimulated with 0.03 units ml^{-1} thrombin for 5 min. HEL cells were harvested, washed with phosphate-buffered saline (PBS) at pH 7.4, and suspended in Hank's balanced salt solution at a density of 5×10^6 cells 0.5 ml^{-1}. After preincubation with the test drugs at 37°C for 2 min, the cells were incubated with 25 μM arachidonic acid for 5 min. The reaction was terminated by cooling in an ice bath. Metabolites of arachidonic acid were extracted using a bond elute C18 column as reported by Raghunath *et al*. (1990). The amounts of 12(S)-HETE and TXB_2 were measured using Titer Zyme enzyme immunoassay kits.

Measurement of 5(S)-HETE in RBL-2H3 cells

RBL-2H3 cells were harvested and suspended in Hank's balanced salt solution at a cell density of 4×10^7 in 500 μl. The cells were preincubated at 37°C for 5 min with 0.1–10 μM OPC-29030 (dissolved in 5 μl ethanol), and incubated for 10 min with 25 μM [1-^{14}C]-arachidonic acid (4.63 kBq 12.5 nmol^{-1} 12.5 μl^{-1} ethanol solution). The reaction was terminated by adding 2 ml of ice-cold stop solution (diethyl ether : methanol : 1 M citric acid = 30 : 4 : 1, by volume). The ethereal extracts were spotted on a silica gel thin layer plate, and the plate was developed at 4°C for 60 min as previously described (Ueda *et al*., 1986). Radioactive 5(S)-HETE was analysed using a Fujix BAS 1000 image analyzer (Tokyo, Japan).

Enzyme activity assay

The crude extract was prepared from human platelets and HEL cells according to the previous report (Mahmud *et al*., 1993). Briefly, 10^9 platelets and 5×10^7 HEL cells were suspended in 2 ml of 50 mM Tris-HCl buffer at pH 7.4 containing 1 mM EDTA. The cell suspension was sonicated at 20 kHz three times each for 20 s in an ice bath using a Tomy ultrasonic disruptor UR-200P (Tokyo, Japan). The sonicate was centrifuged at $100{,}000 \times g$ for 10 min at 4°C to remove cell debris and unbroken cells, and the supernatant was used as the extract containing 12-lipoxygenase and cyclo-oxygenase. A 20-μl aliquot of the supernatant was mixed with 175 μl of 50 mM Tris-HCl buffer at pH 7.4 containing 1 mM EDTA and 2 μl of test compounds followed by incubation at 30°C for 5 min. Reactions with 25 μM [1-^{14}C]-arachidonic acid (1.85 kBq 5 nmol^{-1} 5 μl^{-1} ethanol solution) were carried out at 30°C for 10 min. Extraction and thin layer chromatography were performed as described above. 12-Lipoxygenase activity was determined by measuring the amount of 12(S)-HPETE and 12(S)-HETE, and cyclo-oxygenase activity by measuring TXB_2 amount.

The cytosol fraction of RBL-2H3 cells was prepared as follows. RBL-2H3 cells (1.8×10^8) were suspended in 2 ml of 50 mM Tris-HCl buffer at pH 7.4 containing 1 mM EDTA, and sonicated three times for 15 s in an ice bath. The sonicated sample was centrifuged at $100{,}000 \times g$ for 40 min at 4°C, and the supernatant was used as the cytosol fraction containing 5-lipoxygenase. The enzyme fraction (100 μl) was preincubated at 30°C for 5 min with 2 μl of OPC-29030 dissolved in 100% ethanol at final concentrations of 0.03–30 μM in 200 μl of 50 mM Tris-HCl at pH 7.4 containing 2 mM ATP and 2 mM $CaCl_2$. After preincubation, 25 μM [1-^{14}C]-arachidonic acid (3.7 kBq 10 nmol^{-1} 10 μl^{-1} ethanol solution) was added, and incubation was continued at 30°C for 10 min. Thin layer chromatography and quantification of the products were performed as described above. The protein concentration was detected by the method of Lowry *et al*. (1951) with bovine serum albumin as a standard.

Preparation of platelet cytosol and membrane fractions for Western blot analysis

Human platelets were suspended at 3×10^8 ml^{-1} in 50 mM HEPES-Tyrode buffer at pH 7.4 containing 2 mM of $CaCl_2$. The cells were stimulated with 0.03 or 0.3 units ml^{-1} thrombin

at 37°C for 10 min in the absence or presence of 0.01, 0.1 and 1 μM of OPC-29030. Reaction was terminated by putting samples into liquid nitrogen. The samples were freeze-thawed three times and centrifuged at $100{,}000 \times g$ for 60 min at 4°C. Supernatant solution was used as the cytosol fraction. The pellet was rinsed with the HEPES-Tyrode buffer and used as membrane fraction.

Western blot analysis

Platelet cytosol and membrane fractions were separated on 10% SDS-polyacrylamide gel electrophoresis, and electrophoretically transferred to a PVDF membrane for 2 h. The membrane was washed at room temperature for 20 min with PBS containing 0.1% Triton X-100, blocked with 1% non-fat dry milk in PBS at 4°C for 8 h, and incubated for 1 h with rabbit anti-12-lipoxygenase antibody. The membrane was washed three times each for 15 min in PBS-Triton at room temperature, and then incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG. After washing the membrane three times each for 15 min, the peroxidase-mediated chemiluminescence was detected on an X-ray film as described in the Amersham instruction manual. The density of 12-lipoxygenase bands was analysed using an imaging densitometer (Bio-Rad; model GS-700).

Statistical analysis

The IC_{50} value was calculated by non-linear regression analysis using the Statistical Analysis System (SAS) ver. 6.02.

Results

Effects of OPC-29030 on 12(S)-HETE production and 12-lipoxygenase

Effects of OPC-29030 were examined on 12(S)-HETE production in human platelets stimulated with thrombin or collagen. To determine the optimal concentration, human platelets were stimulated with various concentrations of thrombin (0.01, 0.03, 0.1 and 0.3 units ml^{-1}) and collagen (1, 2 and 4 $\mu g\ ml^{-1}$). We employed 0.03 units ml^{-1} thrombin and 2 $\mu g\ ml^{-1}$ collagen in subsequent experiments, because sub-maximal production of 12(S)-HETE was obtained with these concentrations as examined by an enzyme immunoassay. OPC-29030 in a range from 0 to 10 μM was added to human platelets, and the cells were stimulated with thrombin (Figure 1A). Without OPC-29030, 3×10^9 platelets produced approximately 100 ng of 12(S)-HETE during 5-min incubation. The production of 12(S)-HETE was inhibited by OPC-29030 with an IC_{50} of 0.06 ± 0.01 μM. Essentially the same results were obtained using platelets stimulated by collagen (data not shown). In contrast, OPC-29030 did not inhibit the 12-lipoxygenase activity using the crude extract of platelets up to 10 μM. Approximately 20 nmol of 12-lipoxygenase products (a sum of 12(S)-HETE and 12(S)-HPETE) was produced from 3×10^9 platelets. Effect of OPC-29030 was also examined on HEL cells, stimulated with 25 μM arachidonic acid. Five $\times 10^6$ HEL cells produced approximately 5 nmol of 12(S)-HETE for 5 min. As shown in Figure 1B, OPC-29030 dose-dependently suppressed 12(S)-HETE production with an IC_{50} of 2 μM. In contrast, 12-lipoxygenase activity was not affected up to 10 μM. A lipoxygenase inhibitor, NDGA, completely inhibited the 12-lipoxygenase activity as well as 12(S)-HETE production of the intact cells.

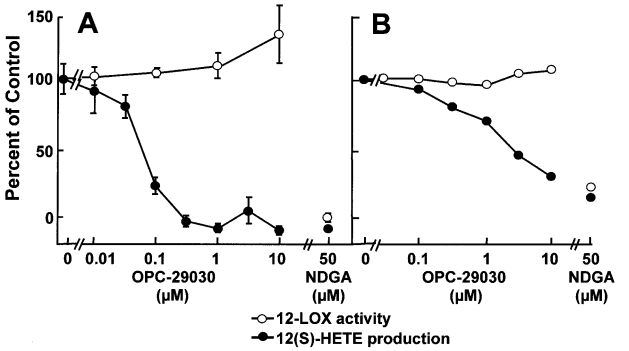

Figure 1 Effects of OPC-29030 on 12(S)-HETE production and 12-lipoxygenase activity of human platelets (A) and HEL cells (B). (A) Human platelets (3×10^8) were stimulated with 0.03 units ml^{-1} thrombin, and 12(S)-HETE was quantified by an enzyme immunoassay ($n = 5$). 12-Lipoxygenase (LOX) activity in the platelet cytosol was determined with [1-^{14}C]-arachidonic acid as a substrate ($n = 3$). (B) HEL cells (5×10^6) were incubated with [1-^{14}C]-arachidonic acid, and a sum of 12(S)-HPETE and 12(S)-HETE was measured as described in Methods. 12-Lipoxygenase activity in the cytosol was determined as in (A). Amount of 12(S)-HETE in non-stimulated cells was taken as 0%, and that of stimulated cells without OPC-29030 as 100%. Incubations with 50 μM NDGA were also performed.

Effects of OPC-29030 on cyclo-oxygenase activity and TXB_2 production

Effect of OPC-29030 on cyclo-oxygenase activity of human platelets was examined using the same samples in Figure 1A. As shown in Figure 2, this compound had no effect on cyclo-oxygenase activity. Production of TXB_2 from endogenous arachidonic acid in human platelets was also determined by an enzyme immunoassay (Figure 2). Approximately 20 nmol of TXB_2 was produced for 5 min without OPC-29030, and the compound had essentially no inhibitory effect. Indomethacin at 20 μM, a cyclo-oxygenase inhibitor, completely inhibited TXB_2 production.

Effect of OPC-29030 on platelet 12-lipoxygenase translocation

Since OPC-29030 inhibited 12(S)-HETE production by intact platelets but not 12-lipoxygenase activity, we assumed that intracellular activation of the 12-lipoxygenase was necessary for the production of 12(S)-HETE. It was reported that a 12-lipoxygenase in rat platelets was translocated from cytosol to membranes in the presence of Ca^{2+} (Baba *et al.*, 1989). We examined the effects of OPC-29030 on the translocation of human platelet 12-lipoxygenase. The cells were preincubated with 0.01, 0.1 and 1 μM OPC-29030 or vehicle, and stimulated with 0.03 or 0.3 units ml^{-1} of thrombin. The cytosol and membrane fractions were prepared by ultra-centrifugation, and the 12-lipoxygenase was quantified by Western blot analysis. As shown in Figure 3A, the 12-lipoxygenase band (72 kDa) was faint in the membranes of unstimulated platelets, and became dense in the thrombin-stimulated platelets. Consistent with this observation, the density of the 12-lipoxygenase band in the cytosol fraction was slightly decreased upon thrombin stimulation. The result indicates that the 12-lipoxygenase is translocated from the cytosol to membranes of human platelets by the thrombin stimulation. The density of the bands was not significantly different between the platelets stimulated by 0.03 and 0.3 units ml^{-1} thrombin (Figure 3A,B). When platelets were preincu-

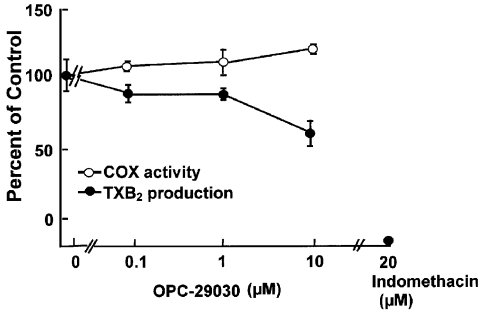

Figure 2 Effects of OPC-29030 on TXB_2 production and cyclo-oxygenase activity in human platelets. TXB_2 was quantified by an enzyme immunoassay ($n=5$). The same samples as in Figure 1A were used for TXB_2 determination. Cyclo-oxygenase (COX) activity was determined with [1-^{14}C]-arachidonic acid as a substrate ($n=3$). Amount of TXB_2 in non-stimulated cells was taken as 0%, and that in stimulated cells without OPC-29030 as 100%. An incubation with 20 μM indomethacin was performed.

bated with OPC-29030 followed by stimulation with 0.03 units ml^{-1} thrombin, the density in membrane fraction decreased depending on the OPC-29030 concentrations with a concomitant increase in cytosol fraction. The results indicate that the translocation of platelet 12-lipoxygenase was dose-dependently inhibited by OPC-29030. The experiments were repeated three times, and a volume (optical density × area) of each band is presented in Figure 3B.

Effects of OPC-29030 on 5(S)-HETE production and 5-lipoxygenase activity of RBL-2H3 cells

RBL-2H3 cells were preincubated with 0–10 μM OPC-29030, and then incubated with arachidonic acid. Without OPC-29030, approximately 2 nmol of 5(S)-HETE was produced by 4×10^7 cells. As shown in Figure 4, 0.1 μM OPC-29030 suppressed 5(S)-HETE production by approximately 50%. However, complete inhibition was not attained even with higher concentrations. On the other hand, OPC-29030 did not inhibit 5-lipoxygenase activity of the RBL-2H3 cytosol up to 10 μM. The 5-lipoxygenase activity and 5(S)-HETE production were completely inhibited by 50 μM NDGA, a non-specific lipoxygenase inhibitor.

A FLAP inhibitor suppressed 12(S)-HETE production in human platelets

It was previously demonstrated that translocation of 5-lipoxygenase through the binding to FLAP which resided in nuclear membrane promoted the 5-HETE production (Brock *et al*., 1995; Vickers, 1995; Woods *et al*., 1993). Since rat platelet 12-lipoxygenase was also shown to translocate from the cytosol to membranes in the presence of Ca^{2+} (Baba *et al*., 1989), we investigated whether the translocation of human platelet 12-lipoxygenase could be mediated by FLAP or a similar protein. For this purpose we used L-655,238 as a FLAP inhibitor (Evans *et al*., 1991). As shown in Figure 5A, this compound inhibited 12(S)-HETE production in thrombin-stimulated human platelets in a dose-dependent manner with an IC_{50} of 171.5±31.8 nM. Essentially identical results were obtained by stimulation with 2 μg ml^{-1} collagen. On the other hand, platelet 12-lipoxygenase activity was not inhibited by L-655,238 up to 200 nM (open circles). In the control experiment, 5(S)-HETE production in RBL-2H3 cells was inhibited by L-655,238 with an IC_{50} of 135.2±11.5 nM (Figure 5B). It should be mentioned that the IC_{50} values for the

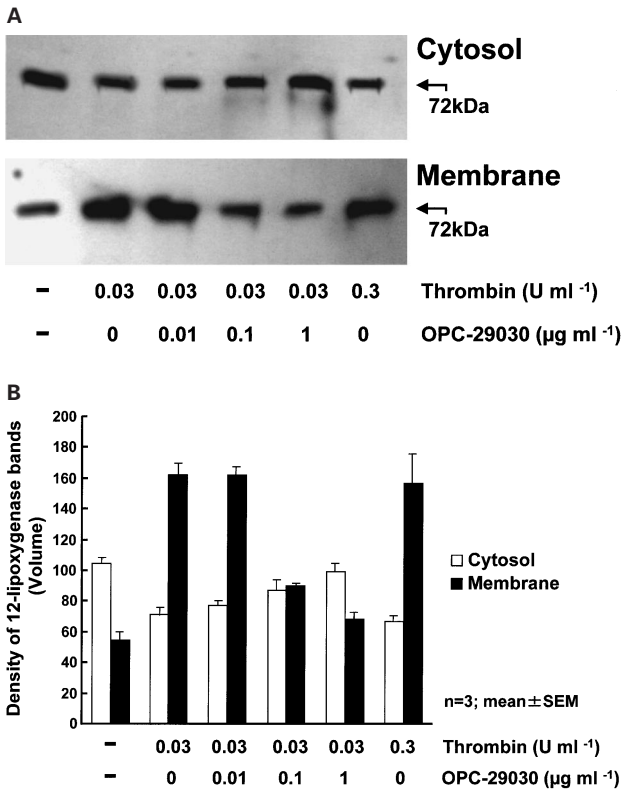

Figure 3 Effects of OPC-29030 on 12-lipoxygenase translocation. (A) Human platelets were pretreated with 0.01, 0.1 and 1 μM OPC-29030 or vehicle, and stimulated with 0.03 or 0.3 units ml^{-1} thrombin. Platelets were freeze-thawed, and cytosol and membrane fractions were prepared. The cytosol and membranes (5 μg each of protein) were resolved by SDS-polyacrylamide gel electrophoresis, and subjected to Western blot analysis for 12-lipoxygenase as described under Methods. Arrows indicate 12-lipoxygenase protein. (B) The experiments were repeated three times, and the density of the bands (volume) were analysed as described in Methods.

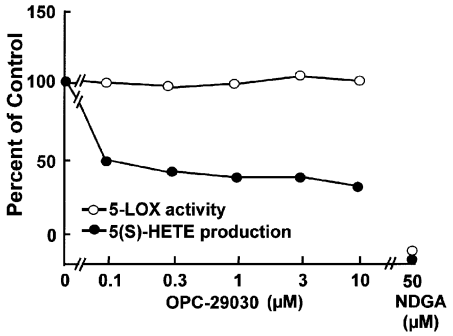

Figure 4 Effects of OPC-29030 on 5(S)-HETE production and 5-lipoxygenase activity of RBL-2H3 cells. Intact 1.8×10^8 cells (5(S)-HETE production) and the cytosol (5-Lipoxygenase; LOX) were incubated with [1-^{14}C]-arachidonic acid in the absence or presence of OPC-29030 at various concentrations, and 5(S)-HETE was quantified as described in Methods. Incubations with 50 μM NDGA were also performed.

production of 12(S)-HETE and 5(S)-HETE were in the same order of magnitiude.

Discussion

This study clearly showed that OPC-29030 suppressed 12(S)-HETE production by intact human platelets, but did not

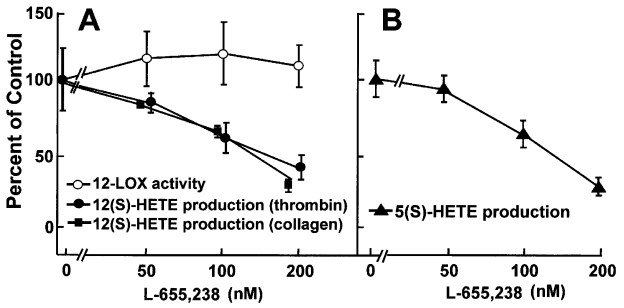

Figure 5 Effects of L-655,238 on 12(S)-HETE production in platelets and 12-lipoxygenase activity (A) and 5(S)-HETE production in RBL-2H3 cells (B). (A) Human platelets were preincubated with various concentrations of L-655,238 at 37°C for 2 min. The cells were stimulated with 0.03 units ml^{-1} thrombin or 2 $\mu g\ ml^{-1}$ collagen, and 12(S)-HETE was measured by an enzyme immunoassay. Platelet 12-lipoxygenase (LOX) activity in the cytosol was determined using [1-^{14}C]-arachidonic acid. Data represent means ± s.e.mean ($n=3$). (B) RBL-2H3 cells were preincubated with various concentrations of L-655,238 at 37°C for 2 min, and then incubated for 10 min with 25 μM [1-^{14}C]-arachidonic acid. Amount of 5(S)-HETE was determined as described in Methods.

inhibit the 12-lipoxygenase activity *per se* (Figure 1A). The results were confirmed with a HEL cell line with megakaryocyte/platelet-like properties (Figure 1B). The IC_{50} value (0.06 μM) for platelet 12(S)-HETE production was two orders of magnitude lower than that (2 μM) in HEL cells. The difference may be attributable to the assay conditions. Namely, endogenous 12(S)-HETE production was measured in human platelets, while 12(S)-HETE synthesis from exogenous arachidonic acid was determined in HEL cells. When we stimulated platelets by 25 μM arachidonic acid instead of thrombin, the IC_{50} of OPC-29030 was increased to 0.8 μM (data not shown).

With regards to the specificity of inhibition by OPC-29030, it should be noted that this compound had no effects on cyclooxygenase activity as well as the production of TXB_2 in human platelets (Figure 2). For the cellular production of 12(S)-HETE and TXB_2 from arachidonic acid in human platelets, arachidonic acid needs to be released from membrane phospholipids by phosholipases (Smith *et al*., 1986). Therefore, no inhibition of TXB_2 production indicated that the release of arachidonic acid was not affected by this compound. It is of interest that 5(S)-HETE production in RBL-2H3 cells was also partially suppressed by this compound without affecting cytosolic 5-lipoxygenase activity (Figure 4). These results suggest that OPC-29030 inhibits a certain intracellular step which is common in the generation of both 12(S)-HETE and 5(S)-HETE.

The fact that OPC-29030 suppressed 12(S)-HETE production in intact cells without 12-lipoxygenase inhibition suggests that this compound inhibits intracellular activation of this enzyme, possibly by the enzyme translocation. Upon stimulation with various agents, the enzyme translocation and activation have been demonstrated for phospholipase A_2 (Channon & Leslie, 1990), protein kinase C (Kraft & Anderson, 1983; Guy *et al*., 1986), 5-lipoxygenase (Vickers, 1995), and 15-lipoxygenase (Van Leyen *et al*., 1998). In the case of 12-lipoxygenase, the enzyme translocation and 12(S)-HETE production were dependent on the Ca^{2+} concentration in rat platelets (Baba *et al*., 1989). Our Western blot analysis showed the 12-lipoxygenase translocation from cytosol to membranes in thrombin-stimulated human platelets, and OPC-29030 inhibited the enzyme translocation in a dose-dependent manner. The decrease in 12-lipoxygenase protein in membrane fractions by OPC-29030 appeared to be correlated with the inhibition of 12(S)-HETE production in Figure 1A. However, almost the same density of bands was observed in 0.3 and 0.03 units ml^{-1} thrombin stimulation (Figure 3B) whereas sub-maximal production of 12(S)-HETE was obtained by 0.03 units ml^{-1} thrombin stimulation (data not shown). The reason for the apparent discrepancy of the results between Western blot analysis and the activity assay is unknown, but it is possible that enzyme translocation is not good enough for the promotion of 12(S)-HETE synthesis.

FLAP, an 18-kDa membrane-associated protein, was shown to be responsible for the 5-lipoxygenase translocation (Miller *et al*., 1990). Thus, FLAP-binding compounds such as MK-886 and L-655,238 have the ability to inhibit the synthesis of 5(S)-HETE and leukotriene (Ford-Hutchinson, 1990). In genetically FLAP-deficient mice, collagen-induced arthritis was markedly reduced (Griffiths *et al*., 1997). It is of particular interest that in addition to the complete loss of leukotriene biosynthesis, the plasma level of 12(S)-HETE was partially reduced in these knock-out mice. The results suggest that the FLAP may work not only for 5-lipoxygenase but for 12-lipoxygenase. A recent report also showed that FLAP was involved in the activation of a *Solanum tuberosum* lipoxygenase (Battu *et al*., 1998). In fact, a FLAP inhibitor (L-655,238) suppressed 12(S)-HETE production in human platelets stimulated with thrombin or collagen (Figure 5A). It should be noted that L-655,238 had no inhibitory effect on platelet 12-lipoxygenase activity (Figure 5A). Based on these findings, we assume that human platelet 12-lipoxygenase translocates to the membranes where FLAP or a similar protein was localized. An anti-platelet agent, OPC-29030, inhibits 12(S)-HETE production possibly by arresting this translocation step. Our appraisal can explain, at least in part, the reason why OPC-29030 suppressed 12(S)-HETE production in intact human platelet without inhibiting cytosolic 12-lipoxygenase activity. 12(S)-HETE plays roles in the expression of adhesive glycoproteins Gp IIb/IIIa (Steinert *et al*., 1995) and P-selectin (Ozeki *et al*., 1998) in human platelets, and OPC-29030 suppressed the expression of these proteins through the inhibition of 12(S)-HETE production (Katoh *et al*., 1998; Ozeki *et al*., unpublished observation). Therefore, this compound will be useful in suppressing platelet functions especially in thrombus formation, and of clinical importance in the treatment and prevention of thrombosis.

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