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Antiplatelet Activity of J78, an Antithrombotic Agent, Is Mediated by TXA_2 Receptor Blockade with TXA_2 Synthase Inhibition and Suppression of Cytosolic Ca^{2+} Mobilization

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D) Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium; EGTA, ethylene glycol bis(β-aminoethyl ether) N,N,N'N'-tetraacetic acid; Fura-2 AM, fura-2 acetoxymethyl ester; HETE, hydroxyl-eicosatetraenoic acid; J78, 2-chloro-3-[2'-bromo, 4'-fluoro-phenyl]-amino-8-hydroxy-1,4-naphthoquinone; U73122, 1-(6-((17β-3-methoxyestra- 1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione and U46619, 9,11-dideoxy-9,11-methanoepoxy-prostaglandin F_2.

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Abstract

We previously reported that 2-chloro-3-[2'-bromo, 4'-fluoro-phenyl]-amino-8-hydroxy-1,4-naphthoquinone (J78), a newly synthesized 1,4-naphthoquinone derivative, exhibited a potent antithrombotic effect, which might be due to antiplatelet rather than anticoagulation activity. In the present study, possible antiplatelet mechanism of J78 was investigated. J78 concentration-dependently inhibited rabbit platelet aggregation induced by collagen (10 μg/ml), thrombin (0.05 U/ml), arachidonic acid (100 μM) and U46619 (9,11-dideoxy-9,11-methanoepoxy-prostaglandin F_2,1 μM), a thromboxane (TX) A_2 mimic, with IC_{50} values of 0.32 ± 0.01, 0.44 ± 0.02, 0.50 ± 0.04 and 0.36 ± 0.02 μM, respectively. J78 also produced a shift to the right of the concentration-response curve of U46619, indicating an antagonistic effect on TXA_2 receptor. J78 concentration-dependently inhibited collagen-induced arachidonic acid liberation. In addition, J78 potently suppressed TXA_2 formation by platelets that were exposed to arachidonic acid in a concentration-dependent manner, but had no effect on the production of PGD_2, indicating an inhibitory effect on TXA_2 synthase. This was supported by a TXA_2 synthase activity assay that J78 concentration-dependently inhibited TXB_2 formation converted from PGH_2. Furthermore, J78 was also able to inhibit the $[Ca^{2+}]_i$ mobilization induced by collagen or thrombin at such a concentration that completely inhibited platelet aggregation. Taken together, these results suggest that the antiplatelet activity of J78 may be mediated by TXA_2 receptor blockade with TXA_2 synthase inhibition and suppression of cytosolic Ca^{2+} mobilization.

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Introduction

Platelet aggregation plays an important role in both physiological haemostatic and pathological thrombotic processes. Once vascular injury occurs, platelets will be activated by endogenous agonists such as ADP, collagen and thrombin, and adhere to the site of injury (Corti et al., 2002; Corti et al., 2003). The formation and release of thromboxane (TX) A_2 is a central component in the platelet response to a variety of agonists. TXA_2 is an eicosanoid, a metabolite of arachidonic acid formed via the cyclooxygenase (COX)-TXA_2 synthase pathway. TXA_2 binds to a G protein-coupled receptor to induce phospholipase Cβ activation which results an increase of $[Ca^{2+}]_i$ and protein kinase C activation, and causes platelets to change shape, extend pseudopods and adhere to platelets on the damaged surface. It also serves as an agonist of the TX receptors on the vascular smooth muscle cell membranes to cause vasoconstriction and proliferation of smooth muscle cells. In platelets, TXA_2 is one of the major COX-1 product of arachidonic acid metabolism. Its biosynthesis is also increased in the smooth muscle cells of patients with atherosclerosis (Fitzgerald et al., 1986). TXA_2 is considered to be one of the most powerful agonists for platelet activation and a major contributor to the thrombus formation. Therefore, inhibition of the synthesis or the action of TXA_2 is a theoretically effective means for treatment of atherothrombotic disorders, which has been demonstrated by the clinical evidences that drugs such as aspirin, picotamide and ridogrel, are benefit for the patients with acute coronary syndromes and myocardial infarction (The RAPT Investigators, 1994; Jneid et al., 2003).

The cytosolic Ca^{2+} mobilization plays a crucial role in platelet activation and aggregation. During platelet activation, the increase of $[Ca^{2+}]_i$ as a result of either Ca^{2+} influx or release from intracellular stores is fundamental to the platelet response to various agonists (Jackson et al.,

2003). Accordingly, agents with inhibition of the cytosolic Ca^{2+} mobilization in platelets may suppress the platelet aggregation (Kim et al., 1999; Shah et al., 1999; Kang et al., 2001).

The compounds with backbone of 1,4-naphthoquinone chemical structure have shown a wide variety of pharmacological effects such as antiviral, antifungal, anticancer, and antiplatelet activities (Chen et al., 2002; Lien et al., 2002). In our previous study, we have reported that 2-chloro-3-[2'-bromo, 4'-fluoro-phenyl]-amino-8-hydroxy-1,4-naphthoquinone (J78), a newly synthesized 1,4-naphthoquinone derivative, displayed a potent antithrombotic effect in mice in vivo and antiplatelet activity in vitro as well as in rat ex vivo, but had no effect on coagulation system. Available results suggest that antithrombotic effect of J78 may be due to antiplatelet activity (Jin et al., 2004). In the present study, we examined possible antiplatelet mechanism of J78 by measurements of the arachidonic acid liberation and formations of TXB_2, prostaglandin (PG) D_2 and 12-hydroxyl-eicosatetraenoic acid (HETE) from exogenous arachidonic acid in platelets. In addition, the antagonistic effect of J78 on TXA_2-mediated platelet aggregation, as well as possible inhibitory effects on TXA_2 synthase activity and cytosolic Ca^{2+} mobilization were also investigated.

Materials and methods

Chemicals

J78 was synthesized as previously described (Jin et al., 2004). In brief, a solution of 1,4-naphthoquinone (0.01 mol) and 2-bromo-4-fluorophenylamine (0.011 mol) in 150 ml of 95% EtOH was refluxed for 5 hr. After the reaction mixture was kept overnight at 4□, precipitate was collected by filtration. Crystallization of the precipitate from MeOH afforded J78. J78 had color: bright red crystal, m.p.: 135-137.0□, IR (KBr, cm^{-1}): 3315, 1494, 1240, 733; 1H-NMR (DMSO-d6): 6.88-7.41 (3H, m, benzene ring), 7.49-7.93 (3H, m), 11.48 (1H, s, OH), 9.27 (1H, s, NH), MS (m/z): 397 (M+), 288. 1,4-Naphthoquinone and 2-bromo-4-fluorophenyl amine, bovine serum albumin (BSA), collagen, dimethylsulfoxide (DMSO), Fura-2 AM and U73122 (1-(6-((17β-3-methoxyestra- 1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) were purchased from Aldrich Chemical Co. (St. Louis, MO, USA). Thrombin and arachidonic acid were purchased from Chrono-Log Co. (Havertown, PA, USA). TXB_2, PGD_2, 12-HETE and U46619 were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). [^{3}H]Arachidonic acid (250 μCi/mmol) was purchased from New England Nuclear (Boston, MA, USA). The other chemicals were of analytical grade.

Animals

New Zealand white rabbits were purchased from Sam-Tako Animal Co. (Osan, Korea) and acclimatized for 1 week at 24□ and 55% humidity, with free access to a commercial pellet diet obtained from Samyang Co. (Wonju, Korea) and drinking water before experiments. The animal studies have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals, Chungbuk National University, Korea.

Washed Platelet Preparation

Blood was withdrawn from the ear aorta of male New Zealand white rabbits and collected directly into 0.15 (v/v) of anticoagulant citrate dextrose (ACD) solution that contained 0.8% citric acid, 2.2% trisodium citrate and 2% dextrose (w/v). Washed platelet was prepared as previously described (Son et al., 2004). Briefly, platelet rich plasma (PRP) was obtained by centrifugation of rabbit blood at 230 x g for 10 min. Platelets were sedimented by centrifugation of the PRP at 800 x g for 15 min and washed with Hepes buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, and 3.8 mM Hepes, pH 6.5) containing 0.35% BSA and 0.4 mM EGTA (ethylene glycol bis(β-aminoethyl ether) N,N,N'N'-tetraacetic acid). The washed platelets were resuspended in Hepes buffer (pH 7.4) and adjusted to 4×10^8 cells/ml.

Measurement of Platelet Aggregation In Vitro

Platelet aggregation was measured by using an aggregometer (Chrono-Log Co., Havertown, PA, USA) according to the turbidimetry method of Born (1963). Briefly, washed platelet suspension of rabbits was incubated at 37□ for 4 min in the aggregometer with stirring at 1000 rpm before aggregation was challenged by the addition of collagen (10 μg/ml), thrombin (0.05 U/ml), arachidonic acid (100 μM) and U46619 (1 μM), respectively. The resulting aggregation, measured as the change in light transmission, was recorded for 10 min. In order to investigate the antagonism of J78 on U46619-induced rabbit platelet aggregation, concentration-response relationships were determined in the absence or presence of a range of concentrations of J78; for these experiments, indomethacin-treated washed rabbit platelets (50 μM for 3 min) were used to prevent any possible contribution of endogenous arachidonic acid metabolites to platelet aggregation. The extent of inhibition of platelet aggregation is expressed as % inhibition (X)

using the following equation: X (%) = (1 - B/A) × 100%, where A is the maximum aggregation rate of vehicle-treated platelets, and B is the maximum aggregation of sample-treated platelets.

Measurement of TXB_2, PGD_2 and 12-HETE Generation

The TXB_2, PGD_2 and 12-HETE generations were measured as previously described (Son et al., 2004). In brief, washed platelets (4×10^8 cells/ml) were preincubated with various concentrations of J78 at 37□ for 3 min, and then further incubated with a mixture of [^{3}H]arachidonic acid and unlabeled arachidonic acid (2 μM, 1 μCi/ml) for 5 min. The reaction was terminated by the addition of stop solution containing 2.6 mM EGTA and 130 μM BW755C (1-Phenyl-3-pyrazolidone (phenidone) and 3-amino-1-(m-(trifluoromethyl)- phenyl)-2-pyrazoline), a COX and lipoxygenase (LOX) inhibitor. Lipids were extracted and separated by thin layer chromatography (TLC) on silica gel G plates (Analtech, Delaware, USA) with the following development system: ethyl acetate/isooctane/acetic acid/H_2O (9:5:2:10, v/v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was determined by liquid scintillation counting (model LS 3801, Beckman, Buckinghamshire, UK).

TXA_2 Synthase Activity Assay

The TXA_2 synthase activity was assayed as previously described (Son et al., 2004). In brief, aliquots of PGH_2, in anhydrous acetone, were pipetted into glass tubes, then the acetone was evaporated under a gentle stream of nitrogen, and PGH_2 was re-dissolved immediately in ethanol. Platelet suspensions were incubated with the test compounds at 37□ for 3 min prior to the addition of 5 μM PGH_2. The final concentration of ethanol was 0.1% (v/v). At 5 min after addition of PGH_2, the incubations were terminated by addition of cooling EGTA (2 mM) and centrifuged at 12000 x g at 4□ for 4 min. The amount of TXB_2 in the supernatants was assayed

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by a commercial enzyme immunoassay kit according to the manufacturers' instructions (Amersham biosciences, Ltd., Little Chalfont, Bucking hamshire, UK). TXA_2 synthase activity is reflected by the production of TXB_2.

Measurement of $[Ca^{2+}]_i$

Cytosolic Ca^{2+} measurements employed the fluorescent dye fura-2, which involved incubating the platelets with cell permeant acetoxymethyl ester. Rabbit platelets (isolated as described above) were incubated with 2 μM fura-2/AM at room temperature for 1 hr (on a rocking platform) in the loading buffer (137 mM NaCl, 27 mM KCl, 0.4 mM NaH_2PO_4, 10 mM HEPES, 12 mM $NaHCO_3$, 5.5 mM dextrose, 0.35% BSA, pH 7.4). Excess fura-2/AM was removed by centrifugation (500 x g for 10 min) and the platelets were suspended in fresh buffer, without added EGTA. Aliquots of platelet suspension (2.5 ml) were added to 4 ml cuvettes containing a teflon coated stirrer bar (Chrono-log, Havertown, PA, USA). Just before $[Ca^{2+}]_i$ measurements were performed, Ca^{2+} was added back to the buffer to a final concentration of 1 mM, and then samples (various concentrations in 25 μl) and agonists were added. The measurements of $[Ca^{2+}]_i$ were performed at room temperature in a MSIII fluorimeter (Photon Technology International, S. Brunswick, NJ, USA) using excitation wavelengths of 340 and 380 as well as an emission wavelength of 505 nm. $[Ca^{2+}]_i$ was calculated by using the SPEX dM3000 software package.

Measurement of Arachidonic Acid Liberation

The arachidonic acid liberation was measured as previously described (Son et al., 2004). In brief, PRP was preincubated with [^{3}H]arachidonic acid (1 μCi/ml) at 37□ for 1.5 hr, and then washed as described above. The [^{3}H]arachidonic acid pre-labeled platelets (4×10^8 cells/ml)

were pretreated with 100 μM BW755C, various concentrations of J78 and U73122 (50 μM) at 37□ for 3 min in the presence of 1 mM $CaCl_2$, and then stimulated with collagen (50 μg/ml). The reaction was terminated by addition of chloroform/methanol/ HCl (200:200:1, v/v/v). Lipids were extracted and separated by TLC on silicagel G plates with the following development system: petroleum ether/diethyl ether/acetic acid (40/40/1, v/v/v). The area corresponding to each lipid was scraped off and the radioactivity was determined by liquid scintillation counting.

Statistical Analysis

The experimental results were expressed as mean ± S.E.M.. A one-way analysis of variance (ANOVA) was used for multiple comparison (Sigma Stat®, Jandel Co., San Rafael, CA, USA). If there was a significant variation between treated-groups, Dunnett's test was applied. Differences with $P < 0.05$ were considered statistically significant.

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Results

Effect of J78 on Rabbit Platelet Aggregation In Vitro

As shown in Fig. 1, J78 concentration-dependently inhibited collagen (10 μg/ml)-, U46619 (1 μM)-, thrombin (0.05 U/ml)- and arachidonic acid (100 μM)-challenged washed rabbit platelet aggregation, with IC_{50} values of 0.32 ± 0.01, 0.36 ± 0.02, 0.44 ± 0.02 and 0.50 ± 0.04 μM, respectively.

Effects of J78 on Conversions of Arachidonic Acid to TXB_2, PGD_2 and 12-HETE in Rabbit Platelet

As shown in Fig. 2, J78 concentration-dependently suppressed TXB_2 generation, which reflected the formation of TXA_2, induced by addition of [^{3}H]arachidonic acid in intact rabbit platelets. The TXB_2 formations were inhibited by 21.8, 35.4, 53.7 and 76.3% at the concentrations of 0.2, 0.4, 0.6 and 0.8 μM, respectively. J78, however, has no effect on PGD_2 generation. These results suggest that J78 may selectively inhibit activity of TXA_2 synthase rather than that of COX, because TXA_2 and PGD_2 are simultaneously produced from arachidonic acid through COX pathway. In addition, J78 has no effect on 12-HETE production (data not shown), suggesting that LOX-pathway was not involved in the antiplatelet effect of J78.

Effect of J78 on TXA_2 Synthase Activity in Rabbit Platelet

The conversion of arachidonic acid to TXA_2 in platelets requires the action of two enzymes, COX and TXA_2 synthase. TXA_2 synthase catalyzes the conversion of PGH_2 to TXA_2 in platelets. By utilizing PGH_2, it is possible to circumvent the COX step during arachidonic acid

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metabolism. The addition of increasing concentrations of PGH_2 to washed rabbit platelet suspensions produced a concentration-dependent increase of TXB_2 (data not shown). Thus, washed rabbit platelet suspensions containing PGH_2 are adequate for the direct evaluation of the TXA_2 synthase inhibitor. In washed rabbit platelet suspensions, the level of TXB_2 in unstimulated platelets was about 15 ng/4 × 10^8 platelets. After incubation of washed platelet suspensions with PGH_2 (5 μM) at 37□ for 5 min, TXB_2 formation was increased to 88.8 ng/4 × 10^8 platelets. As shown in Fig. 3, J78 inhibited the conversion of PGH_2 into TXB_2 by 9.8, 20.3, 45.3 and 63.1% at concentrations of 0.2, 0.4, 0.6 and 0.8 μM in washed rabbit platelet suspensions, respectively. Imidazole, a typical TXA_2 synthase inhibitor, also markedly inhibited the conversion of PGH_2 into TXB_2.

Effect of J78 on U46619-Induced Rabbit Platelet Aggregation

When J78 was preincubated with washed rabbit platelets for 3 min, it reduced platelet aggregation elicited by various concentrations of U46619 under COX blockade with indomethacin in a concentration-dependent manner. At concentrations of 0.36 and 0.80 μM, J78 produced a shift to the right of the concentration-response curve of U46619, suggesting an antagonism on TXA_2 receptor (Fig. 4).

Effect of J78 on $[Ca^{2+}]_i$ in Rabbit Platelet

The representative traces in which two different agonists were added to induce $[Ca^{2+}]_i$ mobilization were shown in Fig. 5. The effect of J78 on $[Ca^{2+}]_i$ mobilization was observed after 3 min incubation with platelet before adding the respective inducers. Collagen induced a slow but stable increase of $[Ca^{2+}]_i$ which reaches the peak level of 300 μM after 5 min. Whereas, thrombin caused a rapid but transient increase in $[Ca^{2+}]_i$. Treatment of the platelet suspension

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with J78 (0.8 μM) almost completely inhibited the elevation of the $[Ca^{2+}]_i$ in response to collagen and thrombin, respectively. The right panels in A and B (Fig. 5) are the average of 3 times separated experiments, similar to that shown in left panels in A and B.

Effect of J78 on Collagen-Induced Arachidonic Acid Liberation in Rabbit Platelet

As shown in Fig. 6, pretreatment of J78 concentration-dependently inhibited collagen-induced arachidonic acid liberation in [^{3}H]arachidonic acid pre-labeled rabbit platelets by 2.6, 16.8, 33.7 and 55.6% at concentrations of 0.2, 0.4, 0.6 and 0.8 μM, respectively. U73122, a phospholipase C inhibitor which was used as a positive control, completely blocked arachidonic acid liberation at a concentration of 50 μM.

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Discussion

Platelet aggregation is a complex process, and it is generally considered that platelet activation is mainly mediated through adhesiveness of platelets to the site of injury and through the action of endogenous agonists like ADP, collagen and thrombin, following by the release of TXA_2 which acts as an amplifying factor in the platelet aggregation (Jackson et al., 2003; Farndale et al., 2004). The important role of TXA_2 has been demonstrated by the clinical effectiveness of aspirin in the prevention of cardiovascular diseases such as acute coronary syndromes (Awtry and Loscalzo, 2000; Catella-Lawson et al., 2001; Jneid et al., 2003). In the present study, we demonstrate that the antiplatelet activity of J78, an antithrombotic agent, may be mediated by TXA_2 receptor blockade with TXA_2 synthase inhibition and suppression of $[Ca^{2+}]_i$ mobilization.

It is well known that U46619, which is a TXA_2 mimic, acts directly on the TXA_2 receptor to induce G protein-coupled phospholipase Cβ activation, resulting an increase of $[Ca^{2+}]_i$ and protein kinase C activation (Jackson et al., 2003). Similarly, arachidonic acid, which acts directly on membrane COX enzyme pathway to produce TXA_2, mediates platelet activation in the same way as U46619 (Parise et al., 1984). From the platelet aggregation study (Fig. 1), J78 inhibited TXA_2-mediated platelet aggregation such as arachidonic acid- and U46619-induced aggregation in a concentration-dependent manner, suggesting that J78 may interfere with the TXA_2 synthesis or its action directly. Therefore, the effect of J78 on the generation of TXA_2 was firstly determined by using [^{3}H]arachidonic acid in intact rabbit platelet. As shown in Fig. 2, J78 concentration-dependently inhibited [^{3}H]TXA_2 formation, whereas had no effect on the production of [^{3}H]PGD_2. These results indicate that J78 may selectively inhibit the activity of TXA_2 synthase rather than that of COX, because TXA_2 and PGD_2 are simultaneously produced

from arachidonic acid through COX pathway in platelets. It was also confirmed by a TXA_2 synthase activity assay that J78 potently reduced PGH_2-, a precursor of the PGs and TXA_2, mediated TXB_2 formation (Fig. 3). Although the inhibition of TXA_2 synthesis by TXA_2 synthase inhibition is theoretically rational to inhibit platelet aggregation, considering that accumulated PGH_2, the precursor of TXA_2, can interact with the same receptor (TXA_2/PGH_2 receptor) as TXA_2 to induce platelet activation, it seems that J78 may also have any inhibitory effect on TXA_2/PGH_2 receptor directly. Therefore, the possible inhibitory effect of J78 on TXA_2/PGH_2 receptor was investigated. Indeed, it completely inhibited platelet aggregation induced by the stable TXA_2 mimic, U46619 in rabbit platelets by shifting, in a parallel way, the concentration-response curve to U46619 to the right (Fig. 4), suggesting that J78 may specifically block platelet TXA_2/PGH_2 receptor, possibly in a competitive manner. It has also been reported that other drugs possessing the dual property of TXA_2/PGH_2 receptor antagonistic and TXA_2 synthase inhibitory effects act also as competitive antagonists of U46619-induced aggregation (Chang et al., 1997; Rolin et al., 2001; Miyamoto et al., 2003). These results correlated well with the in vivo antithrombotic effect of J78 on the murine pulmonary thrombosis (Jin et al., 2004), the lethal effect of collagen plus epinephrine on which is caused by massive occlusion of microcirculation of lung by platelet thromboembolism or by vasoconstriction due to the release of TXA_2 and $PGF_{2\alpha}$ from activated platelets.

In addition, it seems that J78 may also have an inhibitory effect on cytosolic Ca^{2+} mobilization, as J78 inhibited calcium ionophore, A23187-induced platelet aggregation concentration-dependently (Jin et al., 2004). Concomitant with aggregation data, in the platelets loaded with Fura-2 AM, J78 completely blocked the cytosolic Ca^{2+} mobilization induced by collagen and thrombin at a concentration of 0.8 μM which is sufficient to inhibit platelet aggregation completely (Fig. 5). Thrombin and collagen, both of which are strong agonists,

have different platelet aggregation mechanisms (Colman et al., 1994). Thrombin interacts with platelet through a specific receptor belonging to the superfamily of receptors that are coupled to G proteins and phospholipase Cβ, producing diacylglycerol, which stimulates protein kinase C closely linked to secretion. Inositol trisphosphate is also produced and plays a role in increasing $[Ca^{2+}]_i$ (Lapetina, 1990); whereas, collagen induces platelet activation through a tyrosine kinase-based signaling pathway that involves the kinase Syk and phospholipase Cγ2, which results in $[Ca^{2+}]_i$ increase, shape change and granule release; adhesion is partly and aggregation is largely dependent on ADP and TXA_2/PGH_2 release (Farndale et al., 2004). Considering that inhibition of TXA_2 synthesis was only partly effective to inhibit the collagen- or thrombin-mediated platelet aggregation, and several compounds with the same 1,4-naphthoquinone backbone as J78 have been reported to inhibit the rise of $[Ca^{2+}]_i$ by suppression of phosphoinositide breakdown in various cell types including platelet (Chang et al., 1993; Wang and Kuo, 1997; Chen et al., 2002), it is reasonable to speculate that antiplatelet activity of J78 was not only mediated by the TXA_2/PGH_2 receptor antagonism and TXA_2 synthase inhibition, but also by the inhibition of cytosolic Ca^{2+} mobilization, possibly by interfering with the phosphoinositide breakdown. This was indirectly supported by the arachidonic acid liberation assay that J78 inhibited collagen-mediated arachidonic acid liberation from [^{3}H]arachidonic acid prelabelled rabbit platelets in a concentration-dependent manner (Fig. 6). In fact, in lower concentrations (1-20 μg/ml) of collagen-mediated platelet activation, inhibition of phosphoinositide breakdown was able to block the arachidonic acid liberation completely (Balsinde et al., 2002).

Recent studies have demonstrated that injury-induced vascular proliferation and platelet activation are depressed in mice genetically deficient in the TXA_2 receptor or treated with a TXA_2 antagonist (Cheng et al., 2002). Thus, it is conceivable that J78 might be particularly effective for the improvement of atherothrombotic conditions associated with platelet activation.

Such drugs as picotamide and ridogrel that act both as TXA_2 synthase inhibitors and as TXA_2/PGH_2 receptor antagonists have been developed. Picotamide may be an effective drug in patients with peripheral occlusive arterial disease of the lower limbs and cerebral infarction (Coto et al., 1989; Coto et al., 1998). Ridogrel seems to have efficacy in patients with peripheral occlusive arterial disease and acute myocardial infarction (Hoet et al., 1990; Neirotti et al., 1994).

In summary, our results demonstrate that antiplatelet activity of J78 may, at least partly, result from a combination of TXA_2 receptor blockade with TXA_2 synthase inhibition and a suppression of $[Ca^{2+}]_i$ mobilization.

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Footnotes

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Legends for Figure

Fig. 1. Effect of J78 on washed rabbit platelet aggregation. Washed rabbit platelet suspension was incubated at 37□ in an aggregometer with stirring at 1,000 rpm, and then J78 was added. After 3 min preincubation, the platelet aggregation was induced by addition of thrombin (0.05 U/ml), arachidonic acid (100 μM), collagen (10 μg/ml) or U46619 (1 μM), respectively. The aggregation percents were expressed as % of maximum aggregation induced by respective inducers. Data are expressed as mean ± S.E.M. (n = 4).

Fig. 2. Effects of J78 on conversions of arachidonic acid to TXB_2 and PGD_2. Washed rabbit platelets were preincubated with various concentrations of J78 for 3 min without $CaCl_2$, and then further incubated with a mixture of [^{3}H]arachidonic acid and the unlabelled arachidonic acid (2 μM) for 5 min. The [^{3}H]thromboxane B_2 generation was measured as described under Materials and Methods. Data are expressed as mean ± S.E.M. (n = 3). $^*P<0.01$ and $^{**}P<0.005$ vs. corresponding stimulus control.

Fig. 3. Effect of J78 on TXA_2 synthase activity. After preincubation of indomethacin (50 μM) at 37□ for 2 min, platelet suspension containing DMSO (0.1%), J78 or imidazole (50 mM) was further incubated for 3 min, and then 5 μM PGH_2 was added. At 5 min after the addition of PGH_2, the incubations were terminated by addition of cooling EGTA (2 mM) and centrifugation at 12000 x g at 4□ for 4 min. TXB_2 formation in the supernatants was determined by enzymeimmunoassay. TXA_2 synthase activity is reflected by the production of TXB_2, which is presented as mean ± S.E.M. (n = 3). $^*P < 0.05$ and $^{**}P < 0.01$ vs. corresponding stimulus control.

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Fig. 4. Effect of J78 on rabbit platelet aggregation induced by U46619. Indomethacin(50 μM)-treated rabbit platelets for 3 min were used to prevent any possible contribution of endogenous arachidonic acid metabolites to platelet aggregation. Washed rabbit platelets were incubated with J78 or DMSO (0.1%) at 37□ for 4 min, and then U46619 was added to trigger aggregation. The peak level of aggregation was measured for 4 min after the addition of stimulator. The percentage of aggregation was calculated assuming the maximum value of the control (absence of drug) produced by U46619 (1 μM) to be 100%. Each data point is expressed as mean ± S.E.M. (n = 3).

Fig. 5. Effects of J78 on $[Ca^{2+}]_i$ in collagen- and thrombin-stimulated rabbit platelet. Calcium (1 mM) was added to the platelet suspension 10 sec before data collection started (zero time). J78 solution was added to yield a final concentration of 0.8 μM in the platelet suspension. Collagen (10 μg/ml) or thrombin (0.05 U/ml) was added 3 min later. The traces (left panels in A and B) shown are from a representative experiment; similar results were obtained from three times separate experiments and average data are presented in right panels in A and B. $^{**}P<0.01$ vs. corresponding stimulus control.

Fig. 6. Effect of J78 on collagen-induced arachidonic acid liberation in rabbit platelet. [^{3}H]Arachidonic acid-prelabelled platelets were incubated with various concentrations of J78 or U73122 at 37□ for 2 min in the presence of 50 μM BW755C, and then stimulated with 50 μg/ml collagen for 2 min. Liberated [^{3}H]arachidonic acid was determined as described in Materials and Methods. Each point is expressed as mean ± S.E.M. (n = 3). $^{*}P<0.05$ and $^{**}P<0.01$ vs. corresponding stimulus control.

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Fig. 1

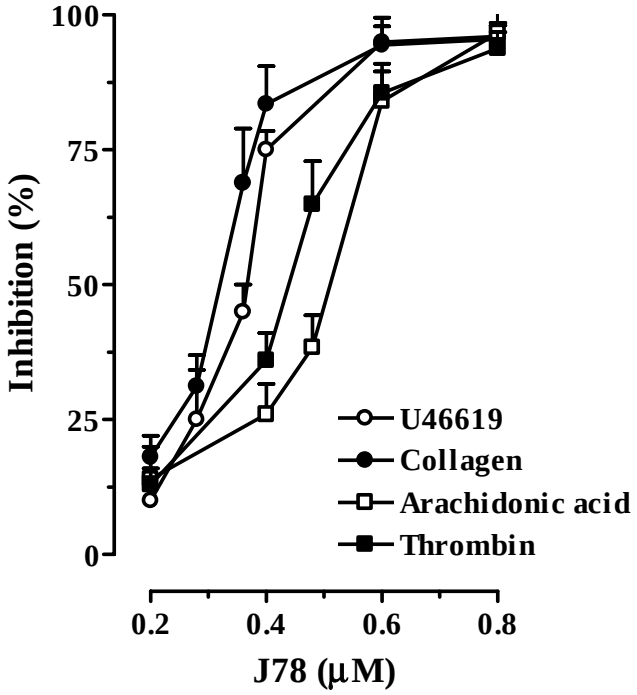

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Fig. 2

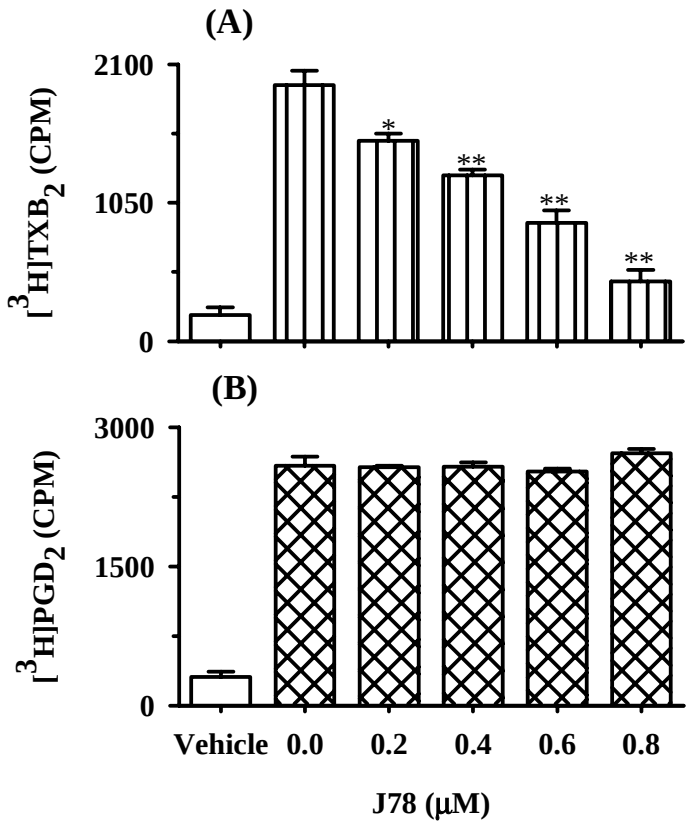

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Fig. 3

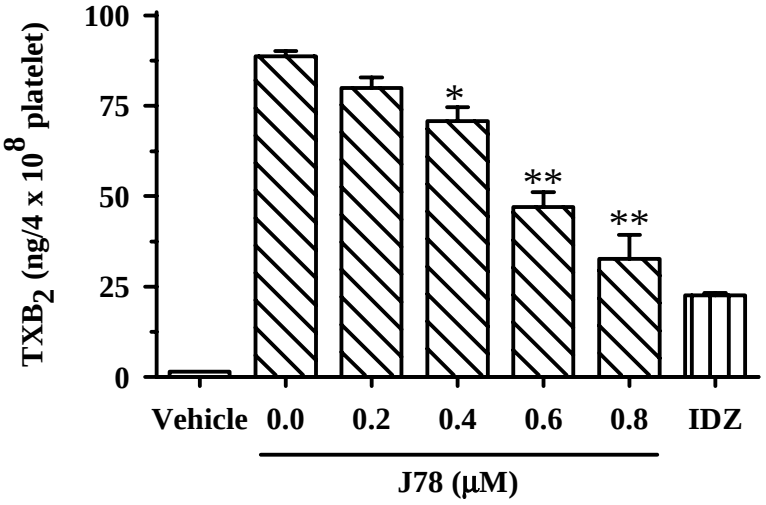

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Fig. 4

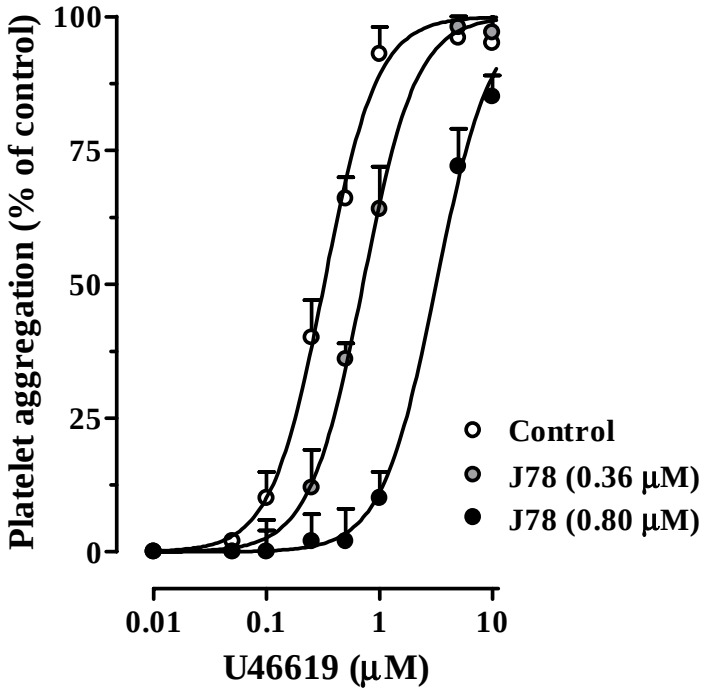

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Fig. 5

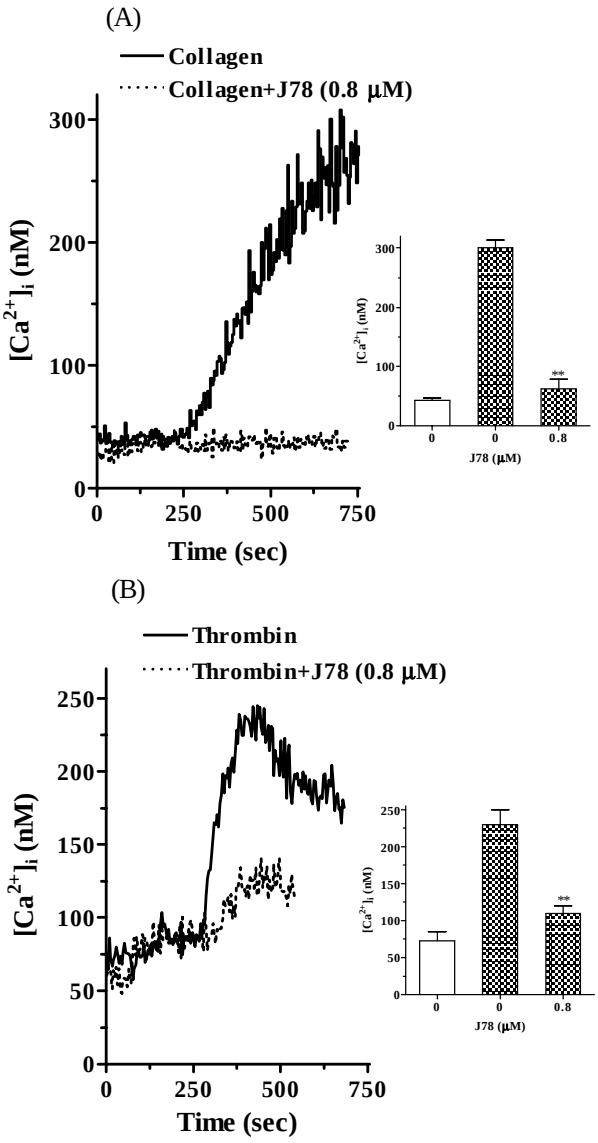

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Fig. 6