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JJK694, a Synthesized Obovatol Derivative, Inhibits Platelet Activation by Suppressing Cyclooxygenase and Lipoxygenase Activities

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Obovatol has various biological activities, including anti-proliferative, neurotrophic, anti-fibrillogenic, anti-platelet, anti-fungal and anti-inflammatory activities. In this study, we investigated the effects of JJK694, a synthesized obovatol derivative, on rabbit platelet activation and its molecular mechanisms. JJK694 significantly inhibited washed rabbit platelet aggregation and serotonin secretion induced by collagen and arachidonic acid, but had little effect on thrombin- or U46619-induced aggregation. These results suggest that JJK694 selectively inhibits collagen- and arachidonic acid-mediated signaling. JJK694 also showed a concentration-dependent decrease in cytosolic Ca^{2+} mobilization, but it had no effect on arachidonic acid liberation. On the other hand, it significantly inhibited the formation of arachidonic acid metabolites, including thromboxane A_2 (TXA_2), prostaglandin D_2, and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), by suppression of cyclooxygenase (COX)-1 and lipoxygenase (LOX) activities. These results indicate that JJK694 hasanti-platelet activities through inhibition of arachidonic acid metabolite production by suppression of COX-1 and LOX activities.

Key words: anti-platelet activity; obovatol derivative; arachidonic acid metabolites; cyclooxygenase; lipoxygenase

Platelets play crucial roles in the development and complications of coronary heart disease. Platelet adhesion after response to injury by plaque rupture or erosion of the atherosclerotic artery leads to the subsequent cascades of thrombus formation, but it also contributes to the formation of pathogenic thrombus associated with acute clinical symptoms of thrombotic disease in atheroma, such as transient ischemic attack, myocardial infarction, acute coronary syndrome, and stroke.[1–3)]

Cyclooxygenase (COX)-1 and lipoxygenase (LOX) are two major enzyme families that catalyze the rate-limiting step in the formation of prostanoids, prostaglandins (PGs), including PGD_2, and thromboxane A_2 (TXA_2) by the COX-1 pathway, and leukotrienes (LTs), including 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE), by the LOX pathway, whose products are significant mediators of pain, fever and inflammation.[4)] TXA_2, formed by platelets, has been reported to be a potent constrictor of blood vessels and an aggregator of platelets, and is activated by various agonists such as collagen, arachidonic acid (AA), adenosine diphosphate (ADP), epinephrine, and the platelet activating factor (PAF).[5,6)] In platelets, TXA_2 is produced mainly *via* COX-1-TXA_2 synthase from AA, which is increased dramatically *via* the action of phospholipase (PL) C and PLA_2-mediated phospholipid hydrolysis when platelets are activated by various inducers.[7–11)] On the other hand, 12-HETE, a parallel metabolite of AA *via* 12-LOX, has also been reported to act as a platelet activator promoting thrombus formation *in vivo*[12,13)] and to give rise to platelet aggregation and aortic smooth muscle cell migration *in vitro*.[14–16)] It is likely that 12-HETE as well as TXA_2 is involved in the initiation and propagation of thrombotic and atherosclerotic disorders. Recently, anti-platelet agents that have inhibitory effects on TXA_2 production and 12-HETE formation have proven beneficial for patients with cardiovascular diseases.

Magnolia extract contains various ingredients, including flavonoids, alkaloids, coumarins, lignans, neolignans, terpenoids, and phenylpropanoids.[17)] Among Magnolia extracts, obovatol is known to have various

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Abbreviations: AA, arachidonic acid; TXA_2, thromboxane A_2; PGD_2, prostaglandin; COX, cyclooxygenase; LOX, lipoxygenase; PLC, phospholipase C; 12-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; JY0695, 5-allyl-3-(4-allylphenoxy)-1,2-phenylene diacetate; U46619, 9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$; BSA, bovine serum albumin; Fura 2/AM, fura 2 acetoxymethyl ester; OPT, *o*-phthalaldehyse; DMSO, dimethyl sulfoxide; ACD, anticoagulant citrate dextrose; TCA, trichloroacetic acid; PRP, platelet-rich plasma

biological activities, including anti-proliferative,[18–20] neurotrophic,[21] anti-fibrillogenic,[22] anti-platelet,[23,24] anti-fungal,[25] and anti-inflammatory activities.[26] Park *et al.* found that obovatol inhibited platelet activation *in vitro* and thrombus formation *in vivo*.[18] Hence, the present study focused on the development of obovatol derivatives as anti-platelet agents. Recently, we prepared a synthetic obovatol derivative and named it JJK694 (Fig. 1A), and investigated the effects of obovatol derivative on platelet activation and possible molecular mechanisms.

Materials and Methods

Materials and animals. JJK694 (5-propyl-3-(4-propylphenoxy) bezene-1,2-diol, Fig. 1A) was kindly provided by Professor Jae-Kyung Jung of the College of Pharmacy, Chungbuk National University, Korea. Collagen, arachidonic acid (AA), ADP, and thrombin were purchased from Chrono-Log (Havertown, PA). U46619 (9,11-dideoxy-9α,11α-methanoepoxy prostaglandin $F_{2\alpha}$), TXB_2, PGD_2, and 12-HETE were from Cayman Chemical (Ann Arbor, MI). Indomethacin, bovine serum albumin (BSA), ethylene glycol-bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), fura-2 acetoxymethyl ester (Fura-2/AM), serotonin creatinine sulfate, *o*-phthalaldehyse (OPT), imipramine, and dimethyl sulfoxide (DMSO) were from Sigma Chemical (St. Louis, MO). [^{3}H] AA (250 μCi/mmol) was from New England Nuclear (Boston, MA). All other chemicals were of analytical grade. New Zealand white rabbits were purchased from Sam-Tako Animal (Osan, Korea). They acclimatized for 1 week at 24 °C at 55% humidity. They had free access to a commercial pellet diet from Samyang (Wonju, Korea) and drinking water before the experiments.

Synthesis and characterization of JJK694. ^{1}HNMR spectra were recorded on a Bruker DPS300 spectrometer in deuteriochloroform ($CDCl_3$). Chemical shifts were expressed in parts per million (ppm, δ) downfield of tetramethylsilane, and are referenced to the deutered solvent ($CHCl_3$). Reagents were purchased from Aldrich Chemical. JJK694 (5-propyl-3-(4-propylphenoxy) bezene-1,2-diol) prepared by saturation of obovatol with ethanol was added Pd/C (10 mg) by a method reported elsewhere. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.14 (d, 2H, $J = 8.4$ Hz), 6.93 (d, 2H, $J = 8.4$ Hz), 6.56 (s, 1H), 6.28 (s, 1H), 2.57 (t, 2H, $J = 7.6$ Hz), 2.41 (t, 2H, $J = 7.6$ Hz), 1.70–1.42 (m, 4H), 0.95 (t, 3H, $J = 7.3$ Hz), 0.88 (t, 3H, $J = 7.3$ Hz).

Washed rabbit platelet preparation and platelet aggregation assay. Blood was withdrawn from the ear artery 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% sodium citrate, and 2% dextrose (w/v). Washed platelets were prepared as described previously.[27] Briefly, platelet rich plasma (PRP) was obtained by centrifugation of rabbit blood at 230 × *g* for 10 min. The platelets were sedimented by centrifugation of the PRP at 800 × *g* for 15 min, and were 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 were adjusted to 4×10^8 cells/mL. Platelet aggregation was measured by aggregometer (Chrono-Log, Havertown, PA) by the turbidimetry method of Born,[28] as described previously.[29] Briefly, the washed platelet suspension was incubated at 37 °C in the aggregometer with stirring at 1,000 rpm, and then DMSO or various concentrations of JJK694 were added. After 3 min of preincubation, platelet aggregation was induced by the addition of collagen (10 μg/mL), AA (100 μM), U46619 (1 μM), or thrombin (0.05 U/mL).

Cell viability. Cell viability of platelets was determined as described previously.[30] In brief, cell death detection of platelets by JJK694 was performed cell counting Kit 8 following the manufacturer's instructions (Wako, Tokyo). *In vitro* viability was determined by measuring reduced formazan, which is a colorimetric assay based on the reduction of tetrazolium salt by cellular NADH or NADPH. Working solution (10 μL) containing WST-8 reagent and JJK694 was added to the PRP (200 μL) containing the platelets (3×10^8 cells/mL) in a 96-well microtiter plate (Disposable Products, Adelaide). Over 2 h at 37 °C, the absorbance of the colored product (formazan dye) was read on a microplate reader (Well Reader SK601, Seikagaku, Tokyo), using a test wavelength of 450 nm against a reference wavelength of 650 nm.

Serotonin secretion assay. Serotonin release was determined as described previously.[27,31] In brief, to prevent re-uptake of secreted serotonin, imipramine (a serotonin re-uptake inhibitor, 5 μM) was added to the platelet suspension. Washed rabbit platelets were treated with various concentrations of JJK694 or DMSO at 37 °C for 3 min prior to the addition of the agonist (collagen 10 μg/mL or AA 100 μM) over 5 min. An aliquot (0.35 mL) of the washed rabbit platelets was mixed with 5 mM EDTA on ice, and the mixture centrifuged at 12,000 × *g* for 2 min. The supernatant was mixed with 6 M trichloroacetic acid (TCA) and centrifuged at 12,000 × *g* for 2 min. An aliquot (0.3 mL) of the TCA supernatant was mixed with 1.2 mL of the solution (0.5% OPT in ethanol diluted 1:10 with 8 N HCl), left in a boiling water bath for 10 min, and then cooled on ice. The excess lipids were extracted with chloroform, and the fluorophore was measured at excitation and emission wavelengths of 360 nm and 475 nm respectively.

Cytosolic calcium mobilization assay. Cytosolic Ca^{2+} mobilization was measured by fluorescent dye fura-2/AM, as described previously,[27,31] which involved incubating the platelets with cell permeant acetoxy methyl ester. Rabbit platelets were incubated with 2 μM fura-2/AM at room temperature for 1 h 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 × *g* for 10 min), and the platelets were suspended in fresh buffer without added EGTA. Aliquots of the platelet suspension (2.5 mL) were inserted into a 4 mL cuvette containing a teflon coated stirrer bar (Chrono-Log, Havertown, PA). Just before $[Ca^{2+}]_i$ measurement was done, Ca^{2+} was added back to the buffer to a final concentration of 1 mM, and then JJK694 (25 μL at various concentration) and collagen (10 μg/mL) and AA (100 μM) were added. Measurement of $[Ca^{2+}]_i$ was done at room temperature in a MSIII fluorimeter (Photon Technology International, South Brunswick, NJ) at excitation wavelengths of 340 and 380 and an emission wavelength of 505 nm. $[Ca^{2+}]_i$ was calculated using the SPEX dM3000 software package.

Arachidonic acid liberation assay. AA liberation was measured as previously described.[27,31] In brief, PRP was preincubated with [^{3}H] AA (1 μCi/mL) at 37 °C for 1.5 h, and then washed as described above. [^{3}H] AA labeled platelets (4×10^8 cells/mL) were pretreated with 100 μM BW755C, a COX-1 and LOX inhibitor, and various concentrations of JJK694 at 37 °C for 3 min in the presence of 1 mM $CaCl_2$, and then stimulated with collagen (10 μg/mL). The reaction was terminated by the addition of chloroform/methanol/HCl (200/200/1, v/v/v). Lipids were extracted and separated by TLC on silica gel G plates by the following development system: petroleum ether/diethyl ether/acetic acid (40/40/1, v/v/v). The area corresponding to each of the lipids was scraped off, and radioactivity was determined by liquid scintillation counting.

Measurement of arachidonic acid metabolites. TXB_2, PGD_2 and 12-HETE generations were measured as previously described.[27,31] In brief, washed rabbit platelets (4×10^8 cells/mL) were preincubated with various concentrations of JJK694 at 37 °C for 3 min and further incubated with a mixture of [^{3}H] AA and unlabeled AA (2 μM, 1 μCi/mL) for 5 min. The reaction was terminated by the addition of stop solution (2.6 mM EGTA, 130 μM BW755C). Lipids were extracted and separated by TLC on silica gel G plates by the following development system: ethyl acetate/isooctane/acetic acid/H_2O (11/5/2/10, v/v/v/v). The area corresponding to each of the lipids was scraped off and radioactivity was measured by liquid scintillation counting.

Statistical analysis. Experimental results were expressed as mean ± SEM. One-way analysis of variance (ANOVA) was used for

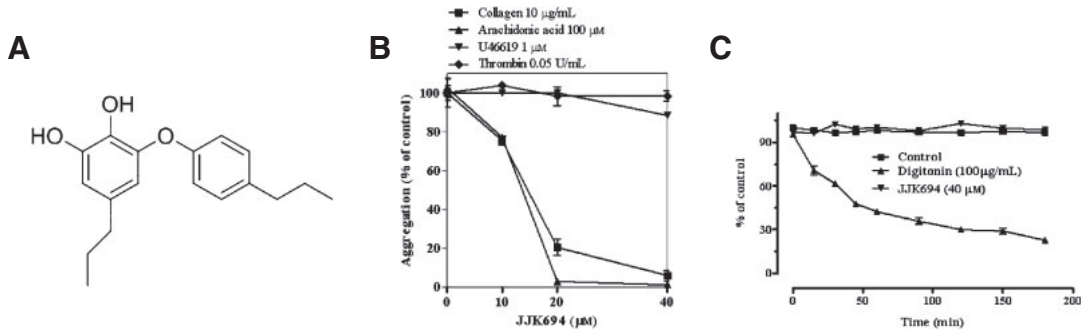

Fig. 1. Chemical Structure of JJK694 and Effects of JJK694 on Washed Rabbit Platelet Aggregation and Cell Viability.

Washed platelets were prepared and platelet aggregation and cell viability were measured as described in "Materials and Methods." (A) Chemical structure of JJK694; (B) Platelet aggregation induced by collagen (10 μg/mL), arachidonic acid (100 μM), U46619 (1 μM), and thrombin (0.05 U/mL); (C) Cell viability under JJK694. The aggregation percentages are expressed as percentages of maximum aggregation by the respective inducers. Data are expressed as mean ± SEM (n = 4).

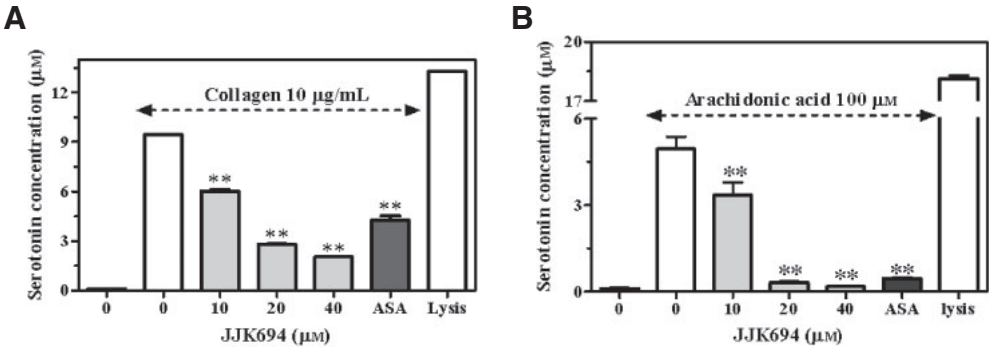

Fig. 2. Effects of JJK694 on Serotonin Secretion.

Washed rabbit platelet suspension was incubated with imipramine (5 μM) and various concentrations of JJK694 at 37 °C for 3 min prior to the addition of collagen (10 μg/mL) (A), arachidonic acid (100 μM) (B). The serotonin concentration was determined by a fluorimetric method, as described in "Materials and Methods." Aspirin (ASA, 100 μM), an anti-platelet agent, was used as positive control. Data are expressed as mean ± SEM (n = 3). $^{**}p < 0.01$ *vs.* stimulus control.

multiple comparison, followed by Dunnett's test (GraphPad, San Diego, CA). Data were considered significant $p < 0.05$.

Results

Effects of JJK694 on washed rabbit platelet aggregation in vitro

To determine the selectivity of JJK694 on various agonist-induced forms of platelet aggregation, agonist concentrations were determined by a preliminary study and set at 10 μg/mL for collagen, 100 μM for AA, 1 μM for U46619, and 0.05 U/mL for thrombin. As shown in Fig. 1B, JJK694 significantly inhibited collagen- and AA-induced washed rabbit platelet aggregation, with IC_{50} values of 13.14 ± 1.25 and 11.85 ± 5.50 μM, respectively, but it had little effect on U46619- or thrombin-induced platelet aggregation. These results indicate that JJK694 selectively affects collagen- and AA-mediated signal transduction in platelet activation. In addition, we confirmed by cell viability assay that this anti-platelet effect of JJK694 on platelets was not due to cellular cytotoxicity (Fig. 1C).

Effects of JJK694 on serotonin secretion

JJK694 inhibited the platelet aggregation induced by collagen and AA, and it reduced the release of serotonin. In this result, JJK694 reduced the release of serotonin in a concentration-dependent manner, with inhibition percentages of 36.2, 70.1, and 78.4% for collagen (Fig. 2A), and 32.6, 93.6, and 96.4% for AA (Fig. 2B) at 10, 20, and 40 μM, respectively. In addition, total serotonin content was expressed as lysis (Fig. 2).

Effects of JJK694 on cytosolic calcium mobilization

Anti-platelet agents that block calcium signaling can exert their beneficial action by inhibition of platelet activation. Agonists, collagen and AA, added to induce $[Ca^{2+}]_i$ mobilization are shown in Fig. 3. Treatment of the platelet suspension with JJK694 inhibited the elevation of $[Ca^{2+}]_i$ in response to collagen by 51.8, 69.2, and 85.0% (Fig. 3A) in response to AA by 53.0, 87.1, and 93.3% (Fig. 3B) at concentrations of 10, 20, and 40 μM, respectively. Bar graphs of right panel in Fig. 3 show the average of three separate experiments.

Effects of JJK694 on arachidonic acid and diacylglycerol liberation

As shown in Fig. 4, pretreatment with JJK694 had no effect on collagen-induced arachidonic acid (A) or diacylglycerol liberation (B) in [^{3}H] AA pre-labeled rabbit platelets. U73122, a PLC inhibitor used as positive control, significantly blocked arachidonic acid liberation at a concentration of 20 μM.

Effects of JJK694 on TXB_2, PGD_2, and 12-HETE formation

JJK694 concentration dependently suppressed TXB_2 (A), PGD_2 (B), and 12-HETE (C) generation induced by

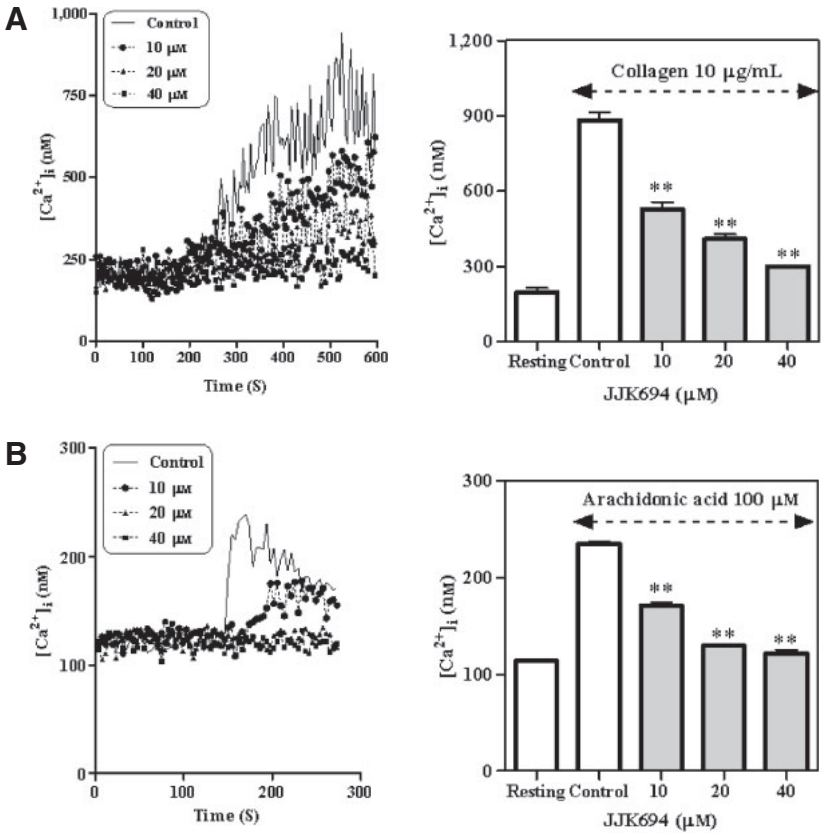

Fig. 3. Effect of JJK694 on Agonist-Stimulated $[Ca^{2+}]_i$.

Calcium (1 mM) was added to the platelet suspension 10 s before data collection (time 0). JJK694 was added to final concentrations of 10, 20, and 40 μM in the platelet suspension. Collagen (10 μg/mL) (A), or arachidonic acid (100 μM) (B) was added 3 min later. Taces are from a representative experiment, with similar results right panel as mean ± SEM (n = 3). $^{**}p < 0.01$ *vs.* stimulus control.

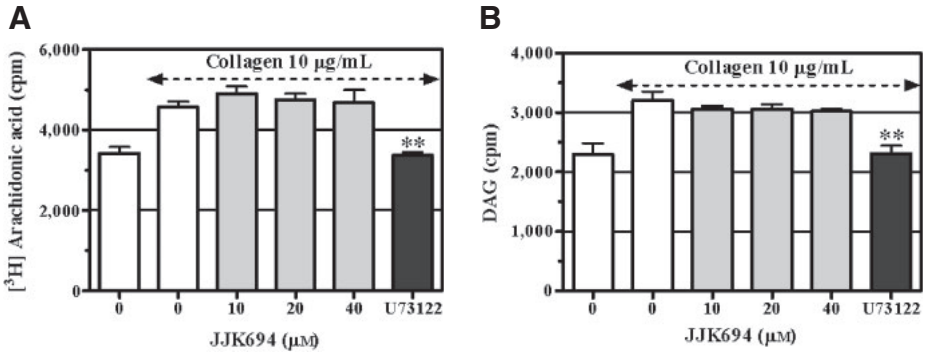

Fig. 4. Effect of JJK694 on Collagen-Induced Arachidonic Acid Liberation.

[^{3}H] Arachidonic acid-prelabeled platelets were incubated with various concentrations of JJK694 at 37 °C for 3 min in the presence of 100 μM BW755C, and then stimulated with 50 μg/mL of collagen for 2 min. Liberated [^{3}H] arachidonic acid was measured as described in "Materials and Methods." Data are expressed as mean ± SEM (n = 3). $^{**}p < 0.01$ *vs.* stimulus control.

addition of [^{3}H] AA to intact rabbit platelets (Fig. 5). These results suggest that JJK694 inhibits COX-1 and LOX activities rather than TXA_2 synthase, because TXA_2, PGD_2, and 12-HETE are simultaneously produced from AA through the COX-1 and LOX pathways. Aspirin (ASA), a COX-1 inhibitor used as positive control, significantly blocked TXB_2 and PGD_2 at a concentration of 100 μM.

Discussion

Platelet activation and aggregation play pivotal roles in atherothrombosis. Inhibition of platelet aggregation by clinical treatment impairs the formation and progression of thrombosis and is therefore of importance in the prevention of complications after an acute coronary.[32] It is generally considered that platelet activation is mediated mainly through adhesion of platelets to the site of injury and through the action of endogenous agonists such as collagen, ADP, and thrombin, followed by the release of TXA_2, which acts as an amplifying factor in the platelet aggregation.[33,34]

We identified recently an anti-platelet effect of obovatol, which appeared to be mainly mediated by inhibition of PLC $\gamma 2$ phosphorylation.[18] However, the inhibitory effect of obovatol derivatives on platelet aggregation and the mechanisms are not understood. Hence, we are investigating the antiplatelet effect of

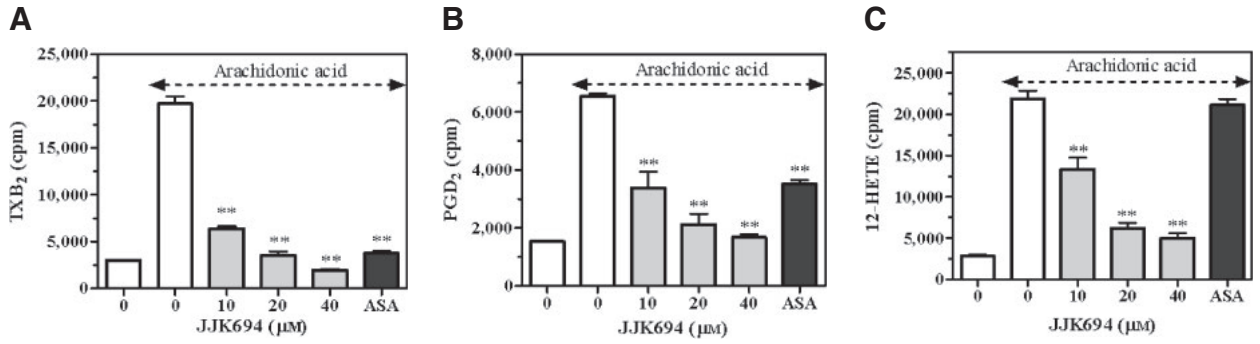

Fig. 5. Effects of JJK694 on Arachidonic Acid Metabolites.

Arachidonic acid-mediated [^{3}H] TXB_2, [^{3}H] PGD_2, and [^{3}H] 12-HETE formations were assayed using [^{3}H] arachidonic acid. [^{3}H] TXB_2 (A), [^{3}H] PGD_2 (B), and [^{3}H] 12-HETE (C) were extracted and separated by TLC on a silica gel G plate. The area corresponding to each lipid was scraped off, and radioactivity was measured by liquid scintillation counting. Aspirin (ASA, 100 μM), an anti-platelet agent, was used as the positive control. Data are expressed as mean ± SEM (n = 3). $^{**}p < 0.01$ *vs.* stimulus control.

JJK694, an obovatol derivative, on washed rabbit platelets. In the present study, JJK694 showed potential as a potent anti-platelet agent in response to collagen and AA. The anti-platelet activity of JJK694 might be mediated by the suppression of COX-1 and LOX activities.

In this study, JJK694 dose-dependently inhibited collagen- and AA-induced platelet aggregation but not U46619- or thrombin-induced platelet aggregation. It inhibited not only platelet aggregation induced by collagen and AA, but also the selective cytosolic calcium mobilization and serotonin secretion induced by collagen and AA. Changes in cytosolic calcium concentration play a central role in platelet activation as an upstream event that further interacts with intracellular targets to evoke functional and structural changes in platelets,[35] leading to the stimulation of enzymes that are not fully functional at the low calcium concentrations present in resting platelets.[27] Serotonin secretion is a marker of platelet activation and aggregation, because it is released from activated platelets during platelet aggregation.[36]

In the progression of platelet activation, AA is converted to various metabolites closely involved in these bidirectional modulations, mainly TXB_2 and PGD_2 by COX-1, and 12-HETE by LOX.[37] TXB_2 and PGD_2 are potent vasoconstrictors and inducers of platelet aggregation, and 12-HETE has been found to induce neutrophil migration.[38]

In the present study, JJK694 completely inhibited the conversion to TXB_2, PGD_2, and 12-HETE at the same time. These effects were due mainly to COX-1 and LOX inhibitory activity. In a previous study, obovatol, the backbone of JJK694, inhibited thrombus formation by antiplatelet activity through suppression of PLCγ2 phosphorylation.[24] Anyway, JJK694 had no effect on PLCγ2 phosphorylation, and JJK694 inhibited COX-1 and LOX activation downstream of PLCγ2 phosphorylation. This indicates that JJK694 was synthesized with the methyl instead of the methylene of obovatol. Based on our data, further analysis of the structure-activity relationships of obovatol derivatives should make it possible to synthesize novel anti-platelet and anti-thrombotic agents.

We suggest that JJK694 selectively inhibits platelet aggregation, perhaps due to inhibition of COX-1 and LOX activities, and that the beneficial effect of JJK694 on thrombotic disease might be also due to its modulation of platelet activity.

Acknowledgments

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