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Antiplatelet Activity of KR-32558, a Novel Selective Sodium/hydrogen Exchanger-1 Inhibitor

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ABSTRACT – We investigated the antiplatelet effect of a newly synthesized guanidine derivative KR-32558, a sodium-hydrogen exchanger-1 (NHE-1) inhibitor, together with the elucidation of the possible mechanisms of action. KR-32558 concentration -dependently inhibited the aggregation of washed rabbit platelets induced by collagen (10 μg/ml) with an IC_{50} value of 85.9 μM, but with much weaker potency against aggregation induced by thapsigargin (0.5 μM) or A23187 (5 μM). And had no effect on platelet aggregation induced by arachidonic acid (100 μM), thrombin (0.05 U/ml) and U46619 (1 μM) up to 100 μM. KR-32558 completely inhibited the $[Ca^{2+}]_i$ mobilization induced by collagen at concentration of 100μìM. Taken together, these observations suggest that KR-32558 selectively inhibited collagen-mediated platelet aggregation by blocking the cytoplasmic calcium mobilization in addition to NHE-1 inhibition.

Key words: KR-32558; sodium-hydrogen exchanger-1; platelet aggregation; $[Ca^{2+}]_i$ mobilization

Introduction

Out of seven sodium/hydrogen exchanger isoforms (NHE), isoform-1 (NHE-1) is reported to regulate the function of cardiovascular system such as heart, platelets, vessel and endothelium (Mentzer *et al.*, 2003; Rosskopf, 1999). With regard to its role in regulating the function of cardiomyocytes, it has been reported that NHE-1 becomes overactive during cardiac ischemia, and contributes to ischemia/reperfusion-induced heart injury via multiple mechanisms of action (Karmazyn *et al.*, 1999). NHE-1 inhibitors such as cariporide, eniporide, and sabiporide have been shown to exert cardioprotective effects in various experimental models of ischemia/reperfusion heart injury either by inhibiting electroneutral exchange of intracellular H^+ for extracellular Na^+ and resultant accumulation of intracellular Ca^{2+} via the reverse mode of sodium/calcium exchanger (NCX) (Mentzer *et al.*, 2003; Karmazyn, 2001) or by delaying mitochondrial matrix acidification and ATP exhaustion during ischemia (Ruiz-Meana *et al.*, 2003).

Vascular injury leads to the activation of platelets and their adhesion to the exposed subendothelium at the site of vascular injury (primary adhesion), which is followed by subsequent platelet-platelet adhesion (aggregation or cohesion) for thrombus growth. This series of successive processes play important role in both physiological haemostatic and pathological thrombotic events and involves a complex multi-step process among a diverse array of adhesive ligands and receptors on the platelet surface (Jackson *et al.*, 2003; Corti *et al.*, 2002; Corti *et al.*, 2003). During platelet activation and aggregation, the $[Ca^{2+}]_i$ increase assumes transient spike mobilization or elevated oscillatory flux as a result of either Ca^{2+} influx or release from intracellular stores and plays a fundamental role in regulating platelet responses to various adhesive and soluble agonists (Jackson *et al.*, 2003). Accordingly, agents with inhibitory effects on the cytosolic Ca^{2+} mobilization in platelets may suppress the platelet aggregation and thrombus growth (Kim *et al.*, 1999; Shah *et al.*, 1999; Kang *et al.*, 2001).

[5-(3,5-dichlorophenyl)furan-2-yl-carbonyl]guanidine (KR-32558, Fig. 1), a newly synthesized guanidine derivative, was shown to inhibit intracellular pH recovery in PS120/NHE-1 (*h* NHE-1 transfected) cells with a significantly greater potency than cariporide and exert potent cardioprotective effects in ischemic rat and dog heart models in vitro and in vivo (personal communication). In the present study, we examined the antiplatelet effects of KR-32558, a newly synthesized potent

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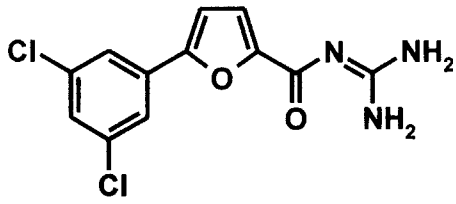

Fig. 1. Chemical structure of KR-32558, whose nomenclature is [5-(3,5-dichlorophenyl)furan-2-yl-carbonyl]guanidine.

selective NHE-1 inhibitor, on rabbit washed platelets activated by various agonists with especial emphasis on the delineation of its mechanisms of action.

Materials and methods

Chemicals

KR-32558 was synthesized at the Medicinal Science Division, Korea Research Institute of Chemical Technology (Daejeon, Korea). Collagen and arachidonic acid (AA) were purchased from Chrono-Log Co. (Havertown, PA, USA). Thrombin, thapsigargin and calcium ionphore, A23187 were purchased from Sigma Chemical Co. (St Louis, MO, USA). Calcium green-1/AM was purchased from Molecular Probes (Eugen, OR, USA). U46619 (9,11-dideoxy-9,11-methanoepoxy-prostaglandin $F_{2\alpha}$) was purchased from Cayman Chemical Co. (Ann Arbor, MI, 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°C 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).

Rabbit platelet preparation

Blood was withdrawn from the ear arteries 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 (Jin *et al.*, 2005). Briefly, platelet rich plasma (PRP) was obtained by centrifugation of rabbit blood at 230×*g* for 10 min. Platelets were sedimented by centrifugation of the PRP at 800×*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(â-aminoethyl ether) N,N,N'N'-tetraacetic acid). The washed platelets were resuspended in Hepes buffer (pH 7.4) and adjusted to 4 $\times 10^8$ cells/ml.

Platelet aggregation in vitro assay

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°C 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), U46619 (1 μM), thapsigargin (0.5 μM) and A23187 (5 μM), respectively. The resulting aggregation, measured as the change in light transmission, was recorded for 10 min. The extent of inhibition of platelet aggregation is expressed as % of control.

$[Ca^{2+}]_i$ measurement

Cytosolic Ca^{2+} measurements employed the fluorescent dye calcium green-1, which involved incubating the platelets with cell permeant acetoxymethyl ester. Rabbit platelets were incubated with 2 μM calcium green-1/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 calcium green-1/AM was removed by centrifugation (500×*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). Prior to $[Ca^{2+}]_i$ measurement, EGTA was added back to the buffer to a final concentration of 4 mM. The measurement of $[Ca^{2+}]_i$ was performed at room temperature in a MSIII fluorimeter (Photon Technology International, S. Brunswick, NJ, USA) using excitation wavelengths of 506 nm as well as an emission wavelength of 533 nm. $[Ca^{2+}]_i$ was calculated by using the SPEX

dM3000 software package.

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).

Results

Effect of KR-32558 on rabbit platelet aggregation in vitro

As shown in Fig. 2, KR-32558 concentration-dependently inhibited rabbit washed platelet aggregation induced by collagen (10 μg/ml) with IC_{50} values of 85.9 μM (Table 1). KR-32558 also dose-dependently inhibited platelet aggregation induced by thapsigargin (0.5 μM), an intracellular Ca^{2+}ATPase inhibitor or A23187 (5 μM), a Ca^{2+} ionophore, However, KR-32558 did not have any inhibitory effect on platelet aggregation induced by arachidonic acid (100 μM), thrombin (0.05 U/ml) and U46619 (1 μM), a thromboxane (TX) A_2 mimic up to 100 μM. Cariporide and sabiporide, selective NHE-1 inhibitors with rapid and slow dissociation kinetics, respectively, had very weak inhibitory effect on collagen-induced platelet aggregation under the same experimental condition.

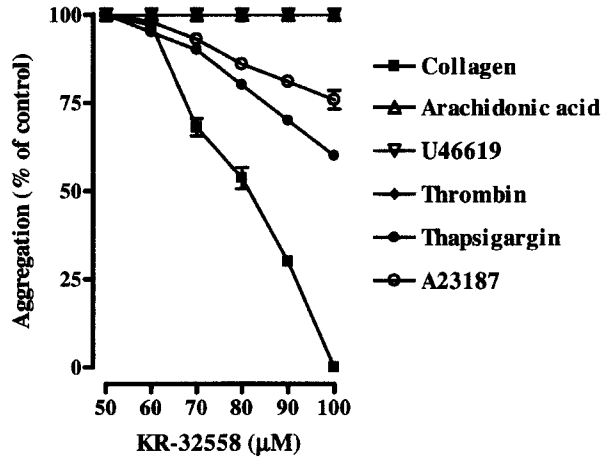

Fig. 2. Effect of KR-32558 on washed rabbit platelet aggregation. Washed rabbit platelets were incubated at 37°C in an aggregometer with stirring at 1,000 rpm, and then KR-32558 was added.
After 3 min preincubation, the platelet aggregation was induced by addition of thrombin (0.05 U/ml), arachidonic acid (100 μM), collagen (10 μg/ml), U46619 (1 μM), thapsigargin (0.5 μM) and A23187 (5 μM), respectively. The aggregation percentage was expressed as % of maximum aggregation induced by respective inducers. Data are expressed as mean ± S.E.M. (n = 4).

Table 1. IC_{50} values of KR-32558

Sample (100 μM)	Inducers	Aggregation (% of control)	IC_{50} (μM)
KR-32558	Collagen	0	85.9
	Arachidonic acid	100	> 200
	U46619	100	> 200
	Thrombin	100	> 200
	Thapsigargin	60 ± 1.1	> 200
	A23187	75.9 ± 2.6	> 200
Cariporide	Collagen	95.4 ± 1.8	> 200
Sabiporide	Collagen	90.2 ± 0.8	98.4

Mean ± S.E.M.
The 50% of inhibition concentration (IC_{50}) was calculated from at least three separate experiments.

Effect of KR-32558 on $[Ca^{2+}]_i$ mobilization

The representative trace, in which collagen was added to induce $[Ca^{2+}]_i$ mobilization, was shown in Fig. 3. The effect of KR-32558 on $[Ca^{2+}]_i$ mobilization was observed after 3 min incubation with platelet before adding collagen (10 μg/ml). Collagen induced a gradual but transient increase of $[Ca^{2+}]_i$ which reached the peak level of 200 nM after 4-6 min. Treatment of the platelet suspension with KR-32558 (100 μM) significantly inhibited the elevation of the $[Ca^{2+}]_i$ in response to collagen.

Discussion

NHE-1 is reported to be the predominant, and most likely the only, plasmalemmal NHE expressed in platelets and play a predominant role in regulating intracellular pH during platelet activation by various agonists like collagen, thrombin and ADP (Rosskopf, 1999; Siffert, 1995). This intriguing finding indicates a close relationship among platelet NHE-1, pH and platelet function. However, due to the lack of experimental evidences by studies using selective NHE-1 inhibitors like cariporide (Klinkhardt *et al.*, 2003), the role of NHE-1 in regulating platelet function, especially with respect to increased platelet aggregability and thrombogenesis was not clarified completely. Platelet plug formation in response to vascular injury is a complex process con-

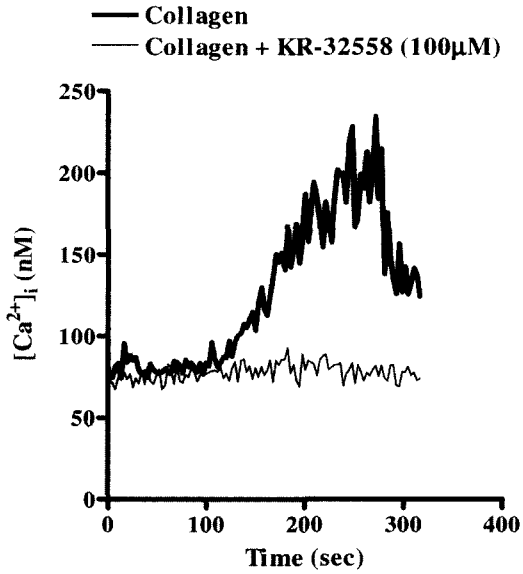

Fig. 3. Effect of KR-32558 on $[Ca^{2+}]_i$ in rabbit platelets. KR-32558 solution was added to yield a final concentration of 100 μM in the platelet suspension. Collagen (10 μg/ml) was added 3 min later.

sisting of three stages of platelet activation - platelet adhesion to subendothelium, subsequentplatelet - platelet adhesion and the growth and stability of the hemostatic plug, where each stage involves an interaction between a diverse array of adhesive (vWF, collagen and others) or soluble (ADP, thrombin and TXA_2) ligands and their receptors on the platelet surface (Jackson *et al.*, 2003; Prevost *et el.*, 2003; Farndale *et al.*, 2004), with soluble agonists acting as amplifying factors in platelet activation and aggregation but to a different degree (Farndale *et al.*, 2004). In the present study, the effect of a newly synthesized potent NHE-1 inhibitor KR-32558 on platelet aggregation was extensively studied using various soluble agonists and adhesive agonist collagen.

Platelet aggregation study (Fig. 1) has shown that KR-32558 exerts differential inhibitory effect against aggregation of washed rabbit platelets induced by various platelet agonists, with the potency rank order of collagen > thapsigargin > A23187 and without any effect against arachidonic acid, thrombin or U46619-induced aggregation.

KR-32558 inhibited collagen-induced platelet aggregation quite effectively compared with its effects against platelet aggregation induced by other types of platelet agonists. In the present study, unlike KR-32558, cariporide and sabiporide, selective NHE-1 inhibitors with rapid and slow dissociation kinetics (Touret *et al.*, 2003), respectively, exerted much weaker inhibitory effect against collagen-induced platelet aggregation (Table 1), indicating significant difference in potency and mode of action among these compounds, despite their classification as NHE-1 inhibitors.

This experiment using calcium green 1/AM-loaded platelets has shown that collagen (10 μg/ml)-induced cytosolic Ca^{2+} mobilization was significantly inhibited by KR-32558 at 100 μM, a concentration that completely inhibited platelet aggregation (Fig. 3), indicating the partial contribution of the blockade of cytosolic Ca^{2+} mobilization to its effects. Collagen is known to induce platelet aggregation via different mechanisms (Colman *et al.*, 1994; Jackson *et al.*, 2003). Collagen activates platelets through GPVI and integrin $\alpha_2\beta_1$, the former inducing phosphorylation of FcR γ-chain through a tyrosine kinase-based signaling pathway involving Src kinases (Syk and Lyn) and Syk, resulting in the recruitment of PI 3-kinase and phospholipase Cγ2 (Jackson *et al.*, 2003). Thus, collagen lead to the activation of different types of phospholipase C, which produces diacylglycerol and inositol trisphosphate, responsible for the release of Ca^{2+} from intracellular Ca^{2+} stores, shape change, granule release and PKC activation (Lapetina, 1990). We could not exclude the possibility that part of the antiplatelet activity of KR-32558 was due to the inhibition of cytosolic Ca^{2+} mobilization accompanied by the blockade of the PLC-catalyzed phosphoinositide breakdown. KR-32558-induced reduction of $[Ca^{2+}]_i$ (shown in Fig. 3) through the blockade of extracellular Ca^{2+} influx or/and intracellular Ca^{2+} release is thought to contribute to the inhibition of platelet aggregation, since both transient and robust cytosolic Ca^{2+} fluxes play a pivotal role in all three stages of thrombogenesis regardless of the activating stimulus (soluble agonist or adhesive substrate) (Jackson *et al.*, 2003). Platelet agonists such as epinephrine, ADP, thrombin, collagen and PMA are known to induce part of the Na^+ influx through the activation of NHE-1, contributing to the platelet activation due to an increase in $[Ca^{2+}]_i$ via a reverse mode of NCX and the procoagulant activity due to release of microvesicles which serve as catalytic sites for the assembly of tenase and prothrombinase complexes (Rosskopf, 1999; Siffert, 1995; Roberts *et al.*, 2004; Stelmach *et al.*,

2002). Accordingly, NHE-1 inhibitors like KR-32558 might be used as promising agents to inhibit platelet aggregation. Thus, it has been recently shown that cariporide, one of the selective NHE-1 inhibitors of first generation, exerted inhibitory effects on the degranulation of human platelets and formation of platelet-leukocyte aggregates (Klinkhardt *et al.*, 2003).

In summary, the results from the present study indicate that KR-32558 exert a significant inhibition of platelet aggregation induced by collagen. These observations suggest that the antiplatelet activity of KR-32558 is mediated by inhibition of cytoplasmic calcuim increase in rabbit platelets. These inhibitory effects on platelet aggregation may contribute to the enhancement of therapeutic potential of this novel NHE-1 inhibitor as cardioprotective agent in ischemic heart disease.

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