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Thromboxane A_2 Synthase Inhibition and Thromboxane A_2 Receptor Blockade by 2-[(4-Cyanophenyl)amino]-3-chloro-1,4-naphthalenedione (NQ-Y15) in Rat Platelets

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ABSTRACT. The effects of 2-[(4-acetylphenyl)amino]-3-chloro-1,4-naphthalenedione (NQ-Y15), a synthetic 1,4-naphthoquinone derivative, on platelet activity and its mechanism of action were investigated. NQ-Y15 caused a concentration-dependent inhibition of the aggregation induced by thrombin, collagen, arachidonic acid (AA), and A23187. The IC_{50} values of NQ-Y15 on thrombin (0.1 U/mL)-, collagen (10 μg/mL)-, AA (50 μM)-, and A23187 (2 μM)-induced aggregation were 36.2 ± 1.5, 6.7 ± 0.7, 35.4 ± 1.7, and 93.1 ± 1.4 μM, respectively. NQ-Y15 also inhibited thrombin-, collagen-, AA-, and A23187-stimulated serotonin secretion in a concentration-dependent manner. However, a high concentration (100 μM) of NQ-Y15 showed no significant inhibitory effect on ADP-induced primary aggregation, which is independent of thromboxane A_2 (TXA_2) production in rat platelets. In fura-2-loaded platelets, the elevation of intracellular free calcium concentration stimulated by AA, thrombin, and 4-bromo-A23187 was inhibited by NQ-Y15 in a concentration-dependent manner. The formation of TXA_2 caused by AA, thrombin, and collagen was inhibited significantly by NQ-Y15. NQ-Y15 inhibited TXA_2 synthase in intact rat platelets, since this agent reduced the conversion of prostaglandin (PG) H_2 to TXA_2. Similarly, NQ-Y15 selectively inhibited the TXA_2 synthase activity in human platelet microsomes, whereas it had no effect on activity of phospholipase A_2, cyclooxygenase, and PGI_2 synthase *in vitro*. NQ-Y15 inhibited platelet aggregation induced by the endoperoxide analogue U46619 in human platelets, indicating TXA_2 receptor antagonism, possibly of a competitive nature. These results suggest that the antiplatelet effect of NQ-Y15 is due to a combination of TXA_2 synthase inhibition with TXA_2 receptor blockade, and that it may be useful as an antithrombotic agent. BIOCHEM PHARMACOL **54**;2: 259–268, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. 1,4-naphthalenedione derivative; platelet; aggregation; secretion; thromboxane A_2 synthase; thromboxane A_2 receptor

Platelet–vessel wall interactions represent important factors in the development of thrombosis and atherosclerosis [1, 2]. When blood vessels are damaged, platelet aggregation occurs rapidly to form hemostatic plugs or arterial thrombi at sites of vessel injury or in regions where blood flow is disturbed. These thrombi are the source of thromboembolic complications of atherosclerosis, heart attacks, strokes, and peripheral vascular disease. Platelet adhesion to subendothelial components, such as collagen, activates signalling pathways that lead to TXA_2 formation and secretion of platelet granule contents. Both substances cause platelet aggregation [3]. The morphological evidence that arterial thrombi are largely composed of platelet aggregates has led many investigators to postulate that platelet aggregation is a major pathogenic mechanism in arterial thrombosis [4]. Thus, inhibition of platelet function represents a promising approach for the prevention of thrombosis.

AA[‖] is a membrane-derived fatty acid that is metabolized by COX to PG endoperoxide intermediates, such as PGH_2. In platelets, endoperoxides are further metabolized to TXA_2 by TXA_2 synthase. TXA_2 is a potent inducer of platelet aggregation and a vasoconstrictor [5]. Levels of this mediator are increased in several thrombotic disorders [6]. Therefore, agents that inhibit the formation or the action

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‖ *Abbreviations:* A23187, 6S-(6α,8β,9β,11α)-5-(methylamino)-2-((3,9,11-trimethyl-8-(1-methyl-2-oxo-2-(1H-pyrrol-2-yl)ethyl)-1,7-dioxaspiro(5,5) undec-2-yl)methyl)-4-benzooxazo ecarboxylic acid; AA, arachidonic acid; $[Ca^{2+}]_i$, intracellular free calcium concentration; COX, cyclooxygenase; 15-HPETE, 15(S)-hydroperoxyeicosa-5Z,8Z,11Z,13E-tetraenoic acid; NQ-Y15, 2-[(4-cyanophenyl)amino]-3-chloro-1,4-naphthalenedione; PG, prostaglandin; PL, phospholipase; RIA, radioimmunoassay; TX, thromboxane; and U46619, 9,11-dioxy-9α,11α-epoxymethanoprostaglandin H_2.

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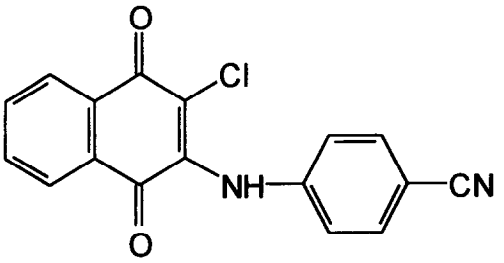

FIG. 1. Chemical structure of NQ-Y15.

of TXA_2 would be potentially useful in the treatment of thrombotic disorders.

In spite of the inhibitory effect of aspirin on platelet COX, the simultaneous inhibition of endothelial COX limits its antithrombotic activity, since it would also inhibit the endothelial production of PGI_2, a powerful antiaggregatory and vasodilatory substance [7].

TXA_2 synthase inhibitors, in addition to selective suppression of TXA_2 formation, cause a redirection of PG endoperoxide metabolism toward antiaggregatory PGs, such as PGD_2 and PGI_2 [8]. However, a limit to this class of drugs is represented by the fact that PGH_2, which accumulates after TXA_2 synthase inhibition, is itself a potent platelet stimulus [8, 9]. Thus, it has been suggested that the combination of TXA_2 synthase inhibitors with TXA_2/PGH_2 receptor antagonists, which block the action of both TXA_2 and PGH_2, may provide an antiplatelet regimen superior to those previously available [10–12]. Drugs such as picotamide [13] and ridogrel (R68070) [14], which combine TXA_2 synthase-inhibitory and TXA_2 receptor-blocking activities in a single molecule, have been described. It was reported that these dual TXA_2 synthase inhibitors/receptor antagonists have inhibitory effects on platelets *in vitro* [13–16] and clinical efficacy in patients with thrombotic disorders [17–22].

In this paper, we found NQ-Y15 (Fig. 1), a synthetic 1,4-naphthoquinone derivative, to possess antiplatelet activity *in vitro* and further investigated that the antiplatelet effect of this compound is due to the combination of TXA_2 synthase inhibition with TXA_2/PGH_2 receptor blockade in a single molecule.

MATERIALS AND METHODS

Materials

NQ-Y15 was synthesized and characterized as described previously [23]. It was dissolved in DMSO so that the final DMSO concentration in reaction mixtures never exceeded 0.5% (v/v). 5-Hydroxy[^{14}C]tryptamine creatinine sulfate (57 mCi/mmol), 1-acyl-2-[1-^{14}C]arachidonyl-GPE (where GPE is *sn*-glycero-3-phosphoethanolamine) (55 mCi/mmol), and a TXB_2 RIA kit were obtained from Amersham (Little Chalfont, Buckinghamshire, England). Thrombin, AA, A23187, 4-bromo-A23187, U46619, BSA, indomethacin, imidazole, acetylsalicylic acid (aspirin), DMSO, EDTA, EGTA, and fura 2/AM were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Collagen and ADP were obtained from the Chrono-Log Co. (Havertown, PA, U.S.A.). PGH_2 was purchased from the Cayman Chemical Co. (Ann Arbor, MI, U.S.A.) and benzylimidazole from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). A TXA_2 synthase assay kit and a PGI_2 synthase assay kit were purchased from Biomol (Plymouth Meeting, PA, U.S.A.). All other reagents were of analytical grade.

Preparation of Platelets

Rat blood was prepared from Sprague–Dawley female rats (Laboratory Animal Center, Seoul National University, Seoul, Korea) weighing 200–250 g. Rats were anesthetized with ethyl ether, and blood anticoagulated with sodium citrate (3.8%; 1:9, v/v) was collected from the abdominal aorta. Human blood, obtained from healthy human volunteers, was purchased from the National Red Cross Blood Center (Seoul, Korea). The blood (320 mL) was anticoagulated with 44.8 mL of CPD solution (0.327 g citrate, 2.63 g sodium citrate, 0.222 g NaH_2PO_4, 2.90 g dextrose, and 0.0275 g adenine dissolved in 100 mL distilled water; Green Cross Pharmaceutical Co., Seoul, Korea). Rat or human blood was centrifuged at 120 *g* for 15 min. The supernatants were pooled and centrifuged at 600 *g* for 15 min at room temperature. The platelet pellets were washed with modified Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 2 mM EGTA, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4) and centrifuged at 600 *g* for 15 min. This washing procedure was repeated twice, and then platelets were gently resuspended in Tyrode-HEPES buffer (129 mM NaCl, 2.8 mM KCl, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 0.8 mM KH_2PO_4, 1 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, 0.35% BSA, pH 7.4). Platelet counts were determined using a ZM Coulter Counter (Coulter Electronics, Hialeah, FL, U.S.A.).

Platelet Aggregation and Serotonin Secretion Assay

Platelet aggregation studies were performed using the turbidimetric method described by Mustard *et al.* [24]. Aggregation was measured by a Lumi-aggregometer (model 450, Chrono-Log Co.) connected to a recorder. To investigate the antagonism of NQ-Y15 on U46619-induced aggregation of human platelets, concentration-response relationships were determined in the absence and in the presence of a range of concentrations of NQ-Y15; for these experiments, indomethacin-treated human washed platelets (50 μM indomethacin for 3 min) were used to prevent any possible contribution of endogenous AA metabolites to platelet aggregation. The secretion of platelet contents from dense granules was measured using [^{14}C]serotonin-incorporated platelets [25]. Washed platelets (8×10^8/mL) were incubated with [^{14}C]serotonin (0.2 μCi/mL) for 30 min at 37°, washed twice more, and then resuspended in modified Tyrode's solution, pH 7.4, including imipramine

(2 μM) to prevent reuptake of [^{14}C]serotonin. Platelets were incubated with NQ-Y15 or vehicle at 37° for 8 min. To determine [^{14}C]serotonin secretion from the platelets, sub-aliquots (450 μL) were withdrawn 6 min after the addition of the agonist and placed in ice-cooled tubes that contained 90 μL of 630 mM formaldehyde and 50 mM EDTA. The secretion of [^{14}C]serotonin from platelets was determined by centrifuging the samples at 12,000 *g* for 2 min in an Eppendorf centrifuge (model 5413) and assaying a 100-μL aliquot of the supernatant by liquid-scintillation counting. Percent [^{14}C]serotonin secretion was determined as described by Holmsen *et al.* [26].

TXB_2 Assay

In the preliminary experiments, TXB_2 production was plateaued above 10 μM AA and 1.0 U/mL of thrombin and was elevated significantly by 10 μg/mL of collagen. Thus, platelets were stimulated with the indicated concentrations of AA, thrombin, and collagen. EDTA (2 mM) and indomethacin (50 μM) were added to a platelet suspension 6 min after the addition of the agonist. TXB_2, a stable metabolite of TXA_2, in the supernatants was obtained after centrifugation at 12,000 *g* in an Eppendorf centrifuge for 2 min and assayed by RIA.

Determination of Intracellular Calcium in Platelets

$[Ca^{2+}]_i$ was determined with fura-2-loaded platelets using the method described by Pollock and Rink [27]. Briefly, platelets (8×10^8/mL) were incubated with fura-2/AM (3 μM) for 45 min at 37° and then centrifuged for 15 min at 800 g; the resultant pellet was washed with EDTA (1 mM)-containing Tyrode-HEPES buffer. After centrifugation, platelets were resuspended gently at a concentration of 2×10^8/mL in Tyrode-HEPES buffer containing 1 mM $CaCl_2$ and were used within 1 hr. Fluorescence was measured at the emission wavelength of 505 nm with the excitation wavelength switched continuously between 340 and 380 nm using a Shimazu RF-5000 spectrofluorimeter (Shimadzu Co., Kyoto, Japan). Fluorescence was measured at 37° in a thermostatically controlled, magnetically stirred cuvette. The ratio of the fluorescence intensities at the two excitation wavelengths was used to determine $[Ca^{2+}]_i$ [28]. $[Ca^{2+}]_i$ was calibrated by lysing the cells with 0.1% (v/v) Triton X-100 in the presence of 1 mM $CaCl_2$ or 10 mM EGTA (pH 9.0).

Assay of PLA_2 Activity

Purified pig spleen cytosolic PLA_2 was provided by Dr. D. K. Kim (College of Pharmacy, Chung Ang University, Seoul, Korea). This enzyme was prepared and purified as described previously [29]. PLA_2 activity was assayed using sonicated liposomes, prepared as described by Kim *et al.* [30]. The standard PLA_2 assay buffer (200 μL) contained 75 mM Tris–HCl (pH 9.0), 5 mM $CaCl_2$, 1 mg/mL fatty acid free BSA, 0.47 nmol of 1-acyl-2-[1-^{14}C]AA-GPE (approximately 55,000 dpm). After preincubating the test compounds with PLA_2 at 37° for 10 min, the reaction was allowed to continue for 30 min. Then the reaction was terminated by adding 1.25 mL of Dole's reagent (78% propan-2-ol, 20% *n*-heptane, and 2% 2 M aqueous H_2SO_4), and the liberated [^{14}C]AA was extracted as follows: 0.55 mL of water was added and the sample was vortexed and then centrifuged at 1200 *g* for 5 min. Then 0.75 mL of the upper phase was transferred to a new tube that contained 100 mg of silica gel and 0.75 mL of *n*-heptane. The sample was vortex-mixed and centrifuged for an additional 5 min each, after which 1.4 mL of supernatant was removed for scintillation counting.

Assay of COX Activity

Microsomes prepared from bovine seminal vesicles were used to assay for COX activity as described by Van Der Ouderaa *et al.* [31]. The COX assay was performed at 37° by monitoring oxygen consumption using an O_2 electrode (Yellow Springs Instrument Co., Yellow Springs, OH, U.S.A.). Reactions were initiated by the addition of microsomes (75 μg/mL) to a reaction mixture that was composed of 1 mL of 0.2 M sodium phosphate buffer, pH 7.4, 10 μM hemin, 160 μg hydroquinone, 300 μg AA, and 20 μL of either NQ-Y15 or indomethacin.

Assay of TXA_2 Synthase Activity in Intact Platelets

Aliquots of PGH_2, in anhydrous acetone, were pipetted into glass tubes. The acetone was evaporated under a gentle stream of nitrogen, and PGH_2 was redissolved immediately in ethanol. Platelet suspensions were incubated with the test compounds at 37° for 8 min prior to the addition of 2 μg/mL PGH_2. The final concentration of ethanol was 0.1% (v/v). At 7 min after the addition of PGH_2, the incubations were terminated by cooling and centrifugation at 12,000 *g* for 1 min at 4°. TXB_2 in the supernatants was determined by RIA. TXA_2 synthase activity is reflected by the production of TXB_2.

Assay of TXA_2 Synthase Activity Using Human Platelet Microsomes

The effect of NQ-Y15 on TXA_2 synthase activity was determined using a TXA_2 synthase assay kit. In brief, human microsomal solutions (0.2 mg/mL, 200 μL) were incubated with DMSO (0.1%, v/v), imidazole (0.5 mmol/20 μL), or various concentrations of NQ-Y15 (20 μL) at 25° for 8 min with shaking. Following this, PGH_2 (20 ng/4 μL) was added, and the mixture was incubated for an additional 3 min. $FeCl_2$ (25 mM, 20 μL) was then added to terminate the reaction, and the reaction mixture was allowed to stand at room temperature for 15 min. After centrifugation at 3000 *g* for 10 min at 4°, TXB_2 in the

supernatant was assayed by RIA. TXA_2 synthase activity is reflected by the production of TXB_2.

Assay of PGI_2 Synthase Activity

The effect of NQ-Y15 on PGI_2 synthase activity was determined using a PGI_2 synthase assay kit. In brief, bovine aortic microsomes (0.14 mg/mL, 200 μL) were incubated with DMSO (0.1%, v/v), 15-HPETE (80 ng/2 μL), or various concentrations of NQ-Y15 (10 μL) at 25° for 8 min with shaking. Following this, PGH_2 (20 ng/4 μL) was added, and the mixture was incubated for an additional 3 min. Then $FeCl_2$ (25 mM, 20 μL) was added to terminate the reaction, and the reaction mixture was allowed to stand at room temperature for 15 min. After centrifugation at 3000 g for 10 min at 4°, 6-keto-$PGF_{1\alpha}$ in the supernatant was assayed by RIA. PGI_2 synthase activity is reflected by the production of 6-keto-$PGF_{1\alpha}$.

Protein Content

For each experiment, the protein content was determined using a Bio-Rad protein® assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Statistical Analysis

Results are expressed as means ± SEM. Statistical significance ($P < 0.05$ or $P < 0.01$) was assessed using Student's *t*-test.

RESULTS

Effects of NQ-Y15 on the Aggregation and Serotonin Secretion of Washed Rat Platelets

Thrombin (0.1 U/mL), collagen (10 μg/mL), AA (50 μM), A23187 (2 μM), and ADP (15 μM) caused 75–90% aggregation of washed rat platelets. NQ-Y15 caused a concentration-dependent inhibition of the aggregation induced by thrombin, collagen, AA, and A23187. However, a high concentration (100 μM) of NQ-Y15 did not show a significant inhibitory effect on ADP-induced primary aggregation. The IC_{50} values of NQ-Y15 on thrombin-, collagen-, AA-, and A23187-induced aggregation were 36.2 ± 1.5, 6.7 ± 0.7, 35.4 ± 1.7, and 93.1 ± 1.4 μM, respectively (Fig. 2). NQ-Y15 also inhibited thrombin-, collagen-, AA-, and A23187-stimulated serotonin secretion in a concentration-dependent manner (Fig. 3).

Aspirin (100 μM), indomethacin (20 μM), or imidazole (1 mM) did not cause any significant alteration of ADP-induced primary aggregation. When a concentration of 1 mM was used, imidazole had no effect on AA- and collagen-induced aggregation. Aspirin (100 μM) significantly inhibited AA- and collagen-induced aggregation (61.8 and 43.6% inhibition, N = 4, respectively).

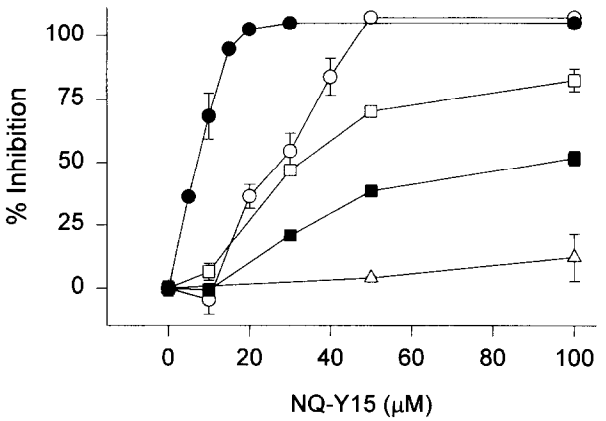

FIG. 2. Effects of NQ-Y15 on the thrombin-, collagen-, AA-, A23187-, or ADP-induced aggregation of washed platelets. Platelets were incubated with NQ-Y15 or vehicle at 37° for 8 min; thrombin (0.1 U/mL, ○), collagen (10 μg/mL, ●), AA (50 μM, □), A23187 (2 μM, ■), or ADP (15 μM, △) was then added to trigger aggregation. The peak level of aggregation was measured 6 min after the addition of stimulator. Percent inhibitions produced by NQ-Y15 were calculated from the reduction of the maximal level of the aggregation tracings in relation to the values obtained in the absence of NQ-Y15. The maximal levels of aggregation were 105–126 mm on the recorded paper. Each data point is expressed as the mean ± SEM (N = 4).

Effects of NQ-Y15 on Platelet TXB_2 Formation

The TXB_2 level of resting platelets was less than 0.5 ng/3 × 10^8 platelets. AA (10 μM), thrombin (1.0 U/mL), and collagen (10 μg/mL) caused a significant elevation of TXB_2 formation. NQ-Y15 significantly inhibited AA-, thrombin-, and collagen-induced TXB_2 formation. Aspirin (100 μM) and imidazole (1 mM) significantly inhibited AA-induced TXB_2 formation although they did not suppress it completely (Table 1). At the concentrations used, it

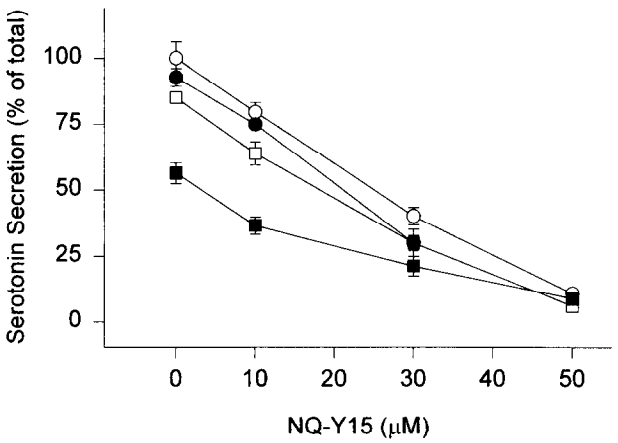

FIG. 3. Effects of NQ-Y15 on serotonin secretion induced by thrombin, collagen, AA, or A23187. Washed platelets were labeled with [^{14}C]serotonin and incubated with NQ-Y15 for 8 min prior to the addition of thrombin (0.1 U/mL, ○), collagen (10 μg/mL, ●), AA (50 μM, □), or A23187 (2 μM, ■). Serotonin secretion was terminated 6 min after the addition of stimulator. The mean value of total radioactivity of [^{14}C]serotonin was 4469 cpm. Each data point is expressed as the mean ± SEM (N = 6).

TABLE 1. Effects of NQ-Y15 on thromboxane B_2 formation in washed rat platelets caused by AA, thrombin, and collagen

	Thromboxane B_2 (ng/3 × 10^8 platelets)		
	Arachidonic acid (10 μM)	Thrombin (1.0 U/mL)	Collagen (10 μg/mL)
Control	65.94 ± 6.24	174.34 ± 28.26	98.44 ± 11.52
NQ-Y15, 10 μM	73.55 ± 13.11	104.31 ± 6.85*	78.33 ± 11.85
30 μM	55.30 ± 4.97	95.57 ± 11.83*	15.30 ± 2.84†
50 μM	34.52 ± 3.90†	73.78 ± 3.33†	8.50 ± 1.81†
Aspirin, 100 μM	36.76 ± 3.57†		
Imidazole, 1 mM	38.16 ± 8.52†		

DMSO (0.1%, control), imidazole, or NQ-Y15 was preincubated with platelets at 37° for 8 min, while aspirin was preincubated for 30 min; then the inducer was added. TXB_2 formation was terminated by EDTA (2 mM) and indomethacin (50 μM) 6 min after the addition of inducer. Values are means ± SEM (N = 6).

*,† Significantly different from the control: *$P < 0.05$, and †$P < 0.01$.

appeared that aspirin and imidazole did not inhibit the activity of COX and TXA_2 synthase completely in rat platelets. Similar results were observed in other studies [32, 33].

Effects of NQ-Y15 on $[Ca^{2+}]_i$ Elevation of Platelets

In fura-2-loaded platelets, AA (50 μM) and 4-bromo-A23187 (2 μM), a nonfluorescent Ca^{2+} ionophore, caused a rapid rise in $[Ca^{2+}]_i$ of platelets. Since 0.1 U/mL of thrombin did not produce an easily measurable increase of $[Ca^{2+}]_i$ 1 min after stimulation, 0.3 U/mL of thrombin had to be used. Pretreatment of platelets with NQ-Y15 (30 μM) for 8 min prior to the addition of stimulators markedly inhibited the rise in $[Ca^{2+}]_i$ (Fig. 4).

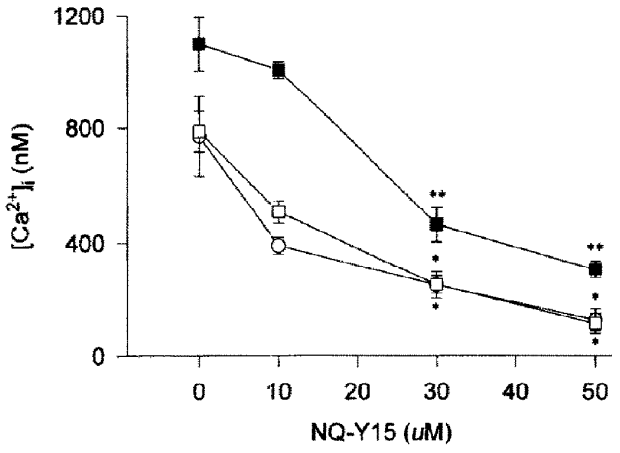

FIG. 4. Effects of NQ-Y15 on the increase of $[Ca^{2+}]_i$ induced by AA, thrombin, or 4-bromo-A23187 in fura-2-loaded platelets. Fura-2-loaded platelets were suspended in a buffer containing 1 mM $CaCl_2$, and the change in $[Ca^{2+}]_i$ was monitored continuously. Platelets were preincubated with DMSO (0.1%) or NQ-Y15 at 37° for 8 min; then AA (50 μM, □), thrombin (0.3 U/mL, ○), or 4-bromo-A23187 (2 μM, ■) was added. The value of $[Ca^{2+}]_i$ was calculated at 1 min after the addition of stimulator. Each data point is expressed as the mean ± SEM (N = 4). Key: (*) $P < 0.05$, and (**) $P < 0.01$ as compared with the control.

Effect of NQ-Y15 on PLA_2 Activity

The effect of NQ-Y15 on PLA_2 activity, a rate-limiting step of TXA_2 production, was examined in purified 100 kDa cytosolic PLA_2 from pig spleen. *p*-Bromophenacylbromide, a direct inhibitor of PLA_2 through covalent binding, inhibited the hydrolysis of 2-[^{14}C]AA-GPE, by approximately 62% at 50 μM (Table 2). However, NQ-Y15 had no effect on PLA_2 activity at the concentration at which it inhibited TXB_2 formation in stimulated platelets.

Effects of NQ-Y15 on COX Activity

COX activity was measured by monitoring oxygen consumption. After incubation of bovine vesicular gland microsomes with AA (75 μg) at 37° for 3 min, the maximal activities in the absence of inhibitor (100%) for COX was 36 μmol O_2/min/mg protein. Indomethacin (8.3 μM) significantly inhibited this oxygen consumption. However, NQ-Y15 had no effect on COX activity (Table 3).

TABLE 2. Effect of NQ-Y15 on the activity of 100 kDa PLA_2 from porcine spleen

		PLA_2 activity (% of control)
Control		100.0 ± 3.1
NQ-Y15,	10 μM	111.1 ± 3.0
	30 μM	108.8 ± 1.4
	50 μM	105.6 ± 0.7
p-Bromophenacylbromide,	50 μM	31.3 ± 2.3*

DMSO (0.1%, control), NQ-Y15, and *p*-bromophenacylbromide were tested for their ability to affect PLA_2 activity. The standard assay buffer (200 μL) contained 75 mM Tris–HCl (pH 9.0), 5 mM $CaCl_2$, 1 mg/mL BSA, 0.72 nmol of 1-acyl-2-[1-^{14}C]AA-GPE, 20 μg of pig spleen PLA_2, and 4 μL of NQ-Y15 or *p*-bromophenacylbromide. After preincubating the test compounds with PLA_2 at 37° for 10 min, the reaction was allowed to continue for 30 min. The reaction was terminated by adding 1.25 mL of Dole's reagent. The liberated [^{14}C]AA was extracted and measured as described in Materials and Methods. The control activity in the experiment was 0.656 nmol/min/mg protein. Values are means ± SEM (N = 4).

* $P < 0.01$, as compared with control.

TABLE 3. Effect of NQ-Y15 on COX activity

		COX activity (% of control)
Control		100.0 ± 5.2
NQ-Y15,	10 μM	100.9 ± 3.5
	30 μM	115.4 ± 16.2
	50 μM	106.0 ± 17.5
	100 μM	106.4 ± 7.8
Indomethacin,	8.3 μM	50.4 ± 8.1*

Microsomes, prepared from bovine seminal vesicles, were used to assay for COX activity. The COX assay was performed at 37° by monitoring oxygen consumption, using an O_2 electrode. Reactions were initiated by the addition of enzyme (75 μg/mL) to a reaction mixture that was composed of 1 mL of 0.2 M sodium phosphate buffer, pH 7.4, 10 μM hemin, 160 μg of hydroquinone, 300 μg of AA, and 20 μL of either NQ-Y15 or indomethacin. The maximal activity in the absence of inhibitor (100%) for COX was 36 μmol of O_2/min/mg protein. Values are means ± SEM (N = 4).
* $P < 0.01$, as compared with control (DMSO, 0.1%).

Effect of NQ-Y15 on TXA_2 Synthase Activity

The conversion of AA 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 AA metabolism. The addition of increasing concentrations of PGH_2 to washed rat platelet suspensions produced a concentration-dependent increase of TXB_2 (data not shown). Thus, washed rat platelet suspensions containing PGH_2 are adequate for the direct evaluation of the TXA_2 synthase inhibitor. In washed rat platelet suspensions, the level of TXB_2 in unstimulated platelets was 0.695 ± 0.03 ng/3 × 10^8 platelets. After incubation of washed platelet suspensions with PGH_2 (2 μg/mL) at 37° for 7 min, TXB_2 formation was 68.47 ± 0.61 ng/3 × 10^8 platelets. Both NQ-Y15 and benzylimidazole, a typical TXA_2 synthase inhibitor, significantly inhibited the conversion of PGH_2 into TXB_2 in washed platelets (Fig. 5). To determine if NQ-Y15 is capable of directly inhibiting TXA_2 synthase, its activity was assayed using a TXA_2 synthase assay kit. When human platelet microsomes were incubated with PGH_2, the specific TXA_2 synthase activity was calculated to be 47.03 ± 2.14 ng of TXB_2/min/mg protein. The conversion of PGH_2 to TXA_2 by human platelet microsomes was inhibited by imidazole. Preincubation of microsomes with NQ-Y15 significantly diminished the yield of TXA_2 in a concentration-dependent manner (Fig. 6).

Effect of NQ-Y15 on PGI_2 Synthase Activity

To determine if NQ-Y15 selectively inhibited TXA_2 synthase activity, PGI_2 synthase activity was assayed using a PGI_2 synthase assay kit. When bovine aortic microsomes were incubated with PGH_2, the specific PGI_2 synthase activity was calculated to be 61.71 ± 2.55 ng of 6-keto-$PGF_{1\alpha}$/min/mg protein. The conversion of PGH_2 to PGI_2 by bovine aortic microsomes was inhibited by 15-HPETE, an inhibitor of PGI_2 synthase. However, NQ-Y15 showed no influence on PGI_2 synthase activity (Table 4).

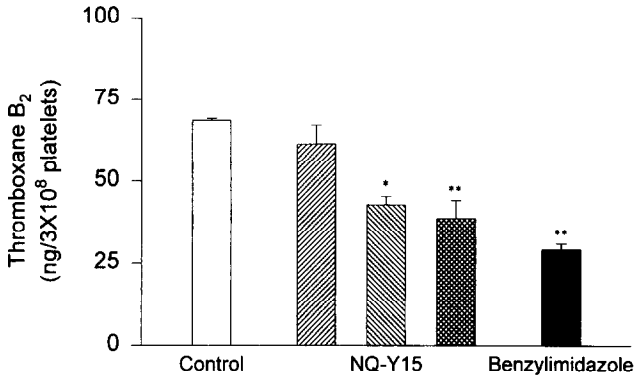

FIG. 5. Inhibitory effect of NQ-Y15 on thromboxane synthase activity in intact platelets. Platelet suspensions containing DMSO (0.1%, □), NQ-Y15 (10 μM, ▨; 30 μM, ▧; 50 μM, ▩), or benzylimidazole (1 mM, ■) were incubated at 37° for 8 min, and then 2 μg/mL of PGH_2 was added. At 7 min after the addition of PGH_2, the incubations were terminated by cooling and centrifugation at 12,000 *g* for 1 min at 4°. TXB_2 in the supernatants was determined by radioimmunoassay. Thromboxane synthase activity is reflected by the production of TXB_2, which is presented as means ± SEM (N = 6). Key: (*) $P < 0.05$, and (**) $P < 0.01$ as compared with the control.

Effects of NQ-Y15 on the Aggregation in Human Washed Platelets Induced by U46619

U46619, a stable TXA_2 mimetic, did not induce aggregation of rat platelets even at 10 μM. However, when U46619 (0.1 μM) was added to rat platelets that had been stimulated previously with collagen (40 μg/mL) in the presence of indomethacin (50 μM), aggregation was in-

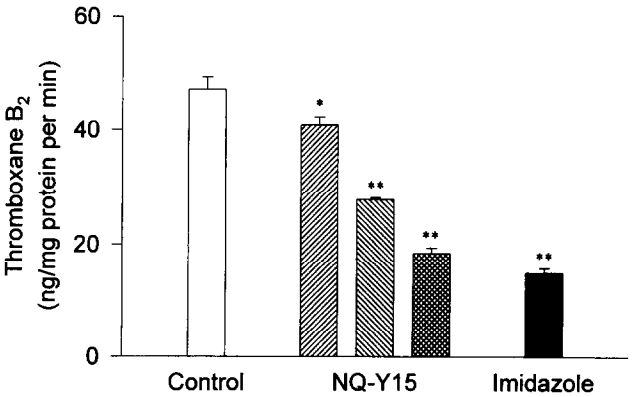

FIG. 6. Inhibitory effect of NQ-Y15 on thromboxane synthase activity in human platelet microsomes. Human microsomal solutions (0.2 mg/mL, 200 μL) were incubated with DMSO (0.1%, □), NQ-Y15 (10 μM, ▨; 30 μM, ▧; 50 μM, ▩), or imidazole (0.5 mmol/20 μL, ■) at 25° for 8 min with shaking. Following this, PGH_2 (20 ng/4 μL) was added and the mixture was incubated for an additional 3 min. Then $FeCl_2$ (25 mM, 20 μL) was added to terminate the reaction, and the reaction mixture was allowed to stand at room temperature for 15 min. After centrifugation at 3000 *g* for 10 min at 4°, TXB_2 in the supernatant was assayed by radioimmunoassay. TXA_2 synthase activity is reflected by TXB_2 production (ng/mg protein per min), which is presented as means ± SEM (N = 6). Key: (*) $P < 0.05$, and (**) $P < 0.01$ as compared with the vehicle control.

TABLE 4. Effect of NQ-Y15 on PGI_2 synthase activity in bovine aortic microsomes

		PGI_2 synthase activity (% of control)
Control		100.0 ± 7.8
NQ-Y15,	30 μM	95.8 ± 3.9
	50 μM	100.4 ± 4.1
	100 μM	106.4 ± 4.4
15-HPETE,	1.15 μM	47.3 ± 4.0*

Bovine aortic microsomes (0.14 mg/mL, 200 μL), were incubated with DMSO (0.1%, v/v), 15-HPETE (80 ng/2 μL), or various concentrations of NQ-Y15 (10 μL) at 25° for 8 min with shaking. Following this, PGH_2 (20 ng/4 μL) was added, and the mixture was incubated for an additional 3 min. $FeCl_2$ (25 mM, 20 μL) was then added to terminate the reaction, and the reaction mixture was allowed to stand at room temperature for 15 min. After centrifugation at 3000 *g* for 10 min at 4°, 6-keto-$PGF_{1\alpha}$ in the supernatant was assayed by RIA. PGI_2 synthase activity is reflected by the production of 6-keto-$PGF_{1\alpha}$. The maximal activity in the absence of inhibitor (100%) for PGI_2 synthase was 61.71 ± 2.55 ng of 6-keto-$PGF_{1\alpha}$/min/mg protein. Values are means ± SEM (N = 6).

* $P < 0.01$, as compared with control (DMSO, 0.1%).

duced about 1 min after the occurrence of shape change (data not shown). A similar response was observed by Hanasaki *et al.* [34]. Since rat platelets were not proper to study the direct effect on U46619-induced aggregation, we carried out this experiment using human washed platelets. When preincubated for 8 min with human washed platelets, NQ-Y15 reduced, in a concentration-dependent manner, platelet aggregation elicited by various concentrations of U46619 under COX blockade with indomethacin. At concentrations between 10 and 50 μM, NQ-Y15 produced a shift to the right of the concentration–effect curve of U46619, suggesting a competitive type of antagonism. Similarly, the IC_{50} values of NQ-Y15 for inhibition of the maximal extent of platelet aggregation induced by U46619 increased with augmenting concentrations of agonist (Fig. 7).

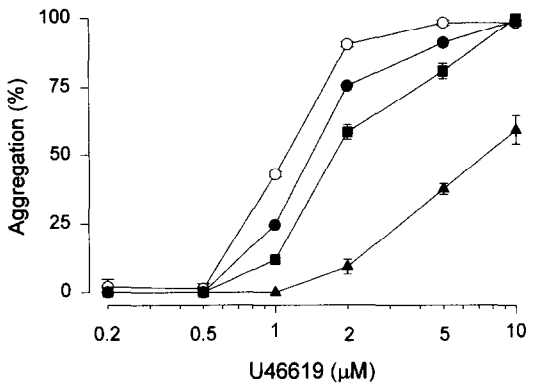

FIG. 7. Effects of NQ-Y15 on the aggregation in human platelets induced by U46619. Indomethacin-treated human washed platelets (50 μM indomethacin for 3 min) were used to prevent any possible contribution of endogenous AA metabolites to platelet aggregation. Human washed platelets were incubated with NQ-Y15 (10 μM, ●; 30 μM, ■; 50 μM, ▲) or DMSO 0.1%, ○) at 37° for 8 min; then U46619 was added to trigger aggregation. The peak level of aggregation was measured 6 min after the addition of stimulator. The percent aggregation was calculated assuming the maximal value of the control (absence of inhibitor) produced by U46619 (10 μM) to be 100%. The maximal level of aggregation was 69 mm. Each data point is expressed as the mean ± SEM (N = 4).

DISCUSSION

The present study shows that NQ-Y15 functions as an inhibitor of platelet aggregation and serotonin secretion induced by a variety of inducers. Its mechanisms of action appear to involve the combination of TXA_2 synthase inhibition with TXA_2/PGH_2 receptor blockade in a single molecule.

In the present study, NQ-Y15 was found to be an effective inhibitor against thrombin-, collagen-, and A23187-induced platelet aggregation and serotonin secretion (Figs. 2 and 3). However, a high concentration (100 μM) of NQ-Y15 failed to inhibit ADP-induced aggregation significantly (Fig. 2). In rat platelets, ADP induces only primary aggregation, which is independent of the production of TXA_2 [35, 36]. We observed that the primary aggregation induced by ADP was not inhibited by indomethacin or imidazole in rat platelets (data not shown). In addition, the formation of TXA_2 caused by thrombin and collagen was inhibited significantly by NQ-Y15 (Table 1) at concentrations that inhibit platelet aggregation and serotonin secretion. These results suggest that the antiplatelet effect of NQ-Y15 is due, in part, to the inhibition of TXA_2 formation and/or action. TXA_2 is a powerful platelet agonist, and its formation by stimulated platelets represents an important amplifying signal for platelet activation [37]. TXA_2 exerts its effects on platelets by binding to a specific receptor [38] and causing PLC activation, an increase in $[Ca^{2+}]_i$, and protein kinase C activation [39, 40].

It is well known that TXA_2 is produced from AA that is cleaved from the *sn*-2-position of phospholipids through the action of PLA_2 in stimulated platelets. In numerous previous studies, it has been shown that platelet aggregation is inhibited by a variety of PLA_2 inhibitors [41–43]. Since the intracellular concentration of free AA is low, the release of AA is thought to be the rate-limiting step in the formation of PGs and the other eicosanoids, including TXA_2 in platelets [44]. Blood platelets contain both secretory PLA_2 and cytosolic PLA_2 [45], which are distinctly different enzymes, having molecular masses of 14 and 85 kDa, respectively. The characteristics of cytosolic PLA_2 [30, 46, 47], such as a requirement for micromolar concentrations of Ca^{2+} for activity, a cytosolic localization, and a specificity for phospholipids containing AA in the *sn*-2 position, are compatible with a role in AA liberation. On the other hand, secretory PLA_2 appears not to be involved in AA liberation during platelet activation [45]. Thus, we examined the effects of NQ-Y15 on PLA_2 activity using purified 100 kDa cytosolic PLA_2 from pig spleen. The cytosolic PLA_2 enzymes from pig spleen are biochemically and immunochemically indistinguishable from those of bovine platelets by a number of criteria [29]. NQ-Y15 had

no effect on PLA_2 activity in the range of concentrations that inhibited the TXA_2 production of stimulated platelets (Table 2). We also found that NQ-Y15 inhibited, in a concentration-dependent manner, platelet aggregation, serotonin secretion, TXA_2 formation, and a rise in $[Ca^{2+}]_i$ caused by AA. The aggregation, serotonin secretion, and rise of $[Ca^{2+}]_i$ in platelets induced by exogenous AA are due to TXA_2 formation [39, 40]. In platelets, AA is further metabolized to TXA_2 in two enzymatic steps including PGH synthase and TXA_2 synthase. PGH synthase exhibits both COX activity, catalyzing PGG_2 formation from AA, and peroxidase activity, which reduces the 15-hydroperoxyl group of PGG_2 to PGH_2 [31, 48]. Nonsteroidal anti-inflammatory drugs, such as aspirin and indomethacin, inhibit COX activity without affecting peroxidase activity, and the COX activity of PGH synthase is the target site of aspirin and related nonsteroidal anti-inflammatory drugs [49]. Considering the fact that AA-induced aggregation is inhibited by COX-inhibiting agents such as epoxyeicosatrienoic acids, miconazole, and girinimbine [50–52], we investigated the effect of NQ-Y15 on COX activity. When COX activity was assayed by measuring oxygen consumption after bovine vesicular gland microsomes were treated with AA, NQ-Y15 was found to have no effect on COX activity (Table 3). These findings suggest that the antiplatelet effects of NQ-Y15 are due, in part, to the inhibition of TXA_2 formation through inhibitory actions at an enzymatic step lower than COX.

The conversion of PGH_2 to TXA_2 is catalyzed by TXA_2 synthase in platelets. In platelet suspensions incubated with PGH_2, NQ-Y15 reduced the formation of TXA_2 (Fig. 5). Similarly, NQ-Y15 inhibited the conversion of PGH_2 into TXA_2 by human platelet microsomes (Fig. 6). These results imply that NQ-Y15 is capable of directly inhibiting TXA_2 synthase activity in platelets. PGI_2 synthase catalyzes the formation of PGI_2 from PGH_2 in endothelial cells. Thus, PGH_2 is the common substrate to TXA_2 synthase and PGI_2 synthase. We assayed PGI_2 synthase activity to elucidate whether NQ-Y15 selectively inhibits the conversion of PGH_2 by TXA_2 synthase without that by PGI_2 synthase. NQ-Y15 had no influence on the PGI_2 synthase activity in bovine aortic microsomes (Table 4). The above results prove that NQ-Y15 selectively inhibits TXA_2 synthase without influence on PLA_2, COX, or PGI_2 synthase *in vitro*.

In addition to inhibition of TXA_2 synthase, NQ-Y15 specifically blocked platelet receptors for TXA_2/PGH_2. Indeed, this compound completely inhibited platelet aggregation induced by the stable TXA_2 analogue U46619 in human platelets by shifting, in a parallel way, the concentration–response curve to U46619 to the right (Fig. 7). Other drugs possessing the dual property of TXA_2/PGH_2 receptor antagonist and TXA_2 synthase inhibitor act also as competitive antagonists of U46619-induced aggregation of human platelets [13, 14]. The IC_{50} value for TXB_2 formation (Table 1) is generally higher than that for platelet aggregation (Fig. 2) because the antiaggregating effect of NQ-Y15 is due to TXA_2/PGH_2 receptor blockade as well as TXA_2 synthase inhibition. Particularly, in collagen (10 μg/mL)-stimulated platelets, NQ-Y15 (10 μM) markedly inhibited aggregation but failed to inhibit TXB_2 formation significantly. Studies on collagen-stimulated rat platelets provided the evidence for a substantial role for TXA_2 in platelet aggregation. A small but significant amount of TXA_2 initially produced by collagen may act as a trigger to provoke subsequent platelet responses (i.e. shape change, aggregation, and secretion) [34]. A series of TXA_2/PGH_2 receptor antagonists completely suppressed U46619 binding to rat platelets as well as collagen-induced platelet aggregation [53]. Therefore, in collagen (10 μg/mL)-stimulated platelets, NQ-Y15 (10 μM) seems to effectively block the binding of a crucial amount of TXA_2 to receptors without having a significant effect on TXA_2 synthase activity.

Unlike COX inhibitors, selective TXA_2 synthase inhibitors selectively suppress the formation of TXA_2 and cause a re-orientation of PG endoperoxide metabolism toward antiaggregatory PGs, such as PGD_2 and PGI_2 [8]. However, a part of the accumulated PG endoperoxides may also activate a receptor, shared with TXA_2, mediating platelet activation and vasoconstriction [8, 9]. Thus, the balance between PGH_2 redirection and activation of TXA_2/PGH_2 receptors by accumulated PG endoperoxides may influence the net effect of TXA_2 synthase inhibitors to inhibit platelet function *in vivo*. In spite of these limitations, ozagrel (OKY-046), a kind of highly selective TXA_2 synthase inhibitor, has been used in the treatment of asthma in Japan and noted as a therapeutic agent for coronary heart diseases, thromboembolic disorders, and asthma [54–56].

Some drugs that act both as TXA_2 synthase inhibitors and as TXA_2/PGH_2 receptor antagonists have been developed. Picotamide and ridogrel have already been administered to humans. Both drugs reduce the formation of TXA_2 and increase the levels of PGI_2 and PGD_2, and competitively inhibit binding and action of TXA_2 and U46619 [13, 14]. Picotamide may be an effective drug in patients with peripheral occlusive arterial disease of the lower limbs [17, 18] and cerebral infarction [19]. Ridogrel seems to have efficacy in patients with peripheral occlusive arterial disease [20], acute myocardial infarction [21], and pregnancy-induced hypertension [22]. Thus, it is conceivable that NQ-Y15 might be particularly effective for the improvement of thrombotic conditions associated with platelet activation.

Based on the results obtained, we conclude that the antiplatelet effect of NQ-Y15 may be due to the combination of TXA_2 synthase inhibition with TXA_2/PGH_2 receptor blockade in a single molecule, which would decrease TXA_2 synthesis and neutralize the stimulatory activity of accumulated PGH_2 and of any residual amounts of TXA_2 still formed.

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