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Thromboxane prostanoid receptor activation impairs endothelial nitric oxide-dependent vasorelaxations: The role of Rho kinase

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ABSTRACT

Activation of thromboxane prostanoid (TP) receptors causes potent vasoconstriction, which contributes to increased vascular tone and blood pressure. The present study examined the hypothesis that stimulation of TP receptor impaired endothelial nitric oxide-mediated vasorelaxation via a Rho kinase-dependent mechanism. The common carotid arteries of Sprague–Dawley rats were isolated and suspended in myograph for measurement of changes in isometric tension. The production of nitric oxide in primary cultured aortic endothelial cells was assayed with an imaging technique and phosphorylated levels of endothelial NOS were determined by Western blot analysis. 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$ (U46619) inhibited isoprenaline-induced relaxations in rings with or without endothelium. Treatment with Rho kinase inhibitors, Y27632 (2 μM) or HA 1077 (10 μM) prevented the effect of U46619 only in rings with endothelium while protein kinase C inhibitors were without effect. Rho kinase inhibitors did not affect isoprenaline-induced relaxations in endothelium-intact rings treated with L-NAME or 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one (ODQ). Isoprenaline stimulated rises in nitric oxide (NO) production in cultured rat endothelial cells. The increased NO production was inhibited by U46619 (100 nM) and this effect was prevented by treatment with Y27632 but unaffected by the absence of extracellular calcium ions. U46619 attenuated isoprenaline-stimulated phosphorylation of eNOS, which was sensitive to inhibition by Y27632 and HA 1077. U46619-mediated effects were abolished by TP receptor antagonist, S18886 and the TP receptor was present in endothelial cells. The present results demonstrate that Rho kinase activation is likely to be the primary mechanism that underlies the U46619-stimulated TP-receptor-mediated inhibition of endothelial NO production and subsequent endothelium-dependent relaxations to isoprenaline.

1. Introduction

Thromboxane, an unstable prostanoid metabolite of arachidonic acid, exerts its action via stimulation of the thromboxane prostanoid (TP) receptor, a G-protein-coupled receptor, resulting in activation of RhoA. RhoA is a member of the Ras superfamily of small GTP binding proteins which activates a serine/threonine protein kinase, Rho kinase/ROCK [1]. Accumulating evidence supports a positive role of RhoA and Rho kinase in the pathogenesis of cardiovascular and renal diseases [2–4] and these two proteins are regarded as important targets for the treatment of cardiovascular disorders [5]. Activation of Rho kinase by thromboxane A_2 not only sensitizes the contractile machinery to Ca^{2+} ions [1,6], but also negatively regulates the production of nitric oxide (NO) [7,8].

Vascular endothelial cells express β-adrenoceptors that contribute to vasodilatation through stimulation of endothelial NO biosynthesis and subsequent activation of cyclic GMP/protein kinase G (PKG). Stimulation of β_2-adrenoceptors increases serine phosphorylation of endothelial NO synthase (eNOS) in cultured endothelial cells [9,10]. Abnormalities in eNOS gene expression or activity, which result in reduced NO production, are thought to

Abbreviations: L-NAME, N^G-nitro-L-arginine methyl ester; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one; TP, thromboxane prostanoid; U46619, 9,11-dideoxy-11α,9α-epoxy-methanoprostaglandin $F_{2\alpha}$.

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contribute to the pathogenesis of atherosclerosis and hypertension [11,12]. Recent research provides compelling evidence in support of an involvement of RhoA or Rho kinase in the regulation of NO production. Activation of RhoA/Rho kinase pathway leads to inhibition of phosphorylation of eNOS at Ser^{1177} in human endothelial cells [7]. However, the exact role of RhoA/Rho kinase in endothelium-derived NO-dependent vasodilatations in intact arteries is largely unclear. Here we hypothesized that β-adrenoceptor-mediated dilatations can be blunted acutely by TP receptor agonist via reduced eNOS activity, which involves activation of Rho kinase. In the present study, we used a stable analogue of thromboxane A_2, U46619, to trigger a sustained contractile response and examined its effect on isoprenaline-mediated endothelium-dependent vasodilatations and mechanisms involved.

2. Materials and methods

2.1. Artery preparation

This investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). This study was approved by the Experimental Animal Ethics Committee, Chinese University of Hong Kong. Male Sprague–Dawley rats (260–280 g) supplied by the Laboratory Animal Service Center, Chinese University of Hong Kong, were sacrificed by cervical dislocation. Both common carotid arteries were dissected out and placed in a dissecting dish filled with an ice-cold oxygenated Krebs solution of the following composition (in mM): NaCl 119, KCl 4.7, $NaHCO_3$ 25, $CaCl_2$ 2.5, $MgCl_2$ 1, KH_2PO_4 1.2, and D-glucose 11. After careful removal of adhering connective tissue, each artery was cut into four 2 mm-wide ring segments. In some rings the endothelial layer was mechanically disrupted by gently rubbing the luminal surface with stainless steel forceps tips. The ring was suspended between two stainless steel wires in a 5-ml chamber on a Multi Myograph (Danish, Myo Technology A/S, Denmark) as previously described [13]. Krebs solution in the bathing chamber was constantly bubbled with 95% O_2–5% CO_2 and maintained at 37 °C (pH ~7.4). Each ring was stretched to 5 mN, a previously determined optimal resting tone for the development of isometric contraction. Thirty minutes after setting up in bathing chambers, all rings were first contracted by phenylephrine (1 μM) and then relaxed by acetylcholine (1 μM). Acetylcholine normally produced over 85% relaxation in rings with endothelium and no relaxation in rings without endothelium. The rings were then rinsed in pre-warmed, oxygenated Krebs solution several times until a stable resting tone returned and finally equilibrated for 60 min. The resting tone was readjusted to 5 mN if necessary. Each experiment was performed on rings prepared from different rats.

2.2. Measurement of isometric force

Phenylephrine (1 μM, submaximal concentration) was used to contract the rings with and without endothelium, the relaxing effect of isoprenaline (3 nM–3 μM) was studied and compared in control and in the presence of L-NAME (100 μM) or 1H-[1,2,4]oxadizolo[4,3-a]quinoxalin-1-one (ODQ, 3 μM). The first group of experiments examined the effect of 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) on isoprenaline-induced relaxations. Cumulative concentration–response curves for isoprenaline were obtained in rings with endothelium. To evaluate the involvement of the TP receptor and Rho kinase, endothelium-intact rings were first exposed for 30 min to each of the following inhibitors: S18886 (TP receptor antagonist, 300 nM), and Y27632 (2 μM) or HA1077 (10 μM) (both are selective inhibitors of Rho kinase), and then contracted with 1 μM phenylephrine plus 100 nM U46619 before the relaxant effect of isoprenaline was studied. To investigate whether the effect was endothelium-dependent, the same experiments were repeated on rings without endothelium. To examine whether the effect of Rho kinase inhibitors was mediated through NO, rings with endothelium were first treated with Rho kinase inhibitor (10 min incubation) and then 100 μM L-NAME or 3 μM ODQ (30 min incubation) prior to the addition of phenylephrine plus U46619. Once a sustained tone was obtained, isoprenaline was applied cumulatively. The concentration of phenylephrine was lowered to 0.3 μM in the presence of L-NAME or ODQ and in rings without endothelium in order to obtain comparable levels of evoked contractions in rings with endothelium. Finally, the inhibitors of protein kinase C were also tested on the U46619-induced inhibition of endothelium-dependent relaxations and the inhibitors included GF109203X (broad-spectrum protein kinase C inhibitor, 2 μM), Go 6976 (specific PKC_α inhibitor, 1 μM), and rottlerin (PKC_δ inhibitor, 10 μM).

2.3. Primary culture of rat aortic endothelial cells

The procedure for primary culture of rat aortic endothelial cells was described elsewhere [14]. Briefly, the aortas were cut open and treated for 15 min with 0.2% collagenase (Type 1A, Sigma, MO, USA) in PBS at 37 °C. The suspension after enzymatic digestion was centrifuged at 600 × *g* for 15 min. The cells were re-suspended in 5 ml culture medium that contained 90% RPMI, 10% FBS supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin in a 25-cm^2 tissue culture flask. After 1-h incubation at 37 °C, the medium was replaced for removal of unattached cells. Attached endothelial cells were cultured in an incubator with 5% CO_2 at 37 °C. Culture medium was changed every 3 days. To avoid possible loss of endothelial properties under the culture condition, only those cells from the first two passages were used. The identity of the primary cultured rat aortic endothelial cells was confirmed by a positive staining of PECAM-1 (Santa Cruz Biotechnology, CA, USA).

2.4. Laser confocal fluorescent microscopy

Fluorimetric measurements were performed on primary rat aortic endothelial cells using an Olympus Fluoview FV1000 laser scanning confocal system (Olympus America Inc., Melville, NY, USA) mounted on an inverted IX81 Olympus microscope, equipped with a 10× objective (NA 0.5). Intracellular NO production was monitored using fluorescent NO indicator 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) [15]. The dye reacts not with NO itself but with NO^+ equivalents, such as nitric anhydride (N_2O_3), which are formed by autoxidation of NO. Cultured rat aortic endothelial cells seeded on glass coverslips were incubated for 30 min at room temperature in normal physiological solution or Ca^{2+}-free solution containing 1 μM DAF-FM DA (Invitrogen, USA). The amount of NO was evaluated by measuring the fluorescence intensity excited at 495 nm and emitted at 515 nm. Changes in intracellular NO production were displayed as a ratio of fluorescence relative to the intensity (F_1/F_0). Normal physiological saline solution (NPSS) contained 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 5 mM HEPES (pH 7.4) while Ca^{2+}-free solution contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 0 mM $CaCl_2$, 10 mM glucose, 5 mM HEPES, and 0.22 mM EGTA (pH 7.4).

2.5. Western blot analysis of eNOS protein

The procedure for immunoblotting eNOS protein was described before [16]. Rat aortas were used because they provided sufficient amount of tissue for Western blot analysis. Arteries were isolated and frozen in liquid nitrogen following similar protocols for force measurement. The arterial tissue was homogenized in ice-cold RIPA

lysis buffer containing 1 μg/ml leupeptin, 5 μg/ml aprotonin, 100 μg/ml phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mM EGTA, 1 mM EDTA, 1 mM sodium fluoride and 2 mg/ml β-glycerolphosphate. The lysates were incubated on ice for 30 min and then centrifuged at 20,000 × *g* for 20 min. The supernatant was collected and analyzed for protein concentration using Lowry method (Bio-Rad, USA). One volume of 2× sample loading buffer containing 5% β-mercaptoethanol was added and the samples were denatured by boiling for 5 min. For each sample, 50 μg of the total protein was electrophoresed under reducing conditions on a 7.5% SDS-polyacrylamide gel. The resolved proteins were electroblotted to immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, USA) using wet transfer at 100 V for 60 min at 4 °C. The membranes were blocked with 1% BSA in 0.5% Tween-20 phosphate-buffered saline (PBST) for 60 min at room temperature and subsequently exposed to a polyclonal rabbit anti-eNOS antibody (1:500, BD Transduction Laboratory) or to a polyclonal rabbit anti-phosphorylated eNOS (1:1000, Upstate) overnight at 4 °C. The membranes were subsequently probed with a horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins antibody (Dako Cytomation) at a dilution of 1:3000 for 1 h at room temperature. The membranes were developed with an enhanced chemiluminescence detection system (ECL reagents; Amersham Pharmacia Biotech, Bucks, UK) and then exposed to X-ray films. eNOS or p-eNOS corresponding to a ~145-kDa band was visualized with reference to molecular weight markers. The signal intensities were quantified using a gel documentation system (FluorChem, Alpha Innotech Corp.) and compared based on the equal loading of the same amount of total protein.

2.6. Immunohistochemistry

Localization of TP receptors were determined by immunohistochemistry. Rat carotid arteries were excised, dissected and fixed in a freshly prepared Carnoy's fixative (absolute methanol:chloroform:glacial acetic acid at a ratio of 6:3:1) overnight. The sections were hydrated and treated with 1.4% hydrogen peroxide in absolute methanol at room temperature for 30 min in order to inhibit endogenous peroxidase activity. After rinsing, the sections were incubated with primary antibodies against TP receptor (Cayman Chemical, MI, USA) and PECAM-1 (Santa Cruz Biotechnology, CA, USA) diluted in PBS supplemented with 2% BSA in a moist chamber at 4 °C overnight. The sections were then incubated with corresponding biotinylated secondary antibodies at room temperature for one hour, followed by a 1 h-incubation of streptavidin conjugated with peroxidase. The sections were developed with DAB according to the manufacturer's instruction (Vector Laboratories). Counter-staining on nucleus was performed with haematoxylin. After dehydration, the sections were mounted with Permount®. Negative control was performed in the absence of primary antibody.

2.7. Chemicals

Phenylephrine, acetylcholine, N^G-nitro-L-arginine methyl ester, isoprenaline, HA 1077, (1)-(R)-trans-4-(1-aminoethyl)-N-(4-pyridyl)cyclohexanecarboxamide dihydrochloride (Y27632) were purchased from Sigma–Aldrich Chemical (MO, USA). Angiotensin II, GF109203X, Go 6976, rottlerin and ODQ were purchased from Tocris (MO, USA). 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619) was purchased from Cayman Chemical (MI, USA). S18886 is a gift from Servier. U46619, ODQ, and GF109203X, rottlerin and Go 6976 were dissolved in dimethyl sulfoxide (DMSO) and all other drugs were dissolved in double-distilled water. Stock solutions were stored at −20 °C. Desired dilution was prepared in Krebs solution shortly before experimentation. DMSO at 0.1% did not influence evoked contractions or relaxations.

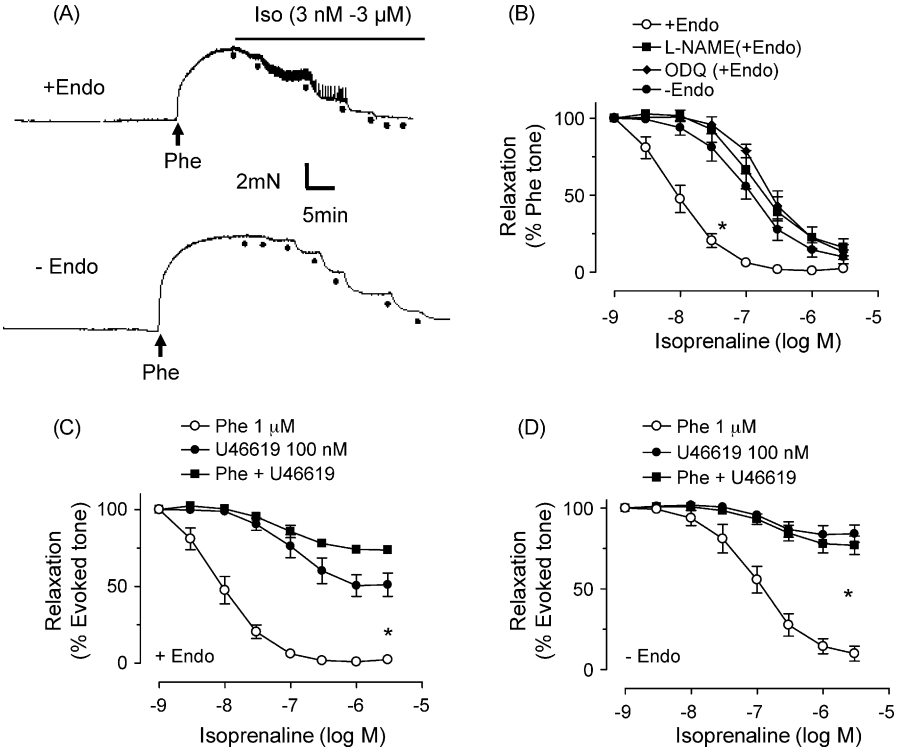

Fig. 1. (A) Representative traces showing isoprenaline-induced relaxations in phenylephrine (Phe)-contracted rat carotid arterial rings with and without endothelium. (B) Concentration–response curves for isoprenaline in control and in the presence of 100 μM L-NAME or 3 μM ODQ, and in rings without endothelium. Markedly attenuated relaxations to isoprenaline in rings contracted by 100 nM U46619 or a combination of phenylephrine plus U46619 in the presence (C) and absence of endothelium (D) (n = 6 in each case). Statistical difference between curves is indicated by $^*P < 0.05$. Data are means ± S.E.M. of 5–7 experiments from different rats.

2.8. Data analysis

Data are means ± SEM for the number (n) of arterial rings from different rats. pD_2 is the negative logarithm of the dilator concentration needed to produce half of the maximal relaxation as determined by non-linear regression curve fitting (Graphpad Prism Software, version 4.0). Concentration–relaxation curves were analyzed by two-way ANOVA followed by Bonferroni post-tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Endothelium-dependent relaxant effect of isoprenaline

Representative traces in Fig. 1A show that isoprenaline produced greater relaxations of phenylephrine-contracted carotid arterial rings with endothelium than rings without endothelium (pD_2: 8.12 ± 0.10 with endothelium and 7.00 ± 0.10 without endothelium, $P < 0.05$, Fig. 1B and Table 1). Isoprenaline-induced relaxations were attenuated by L-NAME or ODQ to the same extent as that found in rings without endothelium while the maximal relaxation was unchanged (Fig. 1B and Table 1).

3.2. Effects of U46619 on isoprenaline-induced relaxations

The relaxations to isoprenaline were significantly less in rings with endothelium pre-contracted by U46619 than by phenylephrine, and the maximal relaxation was progressively reduced (E_{max}: 71.3 ± 8.7% in 30 nM U46619, n = 6; 48.9 ± 7.6% in 100 nM U46619, n = 7; 44.3 ± 10.5% in 300 nM U46619, n = 5, $P < 0.01$, as compared with 97.6 ± 2.1% in 1 μM phenylephrine, n = 5, data not shown). The presence of U46619 attenuated isoprenaline-induced relaxations in rings with and without endothelium (Fig. 1C and D) of similar initial tension (4.53 ± 0.41 mN with endothelium and 3.81 ± 0.41 mN without endothelium). Treatment with 300 nM S18886 prevented the inhibitory effect of U46619 on isoprenaline-induced relaxations (Fig. 2B and C). By contrast, exposure to angiotensin II (100 nM) did not affect isoprenaline-induced relaxations in phenylephrine-contracted rings with endothelium (n = 6, data not shown).

3.3. Effects of inhibitors of Rho kinase and protein kinase C

Y27632 (2 μM) and HA 1077 (10 μM) at concentrations used in the present study are reported to be selective for inhibition of Rho kinase [17]. Both inhibitors did not affect contractions caused by phenylephrine plus U46619 in rings with endothelium (Table 1). Treatment with either Y27632 or HA 1077 partially, but significantly enhanced isoprenaline-induced relaxations in the presence of U46619 only in rings with endothelium ($P < 0.01$ versus control, Fig. 2B, D and E and Table 1). By contrast, treatment with 2 μM GF109203X (broad-spectrum protein kinase C inhibitor), 1 μM Go 6976 (specific PKC_α inhibitor), or 10 μM rottlerin (PKC_δ inhibitor) at effective concentrations [18,19] did not affect U46619-induced inhibition of relaxations to isoprenaline (n = 4, data not shown).

Table 1
pD_2 and E_{max} (%) values for isoprenaline-induced relaxations.

Treatment	Initial tone	pD_2	E_{max} (%)	n
With endothelium				
Control (Phe)	2.07 ± 0.14	8.12 ± 0.10	97.6 ± 2.1	5
+ L-NAME	4.83 ± 0.30	6.87 ± 0.12$^{\#}$	83.9 ± 5.6	5
+ ODQ	6.70 ± 0.07$^{\#}$	86.9 ± 4.9		5
Control (U46619 + Phe)	4.53 ± 0.41	7.08 ± 0.11	26.3 ± 2.9	5
+ S18886	2.92 ± 0.38	7.95 ± 0.08$^{\#}$	95.6 ± 1.9$^{\#}$	5
+ Y27632	3.92 ± 0.16	7.24 ± 0.10	68.4 ± 7.1$^{\#}$	5
+ HA1077	2.95 ± 0.23	7.22 ± 0.05	60.6 ± 8.4§	6
+ Y27623 + L-NAME	3.84 ± 0.21	6.57 ± 0.33	18.9 ± 6.6	6
+ Y23623 + ODQ	3.84 ± 0.50	6.54 ± 0.45	22.6 ± 4.8	6
+ HA1077 + L-NAME	4.30 ± 0.54	6.70 ± 0.15	26.1 ± 5.5	6
Without endothelium				
Control (Phe)	3.81 ± 0.41	7.00 ± 0.10	90.1 ± 4.5	7
Control (U46619 + Phe)	4.07 ± 0.30	6.74 ± 0.20	23.1 ± 5.7	8
+ S18886	3.58 ± 0.22	6.77 ± 0.06	90.7 ± 3.3$^{\#}$	6
+ Y27623	3.94 ± 0.30	6.62 ± 0.18	30.0 ± 5.2	6
+ HA1077	3.71 ± 0.23	5.63 ± 1.99	26.1 ± 5.5	6

Significant difference between control and treatment groups is indicated by $^{\S}P < 0.01$ and $^{\#}P < 0.001$. Data are means ± S.E.M. of n experiments.

3.4. Role of endothelium in U46619-induced effect

Fig. 2E shows that neither Y27632 nor HA 1077 affect the U46619-impaired relaxations to isoprenaline in rings without endothelium (also see Table 1), suggesting that both inhibitors are most likely to act on the endothelium. Y27632 failed to influence isoprenaline-induced relaxations in rings with endothelium after inhibition of NO synthesis by L-NAME or inhibition of NO-dependent guanylate cyclase by ODQ (Fig. 3A and B). Likewise, HA 1077, another Rho kinase inhibitor, did not inhibit the reduced relaxations in the presence of L-NAME (Fig. 3A and C).

3.5. Effect of U46619 on NO production in endothelial cells

Addition of 100 nM isoprenaline triggered a progressive rise in intracellular NO levels in cultured endothelial cells as reflected by the increased dichlorofluorescein fluorescence intensity within a 16-min recording period (Fig. 4A and G) and the elevated NO signal was largely inhibited following 30-min pre-exposure to L-NAME ($P < 0.05$, Fig. 4A). Pretreatment (20 min) with U46619 inhibited the isoprenaline-stimulated rise in NO signal ($P < 0.05$) and this effect was prevented by S18886 (10-min incubation) (Fig. 4B and G). Treatment with 2 μM Y27632 also abolished the inhibitory effect of U46619 (Fig. 4C and G). The isoprenaline-stimulated NO increase was unaffected by removal of extracellular calcium ions (Fig. 4D) and was again inhibited by 100 μM L-NAME or 100 nM U46619 (Fig. 4D and E). Both S18886 and Y27632 abolished the U46619-mediated inhibition of NO production in endothelial cells bathed in a Ca^{2+}-free solution (Fig. 4E and F).

3.6. Protein expression of eNOS

Western blot analysis shows that isoprenaline at 100 nM increased the phosphorylation of eNOS at Ser^{1177} in arteries with endothelium (Fig. 5A). Treatment with 100 nM U46619 prevented isoprenaline-stimulated increase in eNOS phosphorylation while S18886 at 300 nM, Y27632 at 2 μM or HA 1077 at 10 μM abolished the effect of U46619 (Fig. 5A).

3.7. Immunohistochemistry

Immunohistochemical staining shows the expression of the TP receptor on the endothelium of the isolated carotid arteries, which was also stained by PECAM-1, a selective marker for endothelial cells (Fig. 5B).

4. Discussion

The main finding of the present study is that the TP receptor which is present in endothelial cells mediates U46619-induced inhibition of isoprenaline-induced endothelium-dependent relaxations and involves Rho kinase in endothelial cells. The inhibitory effect of U46619 is associated with the reduced activity of eNOS and NO biosynthesis because selective Rho kinase inhibitors but not PKC inhibitors abolished U46619-induced effect

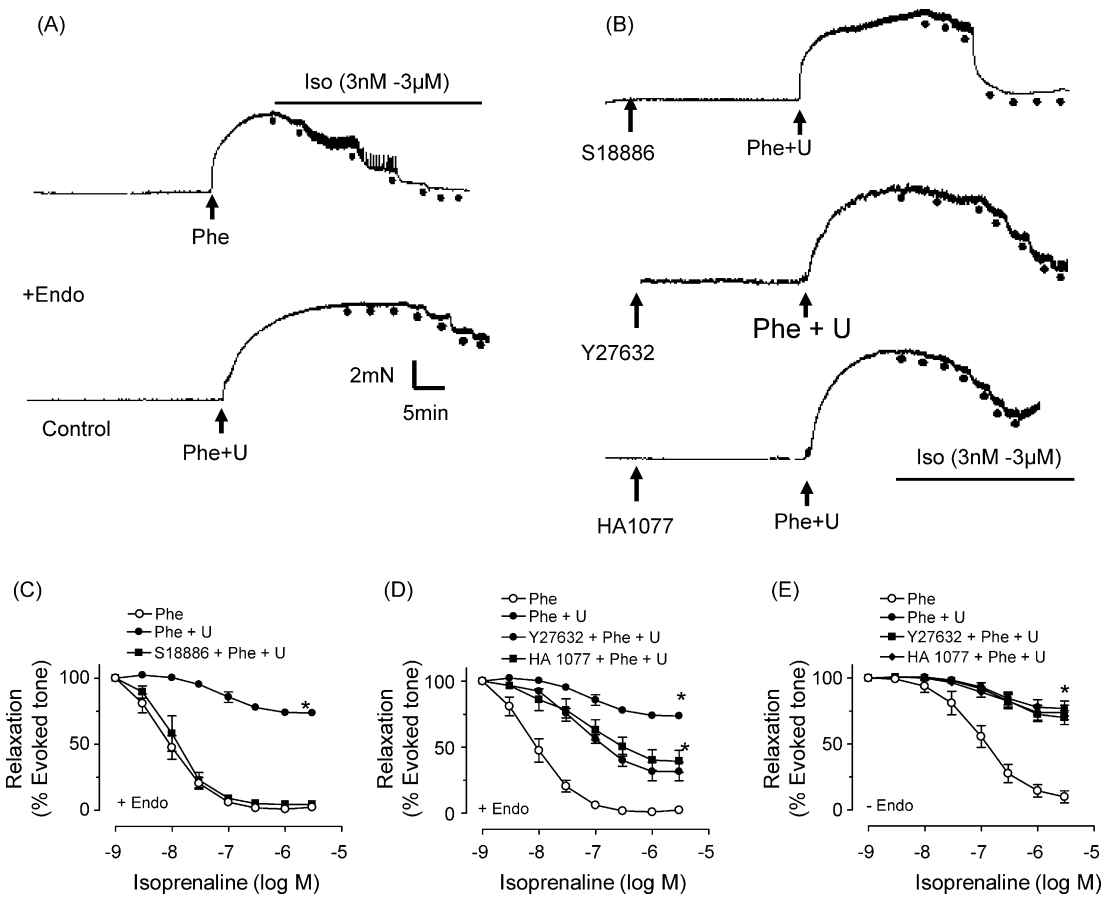

Fig. 2. (A) Representative traces showing isoprenaline-induced relaxations in rings pre-constricted by Phe or Phe + U46619 (Phe + U). (B) Traces showing the reduced relaxations to isoprenaline in the presence of 100 nM U46619 being partially reversed by treatment with 300 nM S18886, 2 μM Y27632 or 10 μM HA1077. Concentration–response curves for isoprenaline in the absence and presence of 100 nM U46619 with and without pretreatment with 300 nM S18886 (C) and of 2 μM Y27632 or 10 μM HA1077 in rings with endothelium (D) or in rings without endothelium (E). Statistical difference between curves is indicated by $^{*}P < 0.05$. Data are means ± S.E.M. of 5–7 experiments.

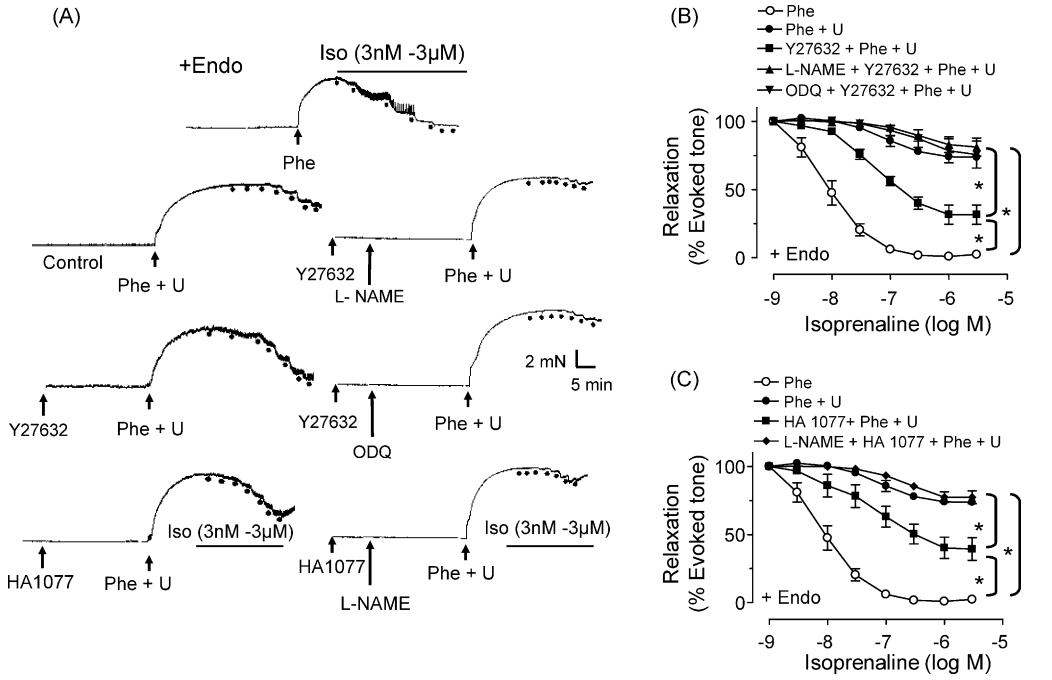

Fig. 3. (A) Representative traces showing isoprenaline-induced relaxations in Phe plus U46619-contracted rings with endothelium in the presence of combined inhibitors: Y27632 plus L-NAME, Y27632 plus ODQ, or HA1077 plus L-NAME. Concentration–response curves for isoprenaline in arteries treated by Y27632 (B) or HA1077 (C). Statistical difference between curves is indicated by $^{*}P < 0.05$. Data are means ± S.E.M. of 5 experiments.

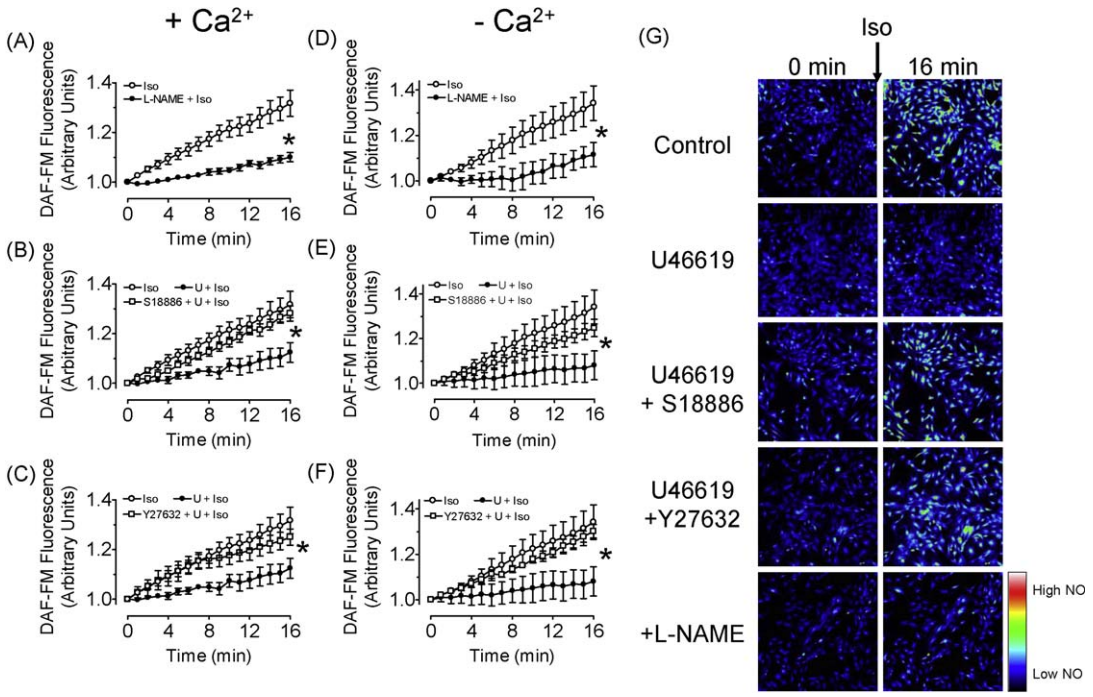

Fig. 4. (A) Isoprenaline (Iso)-stimulated rise in NO levels in cultured rat aortic endothelial cells in control and in the presence of L-NAME. Inhibitory effect of 100 nM U46619 on the stimulated NO generation and reversal of U46619-evoked inhibition by 300 nM S18886 (B) or by 2 μM Y27632 (C). The aforementioned experiments were performed in normal physiological saline solution (NPSS) containing calcium The identical experiments were repeated in Ca^{2+}-free saline solution (n = 3–5, D–F). (G) Representative images showing DAF-FM fluorescence in NPSS. Values are presented as F_1/F_0. Significant difference between curves was indicated by $^{*}P < 0.05$. Data are means ± S.E.M. of 4–7 experiments.

which was also lost upon blockade of NOS/guanylate cyclase by L-NAME or ODQ. In addition, isoprenaline-induced increase in the eNOS activity and NO formation are independent of the presence of extracellular calcium ions.

Stimulation of β-adrenoceptors by isoprenaline caused both endothelium-dependent and -independent relaxations in rat carotid arteries as also reported in other arteries [20]. The former is mediated fully by endothelium-derived NO as isoprenaline produces the same relaxant effects following pharmacological inhibition of NO/cGMP pathway as those in rings without endothelium. β-adrenoceptor agonists also produce vasodilatation through activation of adenylyl cyclase and the consequence of cAMP in vascular smooth muscle cells [21]. Acute (30 min) exposure to U46619 impairs relaxations in both types of rings and the attenuated relaxations were prevented by S18886, a selective TP receptor antagonist [22].

Isoprenaline-induced endothelium-dependent relaxations are attributable to stimulation of endothelial NO biosynthase [9,23]. We have confirmed the involvement of NO by detecting (1) a time-dependent rise in NO signal triggered by isoprenaline in cultured rat endothelial cells loaded with an NO-sensitive fluorescent dye DAF-FM; (2), the increased NO signal being inhibited by L-NAME,

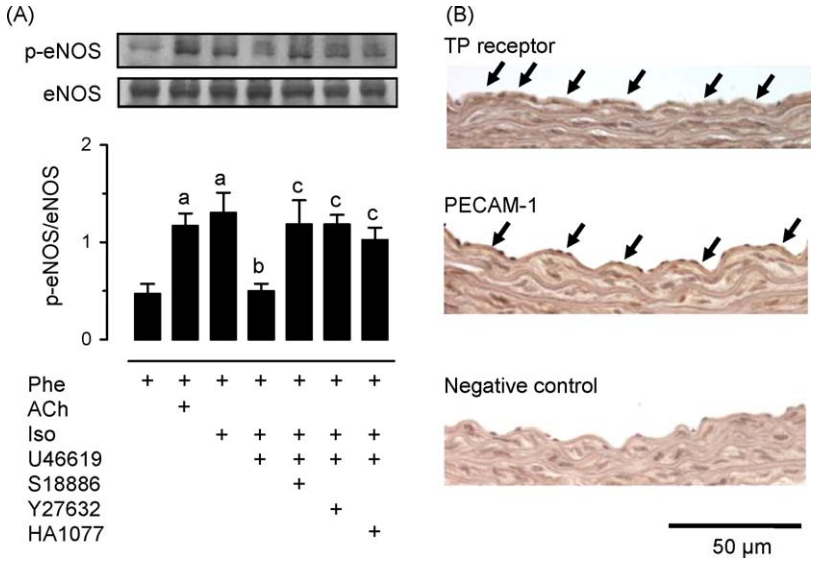

Fig. 5. (A) Western blot showing inhibitory effect of 100 nM U46619 on isoprenaline (100 nM, Iso)-induced increase in phosphorylation of eNOS at Ser^{1177} and prevention of this effect by 300 nM S18886, 2 μM Y27632, and 10 μM HA 1077. Data are means ± S.E.M. of 4–5 experiments. Statistical difference ($P < 0.05$) is indicated by [a]between Phe and other groups; [b]between Iso and U46619 + Iso groups; and [c]between U46619 + Iso and other groups co-treated by U46619. (B) Immunohistochemical staining of the TP receptor and PECAM-1 in endothelial cells of isolated rat carotid arteries. Arrows point to endothelial cells. Similar observations were made on carotid arteries from 4 rats.

and (3) increased serine phosphorylation of eNOS in response to the same concentration of isoprenaline in endothelium-intact rings. The present study shows that addition of isoprenaline elicited rapid and sustained elevation of $[Ca^{2+}]_i$ due to calcium influx in cultured rat endothelial cells (data not shown) and our results are different from those described in human endothelial cells in which β-adrenoceptor-mediated eNOS activation involves no change in $[Ca^{2+}]_i$ [24], indicating a species difference in the regulation of endothelial cell $[Ca^{2+}]_i$. We then examined whether Ca^{2+} was required for the ligand-triggered rise of NO production and found that isoprenaline produced a comparable stimulatory effect on NO production in the presence and absence of extracellular calcium ions and L-NAME prevented NO formation under both ionic conditions. Likewise, isoprenaline-induced increase in eNOS phosphorylation in rings with endothelium was unaffected by the omission of extracellular calcium ions. Taken together, the present findings suggest that isoprenaline-stimulated calcium influx is not coupled to eNOS activation and NO production while other calcium-independent factors such as protein kinase A in relation to β-adrenoceptors may be involved [25,26], which is beyond the focus of the present study.

The present study shows that isoprenaline-induced relaxations were blunted by U46619 in a concentration dependent manner and the action of U46619 is mediated through the TP receptor as it was prevented by S18886. Increased production or/and action of thromboxane A_2 has been implicated in cardiovascular and renal diseases [27,28]. RhoA/Rho kinase plays an important role in the induction of vascular smooth muscle contraction as its inhibitors reduce the vascular tone in several blood vessels from different species [29,30]. Although long-term inhibition of RhoA inhibits vasocontraction by augmenting NO production [8], the precise role of RhoA/Rho kinase in the regulation of endothelial function in the intact arteries remains largely unknown. Previous studies described that RhoA via its downstream effector Rho kinase inhibits eNOS gene expression, eNOS phosphorylation and NO production in cultured human endothelial cell line [7]. The present study shows that pretreatment with two structurally different inhibitors of Rho kinase, Y27632 and HA 1077 reduced the inhibitory effect of U46619 on isoprenaline-induced relaxations in rings with endothelium. Our results clearly suggest that the endothelium is the primary site of action for Rho kinase inhibitors based on the following observations. Firstly, Rho kinase inhibitors failed to improve the U46619-impaired relaxations in rings without endothelium; secondly, inhibition of NO/cGMP pathway by L-NAME or ODQ in rings with endothelium invalidates the effect of Rho kinase inhibitors; and thirdly, Y27632 eliminated the U46619-induced inhibition of NO production in cultured rat endothelial cells. The concentrations used for both inhibitors are known to be selective in inhibiting Rho kinase activity [17]. Y27632, widely used as a specific inhibitor of the Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) family of protein kinases inhibits ROCKs by competing with ATP for its binding to the kinases [31]. Likewise, HA 1077, also known as AT877 or fasudil hydrochloride, potently inhibits ROCK [17]. The precise mechanisms by which TP receptor activation results in blunted isoprenaline-induced endothelium-independent vasodilatation remain unsolved in the present study. It is probable that TP receptor activation may lead to (i) a reduced binding affinity of isoprenaline to β-adrenoceptors; (ii) a reduced cAMP level due to the altered phosphodiesterase activity; and (iii) inhibition of protein kinase A-regulated potassium channels [20].

PKC is required for RhoA-induced ROCK activation leading to eNOS gene suppression in endothelial cells [32]. However, the present results do not indicate an obvious role of PKC in the TP receptor-mediated inhibition of endothelium (NO)-dependent relaxations. Neither non-specific PKC inhibitor nor selective inhibitor for PKC_α and PKC_δ attenuated the effect of U46619 on vasorelaxations.

In summary, the present study demonstrates that TP receptor activation attenuates endothelium-dependent relaxations in response to the β-adrenoceptor agonist via RhoA/Rho kinase-mediated but calcium-independent mechanisms, leading to the reduced eNOS activation and NO production in endothelial cells. As Rho kinase significantly contributes to cardiovascular pathophysiology, it has become an important therapeutic target in cardiovascular medicine [5]. Besides, TP receptor antagonists are known to possess a promising potential for the treatment of thrombosis and atherosclerosis [33]. Our results highlight additional benefit of the TP receptor antagonist and RhoA/Rho kinase inhibitors by preserving eNOS activity and NO production which are commonly impaired during the development of hypertension and atherosclerosis. Nevertheless, further investigation warrants to reveal the calcium-independent pathway linking activation of RhoA/Rho kinase to the inhibition of eNOS activation, especially in human blood vessels.

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