Original Article

The Nitric Oxide-cGMP Pathway Does Not Play an Essential Role in β-Adrenoceptor-Mediated Smooth Muscle Direct Relaxation in the Rat Thoracic Aorta

Shunsuke Shiina Rikako Ui Tomoka Endo
Keisuke Obara Daisuke Chino and Yoshio Tanaka*

Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toho University

ABSTRACT

Background: The smooth muscles of blood vessels express relaxant β-adrenoceptor, which functions as a negative feed-back system against α_1-adrenoceptor-mediated contraction. Although β-adrenoceptor-mediated vascular smooth relaxation is generally thought to be triggered through a cyclic adenosine monophosphate (cAMP)-dependent pathway, a recent report has suggested a principal role for the nitric oxide (NO)-cyclic guanosine monophosphate (cGMP) pathway. Thus, in this study, we examined whether the NO-cGMP pathway played an essential role in β-adrenoceptor-mediated smooth muscle direct relaxation in the rat thoracic aorta.

Methods: The effects of an NO synthase inhibitor (L-NNA) or a soluble guanylyl cyclase inhibitor (ODQ) on the relaxation responses to β-adrenoceptor agonists were examined in endothelium-denuded rat thoracic aortas. The effects of β-adrenoceptor agonists on arterial cGMP content were also examined.

Results: Both L-NNA and ODQ potently suppressed acetylcholine (ACh)-induced, endothelium-dependent relaxation. ODQ also largely suppressed endothelium-independent relaxation induced by an NO donor ((±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide [NOR3]). However, relaxation of the endothelium-denuded aortas in response to the β-adrenoceptor agonists isoprenaline, salbutamol, isoprenaline or CGP-12177A in the presence of propranolol, or noradrenaline was not substantially reduced by L-NNA or ODQ. Neither isoprenaline nor noradrenaline affected arterial cGMP content, whereas NOR3 caused an approximately 30-fold increase in cGMP content.

Conclusions: Our findings suggested that the NO-cGMP pathway had an insignificant effect on endothelium-independent smooth muscle direct relaxation in the rat thoracic aorta in response to β-adrenoceptor agonists of any subtype (β_1, β_2, or β_3).

Toho J Med 2 (3): 95–105, 2016

KEYWORDS: rat thoracic aorta, β-adrenoceptor, vascular relaxation, nitric oxide-cGMP pathway, vascular smooth muscle

Blood vessels constrict when the sympathetic nervous system is activated. This can be explained by the fact that blood vessel smooth muscles contain large amounts of constrictor α (α_1)-adrenoceptor, which is stimulated by adrena-

2–2–1 Miyama, Funabashi, Chiba 274–8510, Japan
*Corresponding Author: tel: +81–(0)47–472–1419
e-mail: yotanaka@phar.toho-u.ac.jp
DOI: 10.14994/tohojmed.2016.012

Received May 9, 2016: Accepted Sept. 9, 2016
Toho Journal of Medicine 2 (3), Sept. 1, 2016.
ISSN 2189–1990, CODEN: TJMOA2

line and noradrenaline to cause blood vessel smooth muscle contraction. However, blood vessel smooth muscles also contain relaxant β-adrenoceptors.[1, 2] In general, the density of β-adrenoceptors is lower than that of constrictor α_1-adrenoceptors. Therefore, stimulation by adrenaline or noradrenaline usually causes blood vessel constriction rather than relaxation. In contrast, when blood vessel smooth muscle α_1-adrenoceptors are inhibited or not activated, blood vessel smooth muscle relaxation, which reflects the stimulation of β-adrenoceptors, can be detected. Therefore, it is reasonable to speculate that the physiological role of β-adrenoceptors is substantial; they function as a negative feedback system to suppress α_1-adrenoceptor-mediated contractions triggered by adrenaline or noradrenaline.[3]

At present, β-adrenoceptors are subdivided into three subtypes: β_1, β_2, and β_3.[4–6] Since the classification by Lands et al in 1967,[7] β_2 has been considered the predominant subtype. However, several reports have indicated a primary role for β_1- rather than β_2-adrenoceptors in some blood vessel smooth muscles.[3, 8–11] Moreover, some blood vessel smooth muscles also contain β_3-adrenoceptors,[3, 5, 11–14] the presence of which was first discovered in fat cells.[15] Our unpublished observations also suggest that in the rat thoracic aorta, the relaxant response to isoprenaline is mediated through both propranolol (Prop)-sensitive and Prop-insensitive β-adrenoceptors that may include the β_3 subtype.

With regard to the intracellular mechanisms responsible for β-adrenoceptor-mediated smooth muscle relaxation, the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) pathway is generally recognized as the most critical route.[16] This presumption is reasonable considering that the β-adrenoceptor subtypes (β_1, β_2, and β_3) are classified as Gs protein-coupled receptors.[4–6] However, in addition to cAMP-dependent pathways, including the cAMP-PKA pathway, cAMP-independent pathways have also been suggested to have important roles in β-adrenoceptor-mediated relaxation of smooth muscles, including blood vessel muscles.[3, 5, 17]

In contrast, some reports have also suggested a substantial role for nitric oxide (NO) in β-adrenoceptor-mediated blood vessel relaxation.[3, 18] The degree of regulation by NO seems to vary depending on the blood vessel type; thus, NO does not mediate all types of blood vessel relaxation in response to β-adrenoceptor agonists.[3] Furthermore, even if the role of NO in β-adrenoceptor-mediated blood vessel relaxation is essential, most reports have indicated that NO is produced in and released from endothelial cells when stimulated with β-adrenoceptor agonists.[3, 19] In contrast to this perception, a recent study has suggested that the primary pathway underlying β_2-adrenoceptor-mediated relaxation in the rat thoracic aorta is the NO-cyclic guanosine monophosphate (cGMP) pathway, rather than the cAMP-PKA pathway.[20] This idea is fascinating, and suggests the presence of a new intracellular signal pathway that is activated by β-adrenoceptor stimulation. However, pharmacological data in support of the indispensability of the NO-cGMP pathway in β-adrenoceptor-mediated vascular relaxation are not necessarily satisfactory. Furthermore, the reproducibility of such data should be cautiously assessed by various research groups to verify the physiological significance of the NO-cGMP pathway in relation to vasorelaxation in response to β-adrenoceptor agonists.

Therefore, the present pharmacological study was carried out to determine whether the NO-cGMP pathway was involved in rat thoracic aortic relaxation caused by β-adrenoceptor agonists. The present findings were unable to verify the NO-cGMP pathway as a substantial signal transduction route for triggering smooth muscle direct relaxation mediated by the stimulation of any β-adrenoceptor subtype (β_1, β_2, or β_3) in a rat conduit artery.

Materials and Methods

Animals

Male Wistar rats (8 − 9 weeks old, 180 − 230 g; Sankyo Labo Service Corp., Inc., Tokyo, Japan) were housed under controlled conditions (lights on: 8 a.m., light off: 8 p.m.; temperature: 20 − 22℃; relative air humidity, 50% ± 5%). Food and water were available to all animals *ad lib*., and only healthy rats were used for experiments. This study was approved by the Toho University Animal Care and User Committee (approval number: 15-51-294, accredited on May 22, 2015; approval number: 16-52-294, accredited on May 16, 2016) and conducted in accordance with the User Guidelines of the Laboratory Animal Center of the Faculty of Pharmaceutical Sciences, Toho University.

Preparation of aortic vascular beds

The rats were anesthetized with pentobarbital sodium (30 mg/kg, intra-peritoneal [i.p.]) or isoflurane (inhalation) and exsanguinated from a carotid artery. A section of the thoracic aorta between the aortic arch and the diaphragm was then quickly removed and immersed in a modified

Krebs-Henseleit solution containing 118 mM NaCl, 4.75 mM KCl, 2.54 mM $CaCl_2$, 1.20 mM $MgSO_4$, 1.19 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM D-(+)-glucose. The aorta was cleaned of loosely adhering fat and connective tissue under a dissecting microscope, and cut into spinal segments approximately 2 mm in width and 20 mm in length. Special care was taken not to damage the intimal surface of the arteries when endothelium-intact preparations were used to record endothelium-dependent relaxation in response to acetylcholine (ACh). Endothelium-free preparations were produced by gently rubbing the intimal surface with filter paper. When the relaxant responses to NA were examined, the region close to the diaphragm was used for recordings.

Recording of isometric tension changes

The spiral aortic segments were then mounted under an optimal resting tension of 1.0 g in a 20-mL organ bath containing the modified Krebs-Henseleit solution (described above) aerated with 95% O_2 and 5% CO_2, and maintained at 35.0±0.5℃ (pH = 7.4). Tension changes in the muscle preparation were isometrically measured using a force-displacement transducer (TB-612T; Nihon Kohden Corp., Tokyo, Japan) connected to an amplifier (AP-621G; Nihon Kohden Corp.) and recorded on a Windows PC through an A/D converter (PowerLab/4sp; ADInstruments Japan Inc., Nagoya, Japan) and associated software (LabChart 7 for Windows; ADInstruments Japan Inc.).

Spiral preparations were equilibrated for 60 − 90 min prior to the first phenylephrine (Phe)-induced contraction, during which time the modified Krebs-Henseleit solution was exchanged every 20 min with a fresh solution. After the equilibration period, the aortic preparation was contracted 2 − 3 times with 10^{-7} M Phe to confirm that the preparation generated a normal level of contraction. At the first Phe-induced contraction, the preparation was challenged with ACh (10^{-5} M) to verify the functional presence of the endothelium. An aortic preparation in which ACh-induced relaxation was not observed was regarded as an endothelium-denuded preparation. In contrast, an aortic preparation in which ACh (10^{-5} M)-induced relaxation exceeded 75% of the Phe-induced contraction was regarded as an endothelium-preserved preparation. With regard to calculation of the degree of ACh-induced relaxation, the tension level before the application of ACh was considered 0% relaxation, and that before the application of Phe was considered 100% relaxation. Every experiment was carried out in the presence of 3×10^{-6} M indomethacin.

Assessment of the effects of NO-cGMP pathway inhibitors on relaxation responses

After the aortic preparation had been contracted 2 − 3 times with 10^{-7} M Phe, as described above, and had been fully recovered by washing with a fresh bath solution, the preparation was again contracted using 10^{-7} M Phe. For recording noradrenaline (NA)-induced relaxation, the contraction was produced by 10^{-5} M Phe instead of 10^{-7} M. When the Phe (10^{-7} or 10^{-5} M)-induced contraction reached a steady-state level, which usually occurred 20 − 30 min after the application of Phe, all muscle relaxants (ACh, (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide [NOR3], and β-adrenoceptor agonists) were cumulatively applied to the bath solution to determine their concentration-response relationships, which were regarded as their control responses. For the relaxant responses induced by ACh, NOR3, and β-adrenoceptor agonists, with the exception of NA, similar procedures were employed with the corresponding preparations in the presence of an NO synthase (NOS) inhibitor, N^G-nitro-L-arginine (L-NNA), or a soluble guanylyl cyclase inhibitor (1*H*-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one [ODQ]); the second responses in the presence of these inhibitors were compared with the control responses (the first responses). In the case of NA, the relaxant responses in the absence or presence of any inhibitor (L-NNA or ODQ) were recorded with different preparations; this was because a high concentration of Phe (10^{-5} M) was used for NA relaxation, and therefore, recordings were difficult to repeat with the same preparation. L-NNA (3×10^{-5} M) or ODQ (10^{-5} M) was applied to the bath solution 40 min (for L-NNA) or 90 min (for ODQ) before cumulative application of muscle relaxants; L-NNA and ODQ were applied 20 and 70 min before stimulation with Phe, respectively. When β-adrenoceptor antagonists or uptake inhibitors (desipramine, 3×10^{-7} M; deoxycorticosterone, 10^{-5} M) were used, they were applied to the bath solution 40 − 50 min before cumulative application of β-adrenoceptor agonists (20 min before stimulation with Phe).

Determination of tissue cGMP content

Spiral segments (2 mm in width and 20 mm in length) were prepared from a 15-mm-long segment of thoracic aorta, as described for similar tension recording studies. In this series of experiments, the endothelium was removed with a filter paper. Each preparation was incubated in an organ bath containing normal Tyrode's solution (20 mL)

containing 158.3 mM NaCl, 4.0 mM KCl, 2.0 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 10.0 mM $NaHCO_3$, and 5.6 mM D-(+)-glucose, which was continuously aerated with 95% O_2 and 5% CO_2 and maintained at 35.0±0.5℃ (pH = 7.4). After a 60-min incubation, the artery segments were exposed to isoprenaline (10^{-6} M), noradrenaline (10^{-4} M), or NOR3 (10^{-7} M) for 5 min. At the end of the protocol, tissues were rapidly frozen in liquid N_2 to terminate the reaction. The frozen arteries were crushed using a frozen cell crusher apparatus (Cryo-Press; Microtec Co., Ltd., Funabashi, Japan), and the obtained crushed powders were mixed in 6% trichloroacetic acid (TCA) solution containing 3-isobutyl-1-methylxanthine (IBMX; 5×10^{-4} M; a phosphodiesterase inhibitor) for 30 s using a vortex mixer. The suspension was left for 60 min at 4℃ and then centrifuged at 2000 × *g* for 15 min at 4℃. The supernatant fractions and tissue pellets were used for the measurement of cGMP and protein content, respectively. The cGMP in the supernatant was extracted four times with water-saturated ether to remove TCA under acidic conditions (HCl) and then lyophilized. The amount of cGMP was measured using an enzyme-immunoassay system (cGMP, Biotrak™ EIA System; GE Healthcare UK, Buckinghamshire, UK). Tissue pellets were dissolved in 1 mL of 1 M NaOH for protein determination by the bicinchoninic acid (BCA) method using a Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The cGMP content was expressed as picomoles per milligram of sample protein (pmol/mg protein).

Drugs

The following drugs were used in the present study: (−)-phenylephrine hydrochloride, salbutamol hemisulfate, (−)-isoproterenol hydrochloride (isoprenaline), (±)-propranolol hydrochloride, (±)-atenolol, (±)-[4-[3-[(1,1-dimethylethyl) amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazol-2-one] hydrochloride ((±)-CGP12177A), L-NNA, desipramine hydrochloride, deoxycorticosterone acetate, and indomethacin (Sigma-Aldrich Co., St. Louis, MO, USA); (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl) oxy]-3-[(1-methylethyl) amino]-2-butanol hydrochloride (ICI-118551), and ODQ (Tocris Bioscience, Ellisville, MO, USA); acetylcholine chloride (Daiichi Sankyo Co. Ltd., Tokyo, Japan); (*R*)-(−)-noradrenaline hydrogen tartrate monohydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan); and NOR3 (Dojindo Laboratories, Kamimashiki, Japan). All other chemicals used in the present study were commercially available and of reagent grade. ODQ and NOR3 were dissolved in 100% dimethyl sulfoxide (DMSO) to produce stock solutions of 10^{-2} M and 10^{-3} M, respectively. Atenolol was dissolved in 0.1 N HCl to produce a stock solution of 10^{-2} M. Deoxycorticosterone and indomethacin were dissolved in 100% ethanol to produce a stock solution of 10^{-2} M. All other drugs were prepared as aqueous solutions and diluted with distilled water. Final DMSO concentrations in the bath medium did not exceed 0.1%. Drugs were added directly to the organ bath and expressed in molar concentration (M) in the bath medium.

Data analysis

The extent of relaxation induced by ACh, NOR3, and β-adrenoceptor agonists (isoprenaline, salbutamol, (±)-CGP 12177A, and noradrenaline) was calculated with respect to the basal tension (100% relaxation) before the application of Phe (10^{-7} or 10^{-5} M) and the steady-state tension level before the application of each relaxant, including β-adrenoceptor agonists (0% relaxation).

The potencies of the vasorelaxants were expressed as pD_2 (pEC_{50}) values (the negative logarithm of the effective agonist concentration that produces a response that is 50% of the maximum response). Data were plotted as a function of the vasorelaxant concentration and fitted to the equation:

$$E = E_{max} \times A^{n_H} / (EC_{50}^{n_H} + A^{n_H})$$

where E is the % relaxation at a given concentration, E_{max} is the maximum response, A is the agonist (relaxant) concentration, n_H is the slope function, and EC_{50} is the effective agonist concentration that produced a 50% response. The curve-fitting was carried out using GraphPad Prism Version 4.00 (GraphPad Software, Inc., San Diego, CA, USA). The EC_{50} values were converted to logarithmic values (pD_2, -logEC_{50}) for statistical analysis.

Data are presented as mean±SEM, and n refers to the number of preparations. The probability (p) of the difference between two sets of values being due to chance was evaluated with GraphPad Prism Version 4.00 by paired or unpaired *t*-tests, unpaired *t*-tests with Welch's correction if necessary, and one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests. Differences with a p value of less than 0.05 were considered statistically significant.

Results

Fig. 1 shows the effects of the NOS inhibitor L-NNA and the soluble guanylyl cyclase inhibitor ODQ on ACh- and

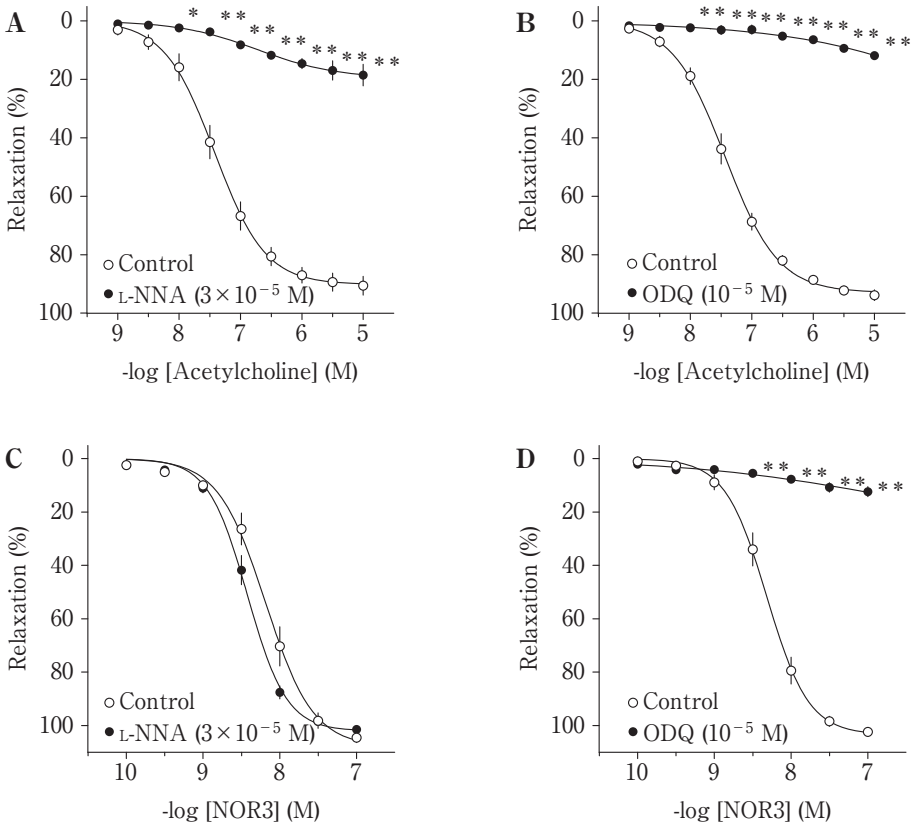

Fig. 1 Effects of NO-cGMP pathway inhibitors on endothelium-dependent relaxation induced by ACh or endothelium-independent relaxation induced by NOR3 in spiral preparations of rat thoracic aortas pre-contracted with phenylephrine (Phe). Rat aortas with **(A, B)** or without **(C, D)** functional endothelium were pre-contracted with Phe (10^{-7} M), and ACh **(A, B)** or NOR3 **(C, D)** was cumulatively applied to the bath solution. L-NNA (3×10^{-5} M) or ODQ (10^{-5} M) was applied 20 min or 70 min before stimulation with Phe, respectively. Vascular relaxation is expressed as a percentage reversal of the Phe-induced sustained tension development just before applying ACh or NOR3. Data are mean ± SEM of n = 5 **(A, B)**, or n = 6 **(C, D)** preparations for each. Significant difference from control values: *p < 0.05, **p < 0.01.
NO: nitric oxide, cGMP: cyclic guanosine monophosphate, ACh: acetylcholine, NOR3: (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide, L-NNA: N^G-nitro-L-arginine, ODQ: 1*H*-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one, SEM: standard error of the mean

NOR3-induced relaxation in endothelium-intact or endothelium-denuded aortas pre-contracted with Phe (10^{-7} M). Both L-NNA (3×10^{-5} M) (Fig. 1A) and ODQ (10^{-5} M) (Fig. 1B) almost completely abolished ACh-induced, endothelium-dependent relaxation. However, endothelium-independent relaxation induced by an NO donor (NOR3) was not affected by L-NNA (3×10^{-5} M) (Fig. 1C), but was nearly abolished by ODQ (10^{-5} M) (Fig. 1D). These findings clearly showed that the activity of NOS was inhibited by L-NNA and that the activity of soluble guanylyl cyclase was inhibited by ODQ.

Fig. 2 shows the effects of L-NNA and ODQ on isoprenaline- and salbutamol (a β_2 adrenoceptor agonist)-induced relaxation in endothelium-denuded aortas pre-contracted with Phe (10^{-7} M). Relaxation induced by isoprenaline (Fig. 2A, B) or salbutamol (Fig. 2C, D) was not significantly affected by either L-NNA (3×10^{-5} M) (Fig. 2A, C) or ODQ (10^{-5} M) (Fig. 2B, D).

Fig. 3 shows the effects of L-NNA or ODQ on relaxation induced by isoprenaline or CGP-12177A (a β_3 partial agonist) in the presence of propranolol (10^{-7} M). Isoprenaline-induced relaxation in the presence of propranolol (10^{-7} M) was not affected by either L-NNA (3×10^{-5} M) (Fig. 3A) or ODQ (10^{-5} M) (Fig. 3B). Relaxation induced by CGP-12177A

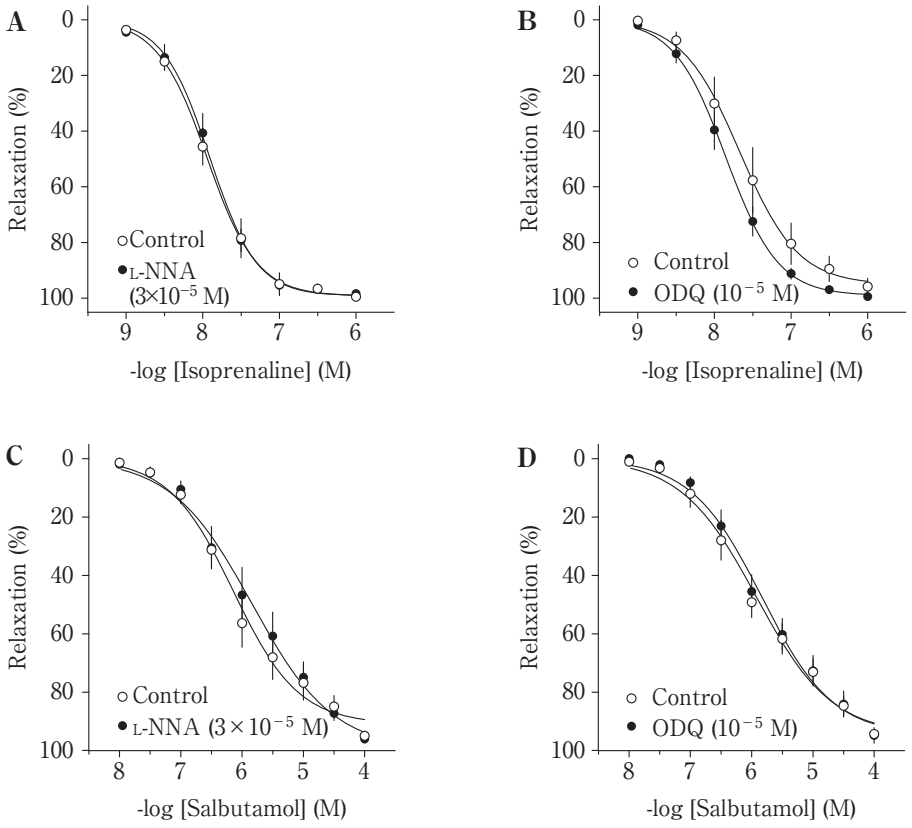

Fig. 2 Effects of NO-cGMP pathway inhibitors on endothelium-independent relaxation induced by isoprenaline or salbutamol in spiral preparations of rat thoracic aortas pre-contracted with phenylephrine (Phe). Rat aortas without functional endothelium were pre-contracted with Phe (10^{-7} M), and isoprenaline **(A, B)** or salbutamol **(C, D)** was cumulatively applied to the bath solution. L-NNA (3×10^{-5} M) **(A, C)** or ODQ (10^{-5} M) **(B, D)** was applied 20 min or 70 min before stimulation with Phe, respectively. Data are mean ± SEM of n = 6 **(A, B, C, D)** preparations for each.
NO: nitric oxide, cGMP: cyclic guanosine monophosphate, L-NNA: N^G-nitro-L-arginine, ODQ: 1*H*-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one, SEM: standard error of the mean

in the presence of propranolol (10^{-7} M) was also not affected by either L-NNA (3×10^{-5} M) (Fig. 3C) or ODQ (10^{-5} M) (Fig. 3D).

Fig. 4A, 4B show relaxation induced by noradrenaline (NA) and the effects of β-adrenoceptor antagonists. In this series of experiments, the endothelium-denuded preparation was pre-contracted with 10^{-5} M Phe to record the NA-induced relaxant component and to mask the contractile component due to NA. NA-induced relaxation was strongly inhibited by propranolol (10^{-7} M) (Fig. 4A) or the β_1-adrenoceptor antagonist atenolol (10^{-6} M) (Fig. 4B). NA-induced relaxation was not reduced by either L-NNA (3×10^{-5} M) (Fig. 4C) or ODQ (10^{-5} M) (Fig. 4D). Instead, NA-induced relaxation was slightly potentiated by both L-NNA (3×10^{-5} M) (Fig. 4C) and ODQ (10^{-5} M) (Fig. 4D).

Fig. 5 shows the effects of L-NNA or ODQ on the relaxation induced by NA in the combined presence of an uptake 1 inhibitor, desipramine (3×10^{-7} M), and an uptake 2 inhibitor, deoxycorticosterone (10^{-5} M). Even in the presence of these uptake inhibitors, NA-induced relaxation was still potentiated significantly by L-NNA (3×10^{-5} M) (Fig. 5A) or ODQ (10^{-5} M) (Fig. 5B).

Fig. 6 shows the effects of isoprenaline and NA on tissue cGMP content. Neither isoprenaline (10^{-6} M) nor NA (10^{-4} M) significantly increased cGMP content. In contrast, NOR3 (10^{-7} M) increased cGMP content from 0.009 ± 0.0004 pmol/mg protein to 0.279 ± 0.030 pmol/mg protein (n = 4 for each, $^{**}p < 0.01$), producing a 31.0-fold increase.

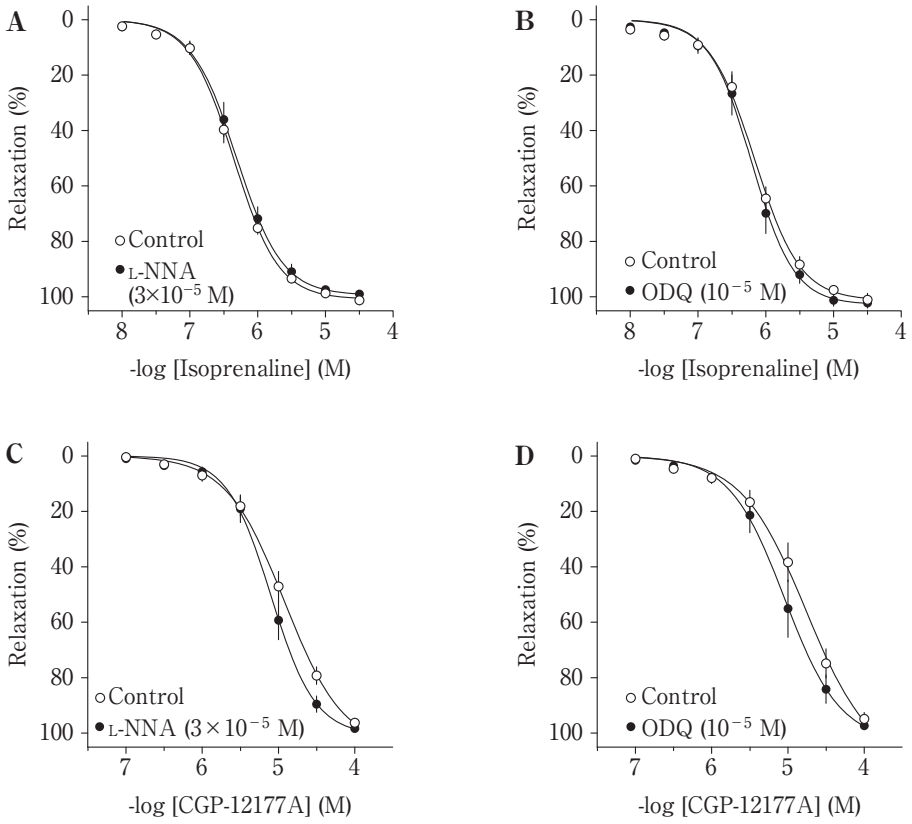

Fig. 3 Effects of NO-cGMP pathway inhibitors on endothelium-independent relaxation induced by isoprenaline **(A, B)** or CGP-12177A **(C, D)** in the presence of propranolol in spiral preparations of rat thoracic aortas pre-contracted with Phe. Rat aortas without functional endothelium were pre-contracted with Phe (10^{-7} M), and isoprenaline **(A, B)** or CGP-12177A **(C, D)** was cumulatively applied to the bath solution in the presence of propranolol (10^{-7} M), which was applied 20 min before stimulation with Phe. L-NNA (3×10^{-5} M) or ODQ (10^{-5} M) was applied 20 min or 70 min before stimulation with Phe, respectively. Data are mean ± SEM of n = 6 **(A, B, C, D)** preparations for each.
NO: nitric oxide, cGMP: cyclic guanosine monophosphate, Phe: phenylephrine, L-NNA: N^G-nitro-L-arginine, ODQ: 1*H*-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one, SEM: standard error of the mean

Discussion

The present study aimed to pharmacologically determine whether the NO-cGMP pathway significantly contributed to β-adrenoceptor-mediated smooth muscle direct arterial relaxation. We designed this study because Flacco et al.[20] proposed that the NO-cGMP pathway, rather than the cAMP pathway, had a significant role in β_2-adrenoceptor-mediated relaxation in both endothelium-preserved and endothelium-denuded rat thoracic aortas. The primary experimental evidence to support their proposal is as follows: aortic relaxation in response to isoprenaline is significantly diminished by an inhibitor of soluble guanylyl cyclase (ODQ), but not by an inhibitor of adenylyl cyclase (SQ22536); relaxation of endothelium-denuded preparations by salbutamol is significantly inhibited by an NO synthase inhibitor, N^G-nitro-L-arginine-methyl ester (L-NAME); L-NAME inhibits isoprenaline-induced relaxation more than endothelium removal; and the expression of eNOS is high in endothelium preparations and aortic smooth muscle cells.[20] However, it is unclear whether the inhibitory effects of ODQ on isoprenaline-induced relaxation are generated in the aortic smooth muscle, in the endothelium, or in both sites, because it is unknown whether the authors used endothelium-intact or endothelium-denuded preparations. The effects of ODQ on relaxation in response to subtype-specific β-adrenoceptor agonists were also not examined

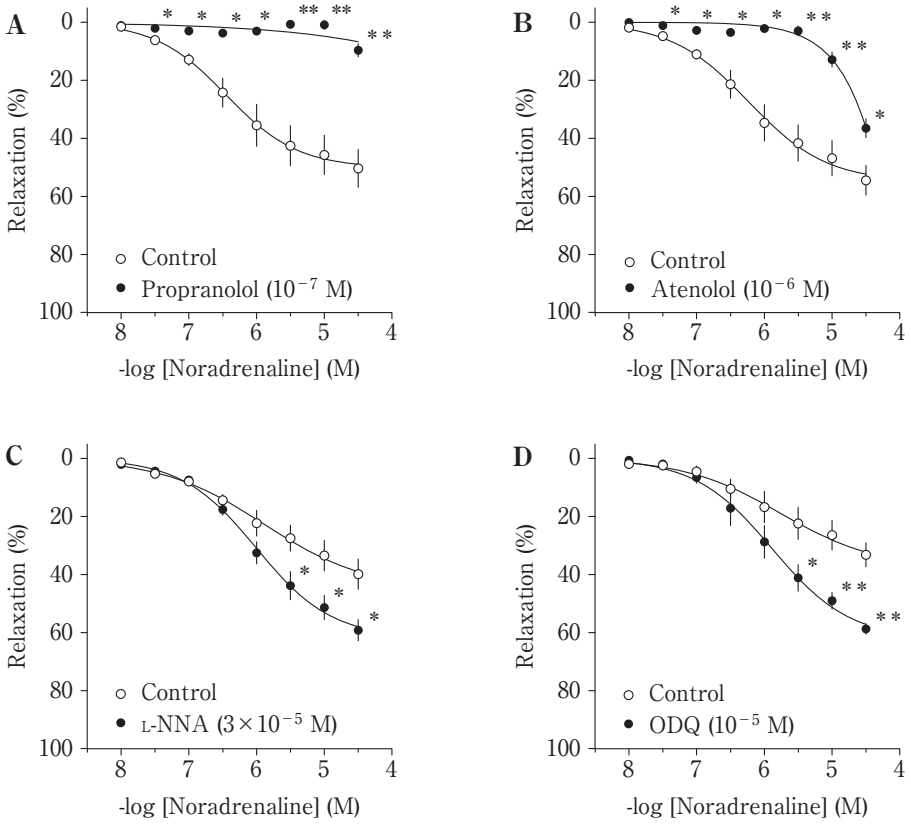

Fig. 4 Effects of β-adrenoceptor antagonists **(A, B)** or NO-cGMP pathway inhibitors **(C, D)** on endothelium-independent relaxation induced by noradrenaline (NA) in spiral preparations of rat thoracic aortas pre-contracted with Phe. Rat aortas without functional endothelium were pre-contracted with Phe (10^{-5} M), and NA was cumulatively applied to the bath solution. β-Adrenoceptor antagonists or NO-cGMP pathway inhibitors were applied at the following times before stimulation with Phe: propranolol (10^{-7} M) **(A)**, 20 min; atenolol (10^{-6} M) **(B)**, 20 min; L-NNA (3×10^{-5} M) **(C)**, 20 min; and ODQ (10^{-5} M) **(D)**, 70 min. Data are mean ± SEM for n = 4 **(A, B, C, D)** preparations for each. Significant difference from control values: *$p<0.05$ and **$p<0.01$.
NO: nitric oxide, cGMP: cyclic guanosine monophosphate, L-NNA: N^G-nitro-L-arginine, ODQ: 1*H*-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one, Phe: phenylephrine, SEM: standard error of the mean

by Flacco et al. Thus, even if the NO-cGMP pathway is coupled with the activation of β-adrenoceptors in the aortic smooth muscle, the significance of its contribution to smooth muscle direct aortic functional relaxation is ambiguous.

Therefore, in the present study, to elucidate the possible role of the NO-cGMP pathway in β-adrenoceptor-mediated smooth muscle direct arterial relaxation, the effects of L-NNA and ODQ on the relaxant response to β-adrenoceptor agonists were investigated in endothelium-denuded rat thoracic aortas. However, L-NNA (3×10^{-5} M) and ODQ (10^{-5} M) did not suppress relaxation induced by isoprenaline (a non-selective β-adrenoceptor agonist) (Fig. 2A, B), salbutamol (a selective β_2-adrenoceptor agonist) (Fig. 2C, D), isoprenaline (which acts as a selective β_3-adrenoceptor agonist in the presence of propranolol) (Fig. 3A, B); CGP-12177A (a partial β_3-adrenoceptor agonist) (Fig. 3C, D), and NA (a selective β_1-adrenoceptor agonist) (Fig. 4 C, D). In addition, neither isoprenaline (10^{-6} M) nor NA (10^{-4} M), which should cause maximal relaxation, significantly increased tissue cGMP content, whereas NOR3 (10^{-7} M) produced an approximate 30-fold increase compared with the control (Fig. 6). These findings indicated that the NO-cGMP pathway did not have an essential role in β-adrenoceptor-mediated smooth muscle direct blood vessel relaxation, although it may play an important role in

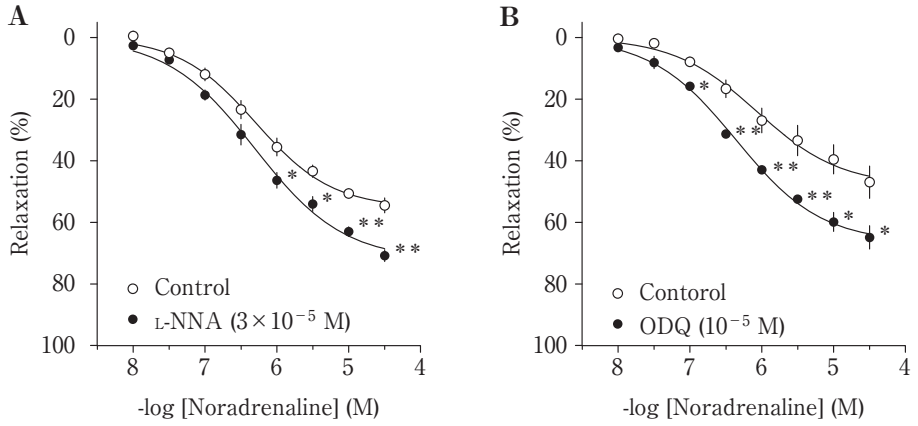

Fig. 5 Effects of NO-cGMP pathway inhibitors on endothelium-independent relaxation induced by noradrenaline (NA) in the combined presence of uptake inhibitors in spiral preparations of rat thoracic aortas pre-contracted with Phe. Rat aortas without functional endothelium were pre-contracted with Phe (10^{-5} M), and NA was cumulatively applied to the bath solution in the combined presence of desipramine (3×10^{-7} M) and deoxycorticosterone (10^{-5} M), which were applied 20 min before stimulation with Phe. L-NNA (3×10^{-5} M) **(A)** or ODQ (10^{-5} M) **(B)** were applied 20 min or 70 min before stimulation with Phe, respectively. Data are mean ± SEM for n = 4 **(A, B)** preparations for each. Significant difference from control values: *p < 0.05 and **p < 0.01.
NO: nitric oxide, cGMP: cyclic guanosine monophosphate, L-NNA: N^G-nitro-L-arginine, ODQ: 1*H*-[1,2,4]-oxadiazolo-[4,3-a]-quinoxalin-1-one, Phe: phenylephrine, SEM: standard error of the mean

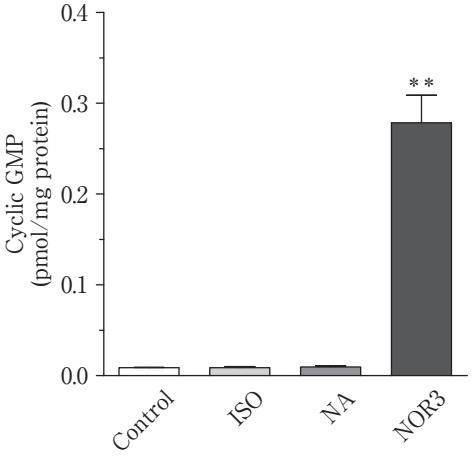

Fig. 6 Effects of β-adrenoceptor agonists and NOR3 on tissue cGMP content. Basal cGMP content (control) was not significantly increased by isoprenaline (ISO, 10^{-6} M) or noradrenaline (NA, 10^{-4} M) whereas it was profoundly increased by NOR3 (10^{-7} M). Data are mean ± SEM of n = 4 experiments (in duplicates). **p < 0.01 significant differences from control (one-way ANOVA followed by Dunnett's multiple comparison test).
NOR3: (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide, cGMP: cyclic guanosine monophosphate, SEM: standard error of the mean, ANOVA: analysis of variance

endothelium-dependent relaxation.

At present, we cannot provide a clear explanation for the difference between our results and those of Flacco et al.[20] The sizes and the ages of the rats used differed between the two studies. Although aging has not been shown to affect the endothelium-dependency of β-adrenoceptor-mediated relaxation in rat thoracic aortas, it reduces the contribution of the β-adrenoceptor-mediated cAMP-PKA pathway while increasing that of potassium channels.[21] Therefore, we investigated this by selecting rats aged 17 weeks (about 300 g) rather than those aged 8 weeks (about 200 g); however, no significant differences were observed. The arterial preparation method or the experimental protocol used may have differed between our experiment and that of Flacco et al, but we were unable to identify definitive differences to explain the discrepancies in the results. Thus, the causes of these differences require further examination.

A plausible alternative explanation for our results is as follows: stimulation of vascular smooth β-adrenoceptors may activate NO-cGMP pathways, which contribute to the relaxant response of this muscle. However, if these NO-cGMP pathways simultaneously suppress β-adrenoceptor-mediated relaxation through some route, the expected in-

hibitory effects of the NO-cGMP pathway inhibitors (L-NNA/ODQ) on β-adrenoceptor-mediated relaxation are apparently undetectable. However, this explanation can be ruled out because significant elevation of tissue cGMP content by isoprenaline (10^{-6} M) or NA (10^{-4} M) was not detected.

When we carefully assessed the effects of L-NNA and ODQ on NA-induced relaxation, we noticed that NA-induced relaxation was potentiated in the presence of L-NNA (Fig. 4C) or ODQ (Fig. 4D). Because the potentiating effects of L-NNA or ODQ on NA-induced relaxation were also observable in the combined presence of desipramine (an uptake 1 inhibitor; 3×10^{-7} M) and deoxycorticosterone (an uptake 2 inhibitor; 10^{-5} M) (Fig. 5), the possibility that potentiation of NA-induced relaxation by L-NNA or ODQ was mediated through inhibition by these inhibitors of NA uptake systems could be ruled out. Furthermore, the potentiating effects of L-NNA or ODQ on NA-induced relaxation were not affected by clorgiline (a selective MAO_A inhibitor; 10^{-5} M) or Ro 41-0960 (a COMT inhibitor; 10^{-5} M) (data not shown). Thus, the effects of these inhibitors (L-NNA or ODQ) on some metabolite systems of NA could also be ruled out. Some universal rules that cannot be ignored may underlie this phenomenon for the following reasons: 1) the potentiating effects of L-NNA/ODQ on NA-induced relaxation in rat aortas were relatively noticeable; 2) ostensible potentiation in the presence of L-NNA or ODQ was also observable in adrenaline-induced relaxation (data not shown); and 3) relaxation induced by both isoprenaline (Fig. 2B) and CGP-12177A (Fig. 3D) was also potentiated by ODQ (10^{-5} M), although statistically significant differences were not detected in the limited number of samples investigated.

One plausible explanation for this phenomenon is the mediation of cGMP-stimulated phosphodiesterases (PDEs), including type II PDE (PDE II).[22–24] PDE II is activated by cGMP, and its selective inhibitors are expected to alleviate pulmonary hypertension because they cause dilatation of the pulmonary artery.[24] For example, if PDE II is activated by NA (possibly through β_1-adrenoceptors) or is conventionally activated independently of NA stimulation, degradation of cyclic mononucleotides (cAMP and cGMP) is promoted, which causes suppression of cyclic mononucleotide-mediated relaxation. In contrast, cyclic mononucleotide-mediated relaxation is expected to be potentiated by NO-cGMP pathway inhibitors such as L-NNA or ODQ, because they inhibit PDE II activity. If we make the assumption that NA-induced relaxation (β_1-adrenoceptor-mediated relaxation) is strongly mediated by cAMP-dependent pathways, potentiation of the relaxant response by L-NNA/ODQ could be explained by possible mediation through PDE II. However, under the present experimental conditions, NA did not significantly increase tissue cGMP content. Therefore, even if the contribution of PDE II is significant, this type of PDE may be activated conventionally by small amounts of cGMP without being stimulated by β-adrenoceptor agonists. Another explanation, which should be examined further, is that significant elevation of tissue cGMP could be induced by NA in the presence of PDE inhibitors in tissue medium.

Signaling pathways that regulate β-adrenoceptor-mediated blood vessel relaxation independently of tissue cAMP contents seem to be physiologically significant and have attracted a lot of attention. Because β-adrenoceptors are classified as Gs protein-coupled receptors, it is reasonable to speculate that some mechanisms relying heavily on the elevation of tissue cAMP content participate in β-adrenoceptor-mediated blood vessel relaxation.[3] However, blood vessel relaxation induced by β_3-adrenoceptor[17] or IP receptor[25] is caused in part by tissue cAMP-independent as well as cAMP-dependent mechanisms. Accordingly, we are currently investigating the relationship between the elevation of tissue cAMP content and the relaxant response to every β-adrenoceptor subtype in rat aortas in order to determine the role of cAMP-independent mechanisms in relaxation. In particular, voltage-dependent K^+ channels may partly account for cAMP-independent mechanisms, and we are further examining this possibility in our ongoing studies.

This study was supported in part by Grants-in-Aid for Scientific Research (C) (23590116 to YT) from the Japan Society for the Promotion of Science (JSPS). Shunsuke Shiina is financially supported in part by a "Nagai Memorial Pharmaceutical Research Encouragement Award". We would like to give special thanks to Ms. Ayaka Kanemura for her research assistance in tissue cGMP measurement. We also would like to thank Editage (www.editage.jp) for English language editing.

Conflicts of interest: The authors have no conflicts of interest to declare.

References

1) Ahlquist RP. A study of the adrenotropic receptors. Am J

Physiol. 1948; 153: 586-600.
2) Allwood MJ, Cobbold AF, Ginsburg J. Peripheral vascular effects of noradrenaline, isopropylnoradrenaline and dopamine. Br Med Bull. 1963; 19: 132-6.
3) Tanaka Y, Matsushita M, Tamura K, Kogo H, Koike K. The β-adrenoceptors in blood vessels: recent knowledge on the vascular smooth muscle receptor subtypes that mediate blood vessel relaxation and the role of endothelium. Curr Top Pharmacol. 2006; 10: 33-42.
4) Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, et al. International Union of Pharmacology nomenclature of adrenoceptors. Pharmacol Rev. 1994; 46: 121-36.
5) Tanaka Y, Horinouchi T, Koike K. New insights into β-adrenoceptors in smooth muscle: distribution of receptor subtypes and molecular mechanisms triggering muscle relaxation. Clin Exp Pharmacol Physiol. 2005; 32: 503-14.
6) Alexander SP, Mathie A, Peters JA. Guide to Receptors and Channels (GRAC), 5th edition. Br J Pharmacol. 2011; 164 Suppl 1: S1-324.
7) Lands AM, Arnold A, McAuliff JP, Luduena FP, Brown TG Jr. Differentiation of receptor systems activated by sympathomimetic amines. Nature. 1967; 214: 597-8.
8) Edvinsson L, Owman C. Pharmacological characterization of adrenergic alpha and beta receptors mediating the vasomotor responses of cerebral arteries in vitro. Circ Res. 1974; 35: 835-49.
9) O'Donnell SR, Wanstall JC. The classification of β-adrenoceptors in isolated ring preparations of canine coronary arteries. Br J Pharmacol. 1984; 81: 637-44.
10) Graves J, Poston L. β-Adrenoceptor agonist mediated relaxation of rat isolated resistance arteries: a role for the endothelium and nitric oxide. Br J Pharmacol. 1993; 108: 631-7.
11) Briones AM, Daly CJ, Jimenez-Altayo F, Martinez-Revelles S, Gonzalez JM, McGrath JC, et al. Direct demonstration of β_1- and evidence against β_2- and β_3-adrenoceptors, in smooth muscle cells of rat small mesenteric arteries. Br J Pharmacol. 2005; 146: 679-91.
12) Brawley L, Shaw AM, MacDonald A. Role of endothelium/nitric oxide in atypical β-adrenoceptor-mediated relaxation in rat isolated aorta. Eur J Pharmacol. 2000; 298: 285-96.
13) Brawley L, Shaw AM, MacDonald A. β_1-, β_2- and atypical β-adrenoceptor-mediated relaxation in rat isolated aorta. Br J Pharmacol. 2000; 129: 637-44.
14) Matsushita M, Horinouchi T, Tanaka Y, Tsuru H, Koike K. Characterization of β_3-adrenoceptor-mediated relaxation in rat abdominal aorta smooth muscle. Eur J Pharmacol. 2003; 482: 235-44.
15) Emorine LJ, Marullo S, Briend-Sutren MM, Patey G, Tate K, Delavier-Klutchko C, et al. Molecular characterization of the human beta 3-adrenergic receptor. Science. 1989; 245: 1118-21.
16) Murray KJ. Cyclic AMP and mechanisms of vasodilation. Pharmacol Ther. 1990; 47: 329-45.
17) Matsushita M, Tanaka Y, Koike K. Studies on the mechanisms underlying β-adrenoceptor-mediated relaxation of rat abdominal aorta. J Smooth Muscle Res. 2006; 42: 217-25.
18) Vanhoutte PM. Endothelial adrenoceptors. J Cardiovasc Pharmacol. 2001; 38: 796-808.
19) Queen LR, Ferro A. β-Adrenergic receptors and nitric oxide generation in the cardiovascular system. Cell Mol Life Sci. 2006; 63: 1070-83.
20) Flacco N, Segura V, Perez-Aso M, Estrada S, Seller JF, Jiménez-Altayó F, et al. Different β-adrenoceptor subtypes coupling to cAMP or NO/cGMP pathways: implications in the relaxant response of rat conductance and resistance vessels. Br J Pharmacol. 2013; 169: 413-25.
21) van der Zypp A, Kang KB, Majewski H. Age-related involvement of the endothelium in β-adrenoceptor-mediated relaxation of rat aorta. Eur J Pharmacol. 2000; 397: 129-38.
22) Mongillo M, Tocchetti CG, Terrin A, Lissandron V, Cheung YF, Dostmann WR, et al. Compartmentalized phosphodiesterase-2 activity blunts β-adrenergic cardiac inotropy via an NO/cGMP-dependent pathway. Circ Res. 2006; 98: 226-34.
23) Keravis T, Lugnier C. Cyclic nucleotide phosphodiesterase (PDE) isozymes as targets of the intracellular signalling network: benefits of PDE inhibitors in various diseases and perspectives for future therapeutic developments. Br J Pharmacol. 2012; 165: 1288-305.
24) Bubb KJ, Trinder SL, Baliga RS, Patel J, Clapp LH, MacAllister RJ, Hobbs AJ. Inhibition of phosphodiesterase 2 augments cGMP and cAMP signaling to ameliorate pulmonary hypertension. Circulation. 2014; 130: 496-507.
25) Tanaka Y, Yamaki F, Koike K, Toro L. New insights into the intracellular mechanisms by which PGI_2 analogues elicit vascular relaxation: cyclic AMP-independent, Gs-protein mediated-activation of MaxiK channel. Curr Med Chem Cardiovasc Hematol Agents. 2004; 2: 257-65.