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Characteristics of attenuated endothelium-dependent relaxation seen in rabbit intrapulmonary vein following chronic nitroglycerine administration

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1 This study was undertaken to determine whether long-term *in vivo* administration of nitroglycerine (NTG) downregulates the endothelium-dependent relaxation induced by acetylcholine (ACh) in the rabbit intrapulmonary vein and, if so, whether the type 1 angiotensin II receptor (AT_1R) blocker valsartan normalizes this downregulated relaxation.

2 In strips treated with the cyclooxygenase inhibitor diclofenac, ACh induced a relaxation only when the endothelium was intact. A small part of this ACh-induced relaxation was inhibited by coapplication of two Ca^{2+}-activated K^+-channel blockers (charybdotoxin (CTX) + apamin) and the greater part of the response was inhibited by the nitric-oxide-synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA).

3 The endothelium-dependent relaxation induced by ACh, but not the endothelium-independent relaxation induced by the nitric oxide donor NOC-7, was significantly reduced in NTG-treated rabbits (*versus* those in NTG-nontreated control rabbits). The attenuated relaxation was normalized by coapplication of valsartan with the NTG.

4 In the vascular wall, both the amount of localized angiotensin II and the production of superoxide anion were increased by *in vivo* NTG treatment. These variables were normalized by coapplication of valsartan with the NTG.

5 It is suggested that long-term *in vivo* administration of NTG downregulates the ACh-induced endothelium-dependent relaxation, mainly through an inhibition of endothelial nitric oxide production in the rabbit intrapulmonary vein. A possible role for AT_1R is proposed in the mechanism underlying this effect.

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Abbreviations: ACh, acetylcholine; AT_1R, type1 angiotensin II receptor; CTX, charybdotoxin; DHE, dihydroethidium; EDHF, endothelium-derived hyperpolarizing factor; E_{max}, maximum effect; L-NNA, N^{ω}-nitro-L-arginine; NOC-7, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene

Introduction

Pulmonary vascular resistance represents the afterload against which the right ventricle operates. Not only the pulmonary arteries but also the pulmonary veins are considered to be important contributors to total pulmonary vascular resistance (Barman & Taylor, 1990; Audi *et al*., 1991). In addition, an increase in pulmonary venous resistance is involved in the development of pulmonary oedema in congestive heart failure (Burkhoff & Tyberg, 1993; Townsley *et al*., 1994). Despite the importance of these functions, little is known about the regulation of pulmonary venous tone, whereas many studies have been conducted on the regulation of pulmonary arterial tone (e.g. Shi *et al*., 1997; Back *et al*., 2002; Norel *et al*., 2004). It is well known that vascular endothelial cells play an important role in the regulation of pulmonary arterial resistance through their release of nitric oxide, endothelium-derived hyperpolarizing factor (EDHF) and prostanoids (Barnes & Liu, 1995; Budhiraja *et al*., 2004; Humbert *et al*., 2004). However, the characteristic features of the endothelium-dependent relaxing factors present in intrapulmonary veins have yet to be fully clarified.

Nitroglycerine (NTG) possesses a more powerful vasorelaxant activity in veins and large coronary arteries than in small coronary arteries or arterioles (Feldman *et al*., 1979; Abrams, 1985). In the former vessel-types, its action leads to a reduction in preload, while in the latter types it enhances the oxygen supply to the heart and hence this agent is widely used in the management of angina pectoris, acute myocardial infarction and congestive heart failure (Abrams, 1996). Despite the beneficial haemodynamic and antiischaemic profile of NTG,

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its usefulness is limited by the development of tolerance during continuous therapy (Parker *et al*., 1991; Parker & Parker, 1998). It was recently found that tolerance develops not only in the relaxation response to nitrovasodilators but also in that to endothelium-derived nitric oxide (referred to hereafter as 'cross-tolerance'; Gori & Parker, 2002a, b). Following studies on conduit arteries, it was suggested that such 'cross-tolerance' develops as a result of (i) neurohormonal adjustments (such as activation of the renin–angiotensin–aldosterone axis and sympathetic nervous system) and (ii) changes intrinsic to the vasculature (Gori & Parker, 2002a, b). However, the characteristics of this 'cross-tolerance' differ between small resistance arteries and large conduit arteries (Zelis & Mason, 1975; Bassenge & Stewart, 1986; Stewart *et al*., 1987; Münzel *et al*., 1995). Furthermore, it is unknown whether 'cross-tolerance' develops in intrapulmonary veins after chronic administration of NTG.

It has been suggested that in conduit arteries, angiotensin II may play a permissive or causal role in the development of 'cross-tolerance' (Münzel & Bassenge, 1996; Berkenboom *et al*., 1999; Kurz *et al*., 1999). Indeed, coapplication of either an angiotensin-converting-enzyme inhibitor or a type 1 angiotensin II receptor (AT_1R) blocker with NTG *in vivo* prevents 'cross-tolerance' developing in both the rat and rabbit aorta (Berkenboom *et al*., 1999; Kurz *et al*., 1999). However, it is unknown whether AT_1R play a significant role in the development of 'cross-tolerance' in intrapulmonary veins (if indeed 'cross-tolerance' develops at all in such vessels).

To attempt to clarify these issues, we examined the effects of long-term *in vivo* administration of NTG (10 days) on acetylcholine (ACh)-induced endothelium-dependent relaxation in isolated rabbit intrapulmonary veins. The rabbits were divided into three groups: an NTG-untreated control group, an NTG-treated group and a group treated with both NTG and the AT_1R blocker valsartan (Criscione *et al*., 1993). First, we pharmacologically characterized the endothelium-dependent relaxation in strips of rabbit pulmonary vein in these three groups by observing the effects of (a) coapplication of the Ca^{2+}-activated K^+-channel blockers charybdotoxin (CTX) and apamin (to inhibit the action of EDHF) and of (b) the nitric-oxide-synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA) on the ACh-induced relaxation (in the presence of the cyclooxygenase inhibitor diclofenac). Next, the changes in the endothelium-independent relaxation to the nitric oxide donor 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7) in the three groups were examined using endothelium-intact strips. Finally, the changes in (i) the amount of localized angiotensin II, (ii) AT_1R expression and (iii) the production of superoxide anion in the vascular wall were histochemically examined in the three groups.

Methods

Animals

All experiments performed in this study conformed to guidelines on the conduct of animal experiments issued by the Graduate School of Medical Sciences in Nagoya City University and were approved by the Committee on the Ethics of Animal Experiments in that institution. Male Japan White albino rabbits (supplied by Kitayama Labes, Ina, Japan), weighing 2.5–3.0 kg were treated by applying transdermal NTG patches (Nitroderm TTS; Novartis Pharma, Tokyo, Japan) to a shaved dorsal thoracic area of the body. Such patches were present continuously for a period of 10 days (each patch being replaced daily with a new one) ('NTG-treated rabbits'). The theoretical delivery of NTG was 5 mg per 24 h. In some of these NTG-treated rabbits, valsartan ($10\,mg\,kg^{-1}$) suspended in 0.5% carboxymethyl cellulose/0.5% Tween solution was administered orally once a day for the same 10-day period ('NTG + valsartan-treated rabbits'). Male rabbits of similar body weight served as controls ('control rabbits').

Tissue preparation

Rabbits were anaesthetized by injection of pentobarbitone sodium ($40\,mg\,kg^{-1}$ given i.v.), and then killed by exsanguination. The lungs were immediately removed, and the second- and third-order intrapulmonary veins (diameter, approximately 0.5–0.8 mm) were immediately excised and placed in Krebs solution, then cleaned by removal of connective tissues. After each vein had been cut open along its long axis using a small scissors, circularly cut strips were carefully prepared so as not to damage the endothelium. In some experiments, the endothelium was carefully removed by gentle rubbing of the intimal surface of the vessel using small pieces of razor blade, as previously described (Yamakawa *et al*., 1997). Diclofenac (3 μM, to inhibit the synthesis of prostanoids), guanethidine (5 μM, to prevent effects due to release of sympathetic transmitters) and famotidine (3 μM, to block histamine H_2 receptors) were present throughout the experiments, unless otherwise described. All the experiments were performed at 37°C.

Recording of mechanical response

Circularly cut strips (1.8–2.3 mm long, 0.3–0.5 mm wide) were mounted horizontally in a small chamber (0.6 ml) and attached to a strain gauge (AE801; SensoNor a.s, Horten, Norway), allowing us to record isometric tension. The strips were superfused with warmed (37°C) Krebs solution at a flow rate of $2\,ml\,min^{-1}$. The transducer was connected to a carrier amplifier (AS2101; NEC-San-ei Instruments, Tokyo, Japan) and the output signal was fed into a Macintosh computer (Apple Co., Tokyo, Japan) through an analogue–digital converter (MacLab; AD Instruments Pty Ltd, Castle Hill, Australia). Drift was less than $3\,\mu N\,h^{-1}$. The resting tension was adjusted to obtain maximum contraction in high-K^+ solution (128 mM) and was not greater than 15 μN.

Concentration–response relationships for histamine were obtained in endothelium-intact strips from each group, the histamine (0.1–30 μM) always being applied cumulatively from low to high concentration. The same relationship was also observed in endothelium-intact strips treated with the nitric-oxide-synthase inhibitor L-NNA (the strips being pretreated for 60 min with 0.1 mM L-NNA, which was then present throughout the experiments).

Endothelium-dependent relaxation was induced by cumulative application of ACh (0.01–10 μM) during the contraction induced by histamine. Unless otherwise stated, the concentration of histamine used was 10 μM. For this set of experiments,

the preparations were first contracted with histamine and then, after a steady-state contraction had been attained, ACh was cumulatively applied from low to high concentration. When the effect of coapplication of the Ca^{2+}-activated K^+-channel blockers CTX (0.1 μM) + apamin (0.1 μM) on the ACh-induced relaxation was to be examined, the toxins were applied concomitantly with histamine. Then, the response to ACh was observed. In preliminary experiments, we confirmed that the ACh-induced relaxation responses recovered completely after a 60-min washout of CTX + apamin. Therefore, after CTX + apamin had been washed out, L-NNA was applied for 60 min in the same preparations to allow us to observe the effect of ACh on histamine-induced contraction in the presence of L-NNA.

The concentration–response relationship for NOC-7 (1 nM–10 μM) was obtained by its cumulative application during the steady-state contraction induced by 10 μM histamine in endothelium-intact strips.

Immunohistochemical examination

Endothelium-intact strips were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 3 h. The strips were then embedded in O.C.T. Compound (Tissue Tek; SAKURA Finetechnical, Tokyo, Japan) and frozen at −80°C. Cryosections were cut at 4 μm thickness on a cryostat (Microtome Cryostat HM 550; MICROM International GmbH, Walldorf, Germany), then mounted on MAS-coated glass slides (Matsunami Glass, Kishiwada, Japan) for immunohistochemistry. After a washout with phosphate-buffered saline (PBS) solution (2.9 mM NaH_2PO_4, 9 mM Na_2HPO_4 and 137 mM NaCl, pH 7.2–7.4), the sections were incubated at 4°C overnight with either guinea-pig anti-angiotensin-II serum (1 : 100 dilution; Peninsula Laboratories, Belmont, CA, U.S.A.) or rabbit anti-AT_1R polyclonal antibody (1 : 100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) as the primary antibody, as previously reported (Itoh *et al*., 2003). After the sections had been rinsed with PBS, they were incubated with the secondary antibody (Alexa Fluor 488 goat anti-guinea-pig or goat anti-rabbit IgG antibody; dilution 1 : 5000; Molecular Probes, Eugene, OR, U.S.A.) for 1 h at room temperature. After a further wash with PBS, the fluorescence of Alexa Fluor 488 was detected by confocal-laser-scanning microscopy (LSM5 PASCAL; Carl Zeiss, Jena, Germany). The specificity of the angiotensin II antibody, which has been reported elsewhere (Kohler *et al*., 1997; Alliot *et al*., 1999; Itoh *et al*., 2003), was verified by its preincubation with angiotensin II peptide (0.1 mM) for 1 h at 37°C in PBS followed by incubation of sections with this mixture overnight at 4°C. The acquisition of images from different groups of rabbits was performed under identical conditions. Fluorescent 12-bit images were acquired and then analysed using commercial software (LSM5 PASCAL).

Western blot analysis

Endothelium-intact strips were homogenized in a solution containing 62.5 mM Tris–HCl (pH 6.8), 10% glycerol and 2% SDS. After centrifugation, the proteins in the supernatant were quantified. Protein samples (40 μg) were heated for 5 min at 100°C in sample buffer (62.5 mM Tris–HCl, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol and 0.0025% bromophenol blue), electrophoresed on 12.5% SDS-polyacrylamide gel, then transferred to nitrocellulose membranes. The membranes were rinsed with PBS solution, then incubated with primary antibody overnight at 4°C. Following a washout with PBS solution, the membranes were incubated for 1 h at room temperature with secondary antibody plus 1% BSA. A polyclonal antibody against AT_1Rs (1 : 100 dilution; Santa Cruz Biotechnology) served as the primary antibody, with peroxidase-conjugated IgG being used as the secondary antibody (dilution 1 : 2000). The signals from the immunoreactive bands were detected by means of an enhanced-chemiluminescence-detection system (SuperSignal West Pico; Pierce Biotechnology, Rockford, IL, U.S.A.) using Hyperfilm (Amersham Pharmacia Biotech, Buckinghamshire, U.K.). The density of the protein was measured by densitometric scanning, as described previously (Itoh *et al*., 1995).

Detection of the production of superoxide anion

Dihydroethidium (DHE), an oxidative fluorescent dye, was used to detect superoxide-anion production in pulmonary veins. DHE (Molecular Probes) is oxidized by intracellular superoxide anion and converted to ethidium, which binds irreversibly to DNA, producing bright red fluorescence. Segments (2.5 mm long) of intrapulmonary veins were incubated in Krebs solution at 37°C for 2 h. After incubation, the segments were frozen in O.T.C. Compound. Then, transverse sections (10 μm thickness) cut on a cryostat (Microtome Cryostat HM 550) were placed on MAS-coated glass slides and incubated in a light-protected chamber at 37°C for 30 min with 2 μM DHE. Images were obtained by confocal-laser-scanning microscopy (LSM5 PASCAL). The excitation wavelength was 488 nm and emission fluorescence was detected through a 585-nm long-pass filter. Identical laser settings were used for the acquisition of images from the various groups of rabbits. Fluorescent 12-bit images were acquired and then analysed using commercial software (LSM5 PASCAL).

Solutions

The composition of the Krebs solution was as follows (mM): 137.4 Na^+, 5.9 K^+, 1.2 Mg^{2+}, 2.6 Ca^{2+}, 15.5 HCO_3^-, 1.2 $H_2PO_4^-$, 134 Cl^-, 11.5 glucose. The solution was bubbled with 95% oxygen and 5% carbon dioxide.

Drugs

The drugs used in the current experiments were as follows: ACh–HCl (Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan), L-NNA, CTX and apamin (Peptides Institute Inc., Osaka, Japan), diclofenac sodium (Sigma Chemical Co., St Louis, MO, U.S.A.), guanethidine (Tokyo Kasei, Tokyo, Japan), histamine dihydrochloride (Wako Pure Chemical, Tokyo, Japan) and NOC-7 (Dojindo Laboratories, Kumamoto, Japan). The AT_1R blocker valsartan was kindly provided by Novartis Pharma K.K. (Tokyo, Japan) and famotidine by Yamanouchi Pharmaceutical Co. (Tokyo, Japan).

NOC-7 was dissolved in 0.1 N NaOH (as a 10 mM stock solution, stored at −80°C) and used within 1 week. The stock solution was diluted in Krebs solution to the required final concentration immediately before use. At the final concentra-

tion in Krebs solution (less than 0.1%), it had no noticeable effect itself on muscle contraction or relaxation. All other drugs were dissolved in ultrapure Milli-Q water (Japan Millipore Corp., Tokyo, Japan).

Statistical analysis

All results are expressed as the mean ± s.e.m., with the n value representing the number of animals used (each animal provided one strip or segment for a given experiment). The pD_2 ($-\log EC_{50}$) values for the concentration–response curves were obtained by fitting the data points for each strip by a nonlinear least-squares method using commercial software (OriginPro; OriginLab Co., Northampton, MA, U.S.A.). Statistical analysis was conducted using either a two-way repeated-measures ANOVA (followed by Scheffé's *post hoc* F test) or a Student's paired or unpaired t-test with an F test using commercial software (Statview; SAS Institute Inc., Cary, NC, U.S.A.). The level of significance was set at $P<0.05$.

Results

Concentration–response relationship for histamine in intrapulmonary veins

Histamine (0.1–30 μM) induced a concentration-dependent contraction in endothelium-intact strips of intrapulmonary vein obtained from the three groups of rabbits (NTG-untreated control rabbits, NTG-treated rabbits and NTG + valsartan-treated rabbits; Table 1). The response to histamine was not significantly different among the three groups ($P>0.05$). The nitric-oxide-synthase inhibitor L-NNA enhanced the histamine-induced maximum contraction (E_{max}) in each group without changing the pD_2 value. The enhancement induced by L-NNA was similar among the three groups (Table 1).

Effects of diclofenac, CTX + apamin and L-NNA on ACh-induced relaxation

To characterize the ACh-induced endothelium-dependent relaxation, the effect of the cyclooxygenase inhibitor diclofenac (to inhibit the production of prostanoids), the Ca^{2+}-activated K^+-channel blockers CTX + apamin (to inhibit the action of EDHF) or the nitric-oxide-synthase inhibitor L-NNA was examined on the ACh-induced relaxation during the contraction induced by 10 μM histamine in endothelium-intact strips of intrapulmonary vein from control rabbits. Diclofenac (3 μM) did not significantly modify the histamine (10 μM)-induced contraction ($n=4$, $P>0.5$), but enhanced the ACh-induced relaxation ($n=4$, $P<0.05$). The E_{max} was 54.5 ± 9.3% and 80.8 ± 2.0% ($n=4$, $P<0.05$) in the absence and presence of diclofenac, respectively. Since high concentrations of ACh (1 and 10 μM) induced a contraction (rather than a relaxation) in two among four strips in the absence of diclofenac, the effect of CTX + apamin or L-NNA was examined in the presence of diclofenac.

In the presence of diclofenac, ACh (0.01–10 μM) produced a concentration-dependent relaxation during the contraction induced by 10 μM histamine in endothelium-intact strips (Figure 1a, left panel, and Figure 1b), while this had no effect on the contraction induced by 10 μM histamine in endothelium-denuded strips ($n=4$, data not shown). In the presence of diclofenac, CTX + apamin did not significantly modify the histamine-induced contraction (Table 2), but attenuated the ACh-induced relaxation (Figure 1a, middle panel, and Figure 1b). After a thorough washout of these toxins, tissues were then pretreated with L-NNA (0.1 mM) for 60 min. Since L-NNA itself significantly enhanced the contraction induced by 10 μM histamine (Table 1), the concentration of histamine applied in the presence of L-NNA was reduced to 2 μM. In this way, the histamine-induced contractions obtained in the absence and presence of L-NNA were amplitude-matched. In the presence of L-NNA, ACh produced a contraction (rather than a relaxation, Figure 1a, right panel, and b).

Effect of long-term in vivo *administration of NTG on ACh-induced relaxation*

The tension induced by 10 μM histamine in endothelium-intact strips in the presence of diclofenac was not significantly different between control rabbits and NTG-treated rabbits (Table 2). In NTG-treated rabbits, the ACh-induced relaxation was significantly diminished, with a reduction in its E_{max} value (*versus* that in control rabbits; Table 2 and Figure 2a). As was the case in control rabbits, CTX + apamin did not significantly modify the histamine-induced contraction but attenuated the ACh-induced relaxation in NTG-treated rabbits (Table 2). It should be noted that the relaxation induced by ACh in the presence of CTX + apamin was significantly smaller in NTG-treated rabbits than in control rabbits (Figure 2b). L-NNA enhanced the contraction induced by 10 μM histamine in NTG-treated rabbits (Table 1), as it had in control rabbits. Therefore, the concentration of histamine employed in the presence of L-NNA was reduced to 2 μM to amplitude-match the histamine-induced contractions (Table 2). In NTG-treated rabbits, application of ACh in the presence of L-NNA produced a contraction of the same magnitude as that obtained under the same conditions in control rabbits (Table 2).

Table 1 Effects of *in vivo* administration of NTG (with or without the AT_1R blocker valsartan) on the contraction induced by histamine in the presence of diclofenac with or without L-NNA in endothelium-intact strips

	n	E_{max} (mN)		pD_2	
		L-NNA (−)	*L-NNA (+)*	*L-NNA (−)*	*L-NNA (+)*
Control rabbit	5	0.172 ± 0.053	0.336 ± 0.055††	5.29 ± 0.06	5.18 ± 0.05
NTG-treated rabbit	5	0.185 ± 0.019	0.344 ± 0.031††	5.17 ± 0.05	5.21 ± 0.12
NTG + valsartan-treated rabbit	5	0.178 ± 0.044	0.306 ± 0.059††	5.24 ± 0.05	5.22 ± 0.11

L-NNA (–), without L-NNA; L-NNA (+), with L-NNA. $^{††}P<0.01$ *versus* 'L-NNA (−)' in same group.

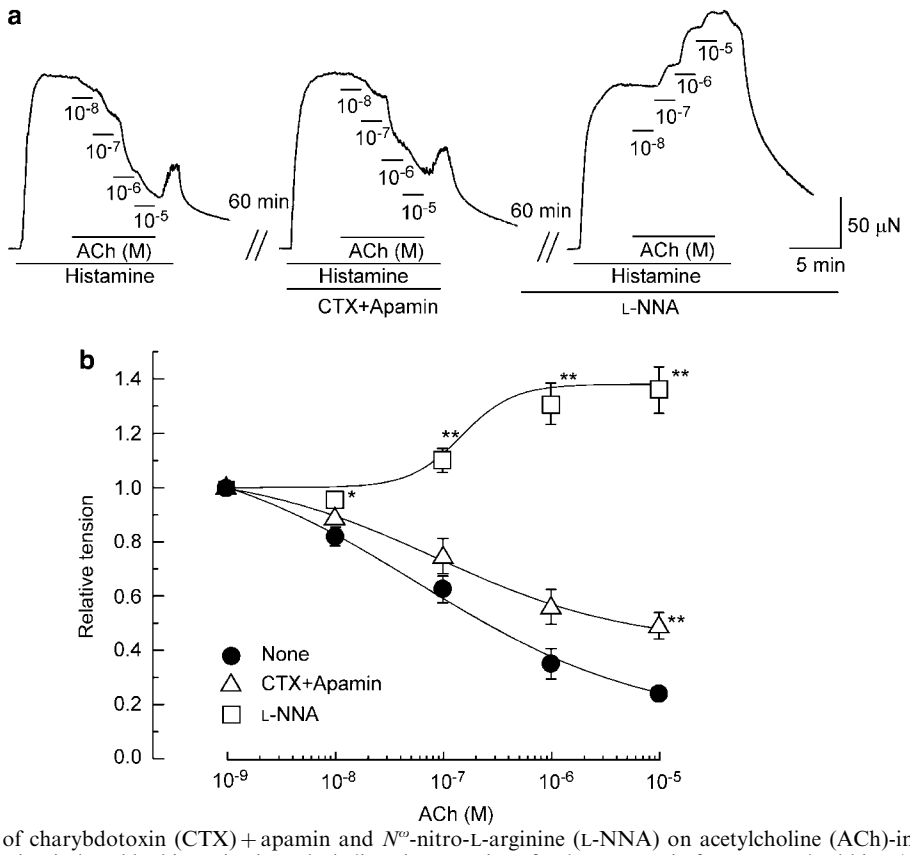


Figure 1 Effect of charybdotoxin (CTX) + apamin and N^{ω}-nitro-L-arginine (L-NNA) on acetylcholine (ACh)-induced responses during the contraction induced by histamine in endothelium-intact strips of pulmonary vein from control rabbits. Actual tracings (a) and summary (b) of the effect of ACh on the contraction induced by histamine in the absence (shown as 'None') and presence of either CTX + apamin ('CTX + Apamin') or L-NNA ('L-NNA'). The amplitude of maintained tonic contraction induced by histamine before application of ACh was normalized as a relative tension of 1.0 for each strip. The concentration of histamine was 10 μM for both 'None' and 'CTX + Apamin', but 2 μM for 'L-NNA' (to match the amplitude of contraction). The cyclooxygenase inhibitor diclofenac was present throughout the experiments. Mean of data from eight strips, with s.e.m. shown by vertical line. $^{*}P<0.05$, $^{**}P<0.01$ *versus* 'None' (two-way repeated-measures ANOVA followed by Scheffé's *post hoc F* test).

Table 2 Effects of *in vivo* administration of NTG (with or without the AT_1R blocker valsartan) on the contraction induced by histamine and the relaxation induced by acetylcholine (ACh) (each in the absence or presence of charybdotoxin (CTX) + apamin or L-NNA) in endothelium-intact strips treated with diclofenac

	n	*Histamine*	*ACh*	
		(mN)	pD_2	E_{max} *(%)*
Control rabbit	8			
None		0.137±0.022	6.99±0.12	75.4±1.2
CTX + apamin		0.147±0.028	6.73±0.36	52.2±6.7†
L-NNA		0.127±0.013	6.67±0.09	−33.7±4.9††
NTG-treated rabbit	6			
None		0.159±0.018	6.41±0.21	40.6±7.4**
CTX + apamin		0.157±0.044	ND	13.1±14.2*
L-NNA		0.141±0.024	6.87±0.08	−45.7±1.7††
NTG + valsartan-treated rabbit	7			
None		0.142±0.023	7.06±0.18	75.3±2.6
CTX + apamin		0.167±0.051	7.02±0.08	57.8±4.3†
L-NNA		0.144±0.023	6.64±0.15	−35.1±7.6††

The concentration of histamine was 10 μM for both 'None' and 'CTX + apamin', but 2 μM for 'L-NNA' in all three groups of rabbits. $^{*}P<0.05$, $^{**}P<0.01$ *versus* corresponding values in Control rabbit. $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ *versus* 'None' in same group. ND, not determined (since E_{max} was very small).

Effect of coapplication of a AT_1R blocker and NTG on ACh-induced relaxation

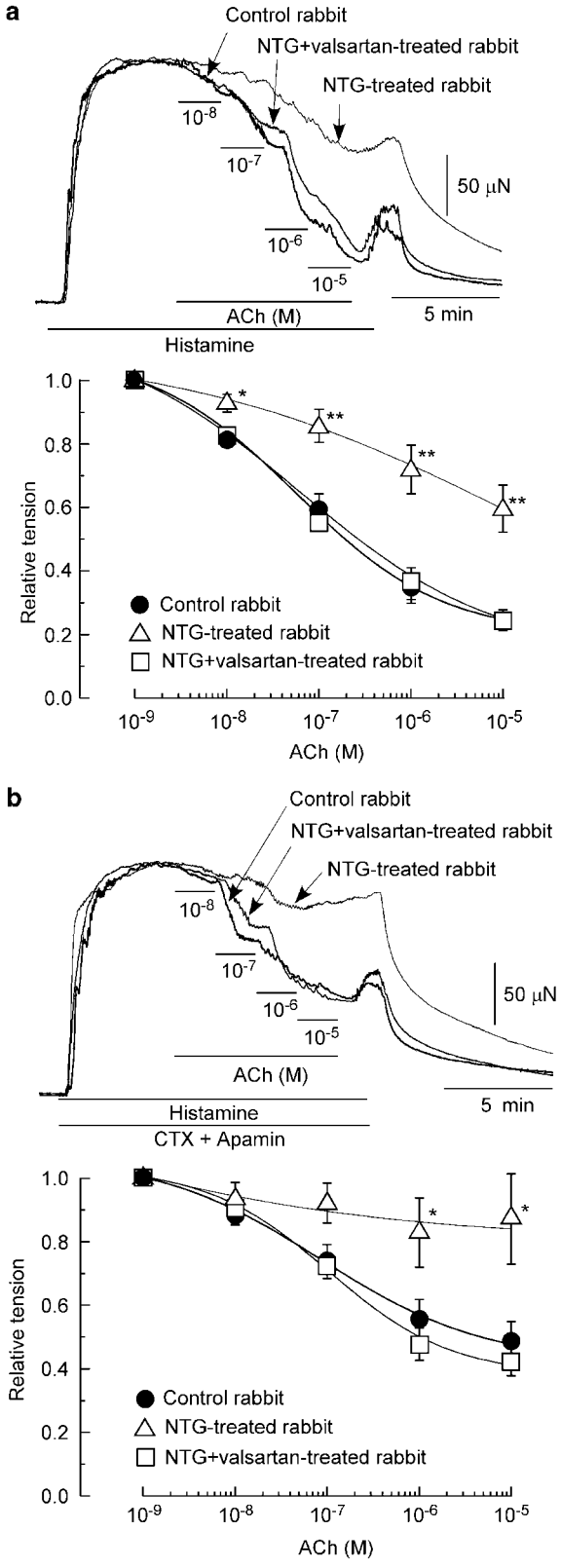
The tension induced by 10 μM histamine in endothelium-intact strips from rabbits cotreated *in vivo* with the AT_1R blocker valsartan and NTG was similar whether the strip had been obtained from a control rabbit or an NTG-treated rabbit (Table 2). Valsartan normalized the ACh-induced endothelium-dependent relaxation whether it was applied in the absence or presence of CTX + apamin (Figure 2a and b).

Effect of long-term in vivo *administration of NTG on NOC-7-induced relaxation*

During the contraction induced by 10 μM histamine in endothelium-intact strips, the relaxation response to the nitric oxide donor NOC-7 (1 nM–10 μM) was similar among the three groups of rabbits (Figure 3 and Table 3).

Effect of long-term in vivo *administration of NTG on the distribution of angiotensin II and AT_1R in the vascular wall*

The amount of angiotensin II localized in the walls of intrapulmonary veins was estimated from the intensity of the immunostaining obtained using angiotensin II antibody, as reported previously (Itoh *et al*., 2003). No staining, or only



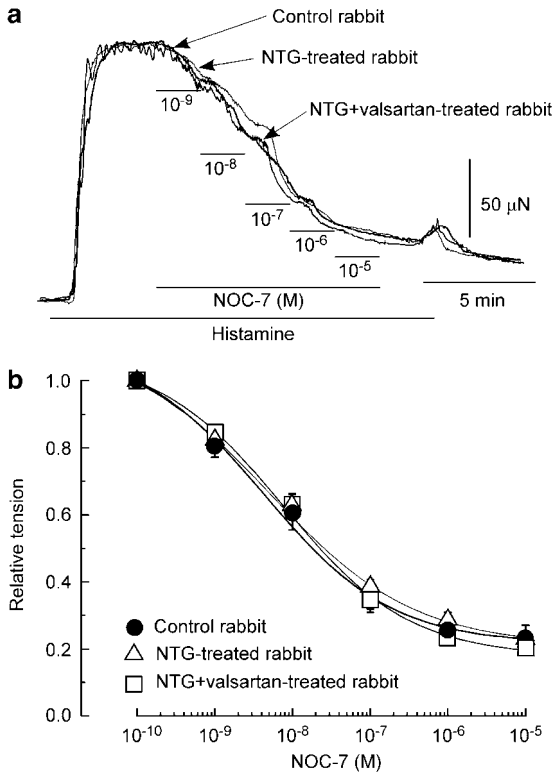


Figure 3 Effect of *in vivo* treatment with NTG (with or without valsartan) on the relaxation induced by the nitric oxide donor NOC-7 during histamine-induced contraction in endothelium-intact strips. Actual tracings (a) and summary (b) of the effects of NOC-7 in Control, NTG-treated and NTG + valsartan-treated rabbits. The amplitude of maintained tonic contraction induced by histamine before application of ACh was normalized as a relative tension of 1.0 for each strip. Mean of data from five strips, with s.e.m. shown by vertical line.

Figure 2 Effect of *in vivo* treatment with NTG (with or without the AT_1R blocker valsartan) on ACh-induced relaxation in endothelium-intact strips. Concentration-dependent effects of ACh during the contraction induced by histamine in the absence (a) or presence (b) of CTX + apamin in control, NTG-treated and NTG + valsartan-treated rabbits. Upper panel, actual tracings; lower panel, summary of the effects. The amplitude of maintained tonic contraction induced by histamine before application of ACh was normalized as a relative tension of 1.0 for each strip. The cyclooxygenase inhibitor diclofenac was present throughout the experiments. Mean of data from six to eight strips, with s.e.m. shown by vertical line. $^*P<0.05$, $^{**}P<0.01$ *versus* control rabbit (two-way repeated-measures ANOVA followed by Scheffé's *post hoc F* test).

Table 3 Effects of *in vivo* administration of NTG (with or without the AT_1R blocker valsartan) on the contraction induced by 10 μM histamine and the relaxation induced by the nitric oxide donor NOC-7 in endothelium-intact strips

	n	*Histamine* (mN)	*NOC-7* pD_2	E_{max} (%)
Control rabbit	6	0.143±0.019	8.14±0.17	75.4±3.9
NTG-treated rabbit	6	0.120±0.042	8.05±0.12	75.8±1.0
NTG + valsartan-treated rabbit	6	0.133±0.020	8.04±0.15	79.8±1.8

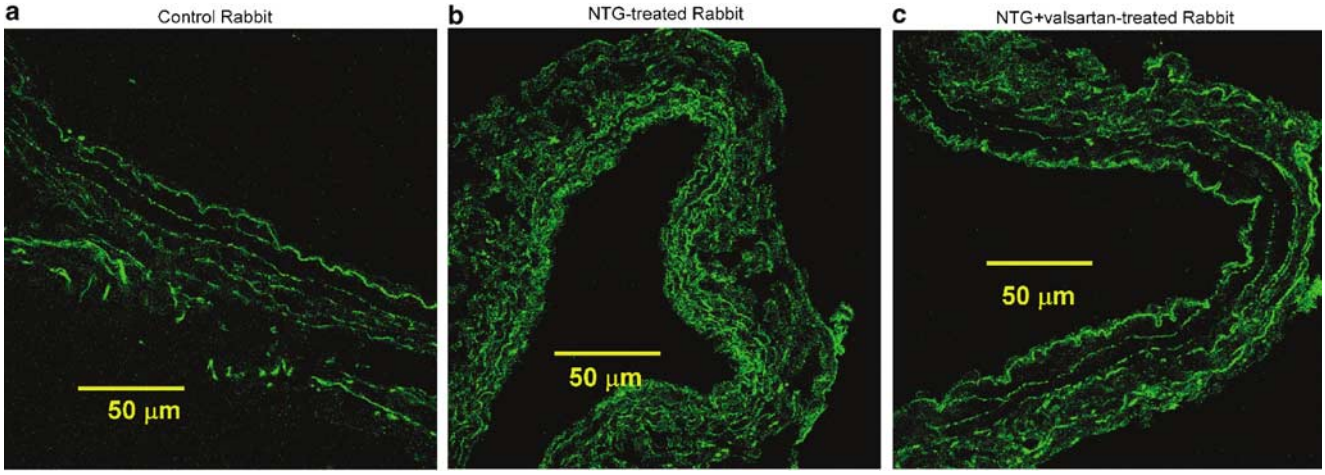


Figure 4 Immunostaining for angiotensin II in rabbit intrapulmonary vein. Immunofluorescence staining against angiotensin II in preparations from control rabbit (a), NTG-treated rabbit (b) and NTG + valsartan-treated rabbit (c). Fluorescence can be clearly seen in the vascular wall in the NTG-treated rabbit. Similar observations were made in other sections obtained from four preparations from four other animals.

very faint staining, was observed in control rabbits (Figure 4a), but this was very much increased in NTG-treated rabbits (Figure 4b). Coapplication of the AT_1R blocker valsartan with the NTG normalized the staining (Figure 4c).

Figure 5a shows the distribution of AT_1R in the walls of intrapulmonary veins obtained from a control rabbit (left panel) and an NTG-treated rabbit (right panel). The AT_1R were distributed mainly within endothelial cells and smooth muscle cells. The amount of AT_1R estimated from Western blots was similar between control and NTG-treated rabbits (Figure 5b and c).

Effect of long-term in vivo *administration of NTG on the production of superoxide anion in the vascular wall*

In control rabbits, a very weak production of superoxide anion was detected in a few endothelial cells (Figure 6a). In NTG-treated rabbits, a greatly increased superoxide-anion production was observed in many endothelial cells and also in some smooth muscle cells (Figure 6b). Its production was normalized in NTG + valsartan-treated rabbits (Figure 6c).

Discussion

In the present experiments, we found that in strips of rabbit intrapulmonary vein, ACh produced a relaxation only when the endothelium was intact. The cyclooxygenase inhibitor diclofenac enhanced the ACh-induced endothelium-dependent relaxation, suggesting that prostanoids derived from endothelium may not contribute to the ACh-induced relaxation in rabbit intrapulmonary vein. In the presence of diclofenac, a small part of the ACh-induced relaxation was inhibited by coapplication of the Ca^{2+}-activated K^+-channel inhibitors CTX + apamin (a manoeuvre that inhibits the action of EDHF: Hinton & Langton, 2003), and the greater part of the response was inhibited by the nitric-oxide-synthase inhibitor L-NNA. These results indicate that in rabbit intrapulmonary veins treated with diclofenac, ACh produces an endothelium-dependent relaxation, mainly through an increased endothelial-cell production of nitric oxide.

It is thought that an increase in the production of superoxide anion by vascular cells plays a crucial role in mediating the downregulation of the relaxations mediated by endothelium-derived nitric oxide in both the rat and rabbit aorta (Münzel *et al*., 1995; Laursen *et al*., 1996; Berkenboom *et al*., 1999). For this, there are several possible underlying mechanisms: (i) a decrease in the activity of endothelial nitric oxide synthase (Münzel *et al*., 2000; Gori & Parker, 2002a, b), (ii) a decrease in the bioavailability of nitric oxide (Münzel *et al*., 1997; Mihm *et al*., 1999), (iii) a desensitization of soluble guanylyl cyclase (Molina *et al*., 1987; Mollnau *et al*., 2002) and/or (iv) a reduction in cGMP-mediated relaxation (Soff *et al*., 1997; Nakano *et al*., 2004). In the present experiments, we found that in rabbit intrapulmonary veins, 10 days *in vivo* treatment with NTG reduced the endothelium-dependent relaxation induced by ACh, but not the endothelium-independent relaxation induced by the nitric oxide donor NOC-7. This suggests that such NTG treatment downregulates ACh-induced endothe-

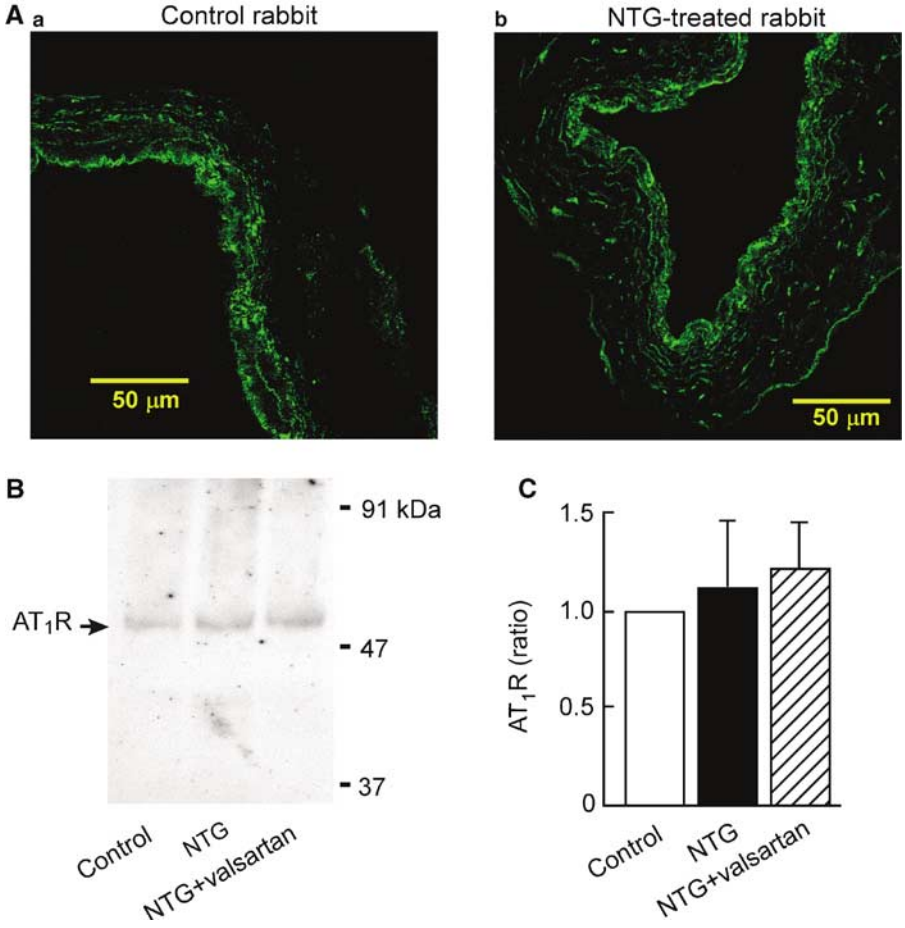


Figure 5 Immunostaining and Western blot analysis of AT_1R in rabbit intrapulmonary vein. (A) Immunofluorescence staining against AT_1R in preparations from control rabbit (Aa) and NTG-treated rabbit (Ab). Similar observations were made in other sections obtained from four preparations from four other animals. (B) Western blot analysis of AT_1R. (C) Summary of data obtained from four samples from four different animals in each group. AT_1R was detected as a single band with an approximate molecular mass of 50 kDa. Data are shown as mean ± s.e.m. Student's unpaired *t*-test revealed no significant differences.

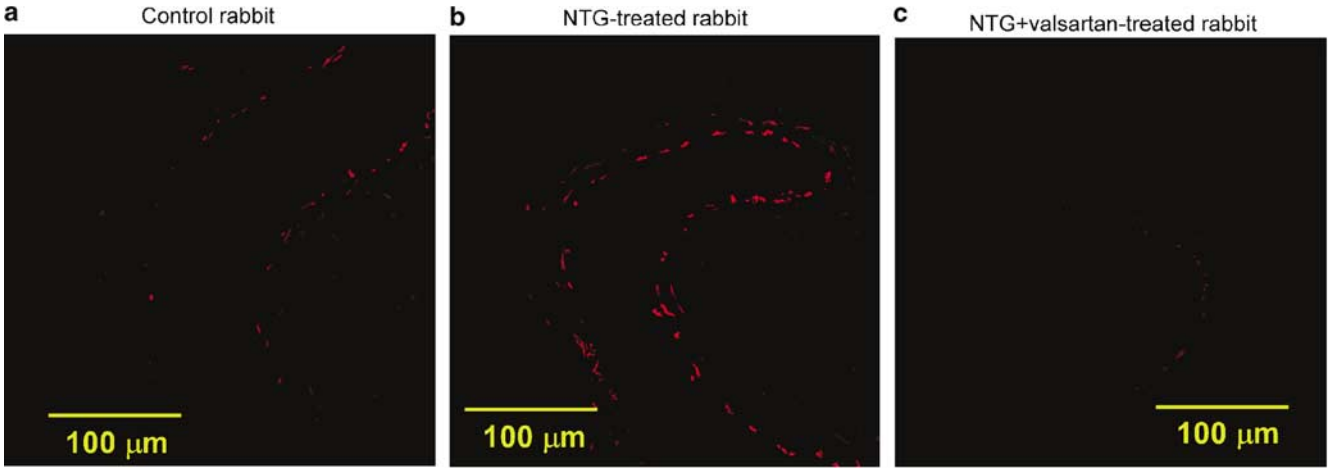


Figure 6 Effect of *in vivo* treatment with NTG (with or without valsartan) on the production of superoxide anion in rabbit pulmonary vein. (a) A weak superoxide anion production (detected by DHE) seen in a few endothelial cells in a control rabbit. (b) An increased superoxide-anion production seen in many endothelial cells and some smooth muscle cells in an NTG-treated rabbit. (c) The increased production of superoxide anion (b) was normalized when the AT_1R blocker valsartan was coapplied with NTG. Similar observations were made in other sections obtained from four preparations from four other animals.

lium-dependent relaxation by inhibiting the synthesis of nitric oxide within endothelial cells in rabbit intrapulmonary veins (rather than by inhibiting the action of nitric oxide). The present result would seem to be at least partly consistent with previous findings made in the rat and rabbit aorta, in which *in vivo* NTG treatment for 3 days reduced the relaxations induced

not only by the endothelium-dependent agent ACh but also by the endothelium-independent agents SIN-1 and sodium nitroprusside (Molina *et al*., 1987; Münzel *et al*., 1995). This suggests that in the case of conduit arteries, not only an inhibition of nitric oxide production but also a decreased bioavailability of nitric oxide, a decrease in soluble guanylyl cyclase activity and/or a reduction in cGMP-mediated relaxation may be involved in the downregulation of endothelium-dependent relaxation seen in vessels obtained from animals treated *in vivo* with NTG (Molina *et al*., 1987; Soff *et al*., 1997; Mollnau *et al*., 2002). Thus, the mechanisms underlying the downregulation of endothelium-dependent relaxation that is induced by long-term *in vivo* administration of NTG may differ between conduit arteries and intrapulmonary veins.

Regarding the development of nitrate tolerance, attention has been paid to the possible role of circulating, as well as local, angiotensin II (Münzel & Bassenge, 1996; Gori & Parker, 2002a, b). Indeed, (i) *in vivo* treatment with NTG activates the renin–angiotensin–aldosterone axis as a neurohormonal counter-regulatory mechanism in both animals and humans (Münzel & Bassenge, 1996; Gori & Parker, 2002a, b) and (ii) *in vivo* administration of angiotensin II increases the production of superoxide anion by the rat aorta *via* an activation and upregulation of membrane-bound NAD(P)H oxidase (Mollnau *et al*., 2002). In the present experiments, we found that *in vivo* treatment with NTG increased the amount of immunoreactive angiotensin II in the vascular wall without a significant change in AT_1R expression, and that this treatment enhanced the production of superoxide anion by the endothelial cells. Furthermore, consistent with previous findings in the rat and rabbit aorta (Münzel & Bassenge, 1996; Berkenboom *et al*., 1999; Kurz *et al*., 1999), an *in vivo* AT_1R blockade normalized both the increased production of endothelial superoxide anion and the downregulated ACh-induced endothelium-dependent relaxation seen in NTG-treated rabbits. Taken together, these results suggest that in rabbits treated *in vivo* with NTG, ACh-induced nitric oxide production by the intrapulmonary veins is inhibited *via* an AT_1R-mediated increase in the endothelial-cell production of superoxide anions.

In conclusion, in rabbit intrapulmonary veins, ACh produces an endothelium-dependent relaxation through an action mediated by endothelium-derived nitric oxide. Chronic *in vivo* administration of NTG increases the production of superoxide anion by the endothelial cells *via* an action mediated by AT_1R and thereby downregulates endothelium-dependent relaxation. Thus, it is suggested that AT_1Rs may play an essential role in the development and/or maintenance of the attenuated endothelium-dependent relaxation seen in intrapulmonary veins obtained from rabbits given long-term *in vivo* treatment with NTG.

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