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RESEARCH PAPER

Effects of chronic *in vivo* administration of nitroglycerine on ACh-induced endothelium-dependent relaxation in rabbit cerebral arteries

Y Watanabe[1], N Kusama[1,2] and T Itoh[1]

[1]*Department of Pharmacology, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan and* [2]*Department of Anesthesiology and Medical Crisis Management, Graduate School of Medical Sciences, Nagoya City University, Nagoya, Japan*

Background and purpose: In the setting of nitrate tolerance, endothelium-dependent relaxation is reduced in several types of peripheral vessels. However, it is unknown whether chronic *in vivo* administration of nitroglycerine modulates such relaxation in cerebral arteries.
Experimental approach: Isometric force and smooth muscle cell membrane potential were measured in endothelium-intact strips from rabbit middle cerebral artery (MCA) and posterior cerebral artery (PCA).
Key results: ACh (0.1–10 μM) concentration-dependently induced endothelium-dependent relaxation during the contraction induced by histamine in both MCA and PCA. Chronic (10 days) *in vivo* administration of nitroglycerine reduced the ACh-induced relaxation in PCA but not in MCA, in the presence of the cyclooxygenase inhibitor diclofenac (3 μM). In the presence of the NO-synthase inhibitor N^{ω}-nitro-L-arginine (L-NNA, 0.1 mM) plus diclofenac, in MCA from both nitroglycerine-untreated control and -treated rabbits, ACh (0.1–10 μM) induced a smooth muscle cell hyperpolarization and relaxation, and these were blocked by the small-conductance Ca^{2+}-activated K^+-channel inhibitor apamin (0.1 μM), but not by the large- and intermediate-conductance Ca^{2+}-activated K^+-channel inhibitor charybdotoxin (0.1 μM). In contrast, in PCA, ACh (<3 μM) induced neither hyperpolarization nor relaxation under these conditions, suggesting that the endothelium-derived relaxing factor is NO in PCA, whereas endothelium-derived hyperpolarizing factor (EDHF) plays a significant role in MCA.
Conclusions and implications: It is suggested that in rabbit cerebral arteries, the function of the endothelium-derived relaxing factor NO and that of EDHF may be modulated differently by chronic *in vivo* administration of nitroglycerine.
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Keywords: middle cerebral artery; posterior cerebral artery; endothelium-dependent relaxation; nitric oxide; endothelium-derived hyperpolarizing factor; nitroglycerine; nitrate tolerance; rabbit

Abbreviations: EDHF, endothelium-derived hyperpolarizing factor; K_{Ca} channel, Ca^{2+}-activated K^+ channel; L-NNA, N^{ω}-nitro-L-arginine; MCA, middle cerebral artery; NOC-7, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene; PCA, posterior cerebral artery

Introduction

Organic nitrates, such as nitroglycerine, have been widely used in the management of ischaemic heart diseases. However, despite their undoubted beneficial haemodynamic and anti-ischaemic effects, their usefulness is limited by the development of tolerance during continuous therapy (referred to as 'nitrate tolerance'; Parker *et al.*, 1991; Parker and Parker, 1998). Furthermore, in the setting of nitrate tolerance, not only the relaxing response to nitrates, but also the relaxation mediated by endothelium-derived NO is reduced in coronary and forearm arteries in humans (referred to as 'cross-tolerance'; Caramori *et al.*, 1998; Gori *et al.*, 2001). Such cross-tolerance has been documented most commonly in peripheral conduit vessels in experimental animals (Münzel *et al.*, 1995; Laursen *et al.*, 1996; Berkenboom *et al.*, 1999). However, we recently found that chronic *in vivo* administration of nitroglycerine reduced endothelium-derived NO-mediated responses in the rabbit mesenteric resistance artery and intrapulmonary vein (Yamamoto *et al.*, 2005; Kusama *et al.*, 2005a), suggesting that cross-tolerance also develops in peripheral resistance arteries and small veins. Further, such treatment also reduced agonist-induced endothelial cell hyperpolarization in rabbit aortic valve (Kusama *et al.*, 2005b). Endothelial-cell hyperpolarization plays a pivotal role in the action of endothelium-derived

Correspondence: Dr Y Watanabe, Department of Pharmacology, Graduate School of Medical Sciences, Nagoya City University, Nagoya 467-8601, Japan.
E-mail: yoshiw@med.nagoya-cu.ac.jp
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hyperpolarizing factors (EDHFs; Busse *et al*., 2002; Griffith, 2004; von der Weid and Coleman, 2005), which have been suggested to function as additional, important endothelium-derived vasorelaxing factors, especially in resistance vessels (Kuriyama *et al*., 1998). We also found recently that chronic *in vivo* administration of nitroglycerine reduced ACh-induced endothelium-dependent smooth muscle cell hyperpolarization in rabbit coronary artery (Kusama *et al*., 2006). These findings seem to suggest that long-term *in vivo* administration of nitroglycerine downregulates not only endothelium-derived NO-mediated relaxation, but also EDHF-mediated relaxation in peripheral arteries.

It has been found that in human intracranial cerebral arteries, the vasodilator effect of nitrate is reduced upon repeated administration (Christiansen *et al*., 2000), suggesting that nitrate tolerance also develops in cerebral arteries. However, little attention seems to have been paid to the possibility of the development of endothelial dysfunction in cerebral arteries, as seen in peripheral arteries, during long-term nitrate therapy. The blood supply to the brain depends on two different cerebrovascular systems: the carotid and vertebrobasilar systems. The terminal branches of these two systems are the middle cerebral artery (MCA) and posterior cerebral artery (PCA), respectively, and these vessels thereby represent the major blood suppliers to the cerebrum. In the present study, we set out to clarify whether long-term *in vivo* administration of nitroglycerine in rabbits might induce changes in ACh-induced, endothelium-dependent relaxation in MCA and PCA. We found that such nitroglycerine administration modulates ACh-induced endothelium-dependent relaxation differently in these two arteries.

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 albino rabbits (supplied by Kitayama Labes, Ina, Japan), weighing 2.5–3.0 kg, were treated by applying transdermal nitroglycerine patches (Nitroderm TTS; Novartis Pharma, Tokyo, Japan), as described previously (Nakano *et al*., 2004). Such patches were present continuously for a period of 10 days (each patch being replaced daily with a new one) ('nitroglycerine-treated rabbits'). Using this protocol, we demonstrated previously the presence of nitrate tolerance by showing that the relaxing response to nitroglycerine was significantly reduced in the smooth muscle of mesenteric resistance arteries taken from such rabbits (Nakano *et al*., 2004). Male rabbits of a similar body weight served as controls ('nitroglycerine-untreated control rabbits').

Tissue preparation

Rabbits were anaesthetized by injection of pentobarbitone sodium (50 mg kg^{-1} given i.v.), then killed by exsanguination. The brain was immediately removed and placed in a chamber filled with Krebs solution. Distal parts of the MCA trunk and those of the ambient segment of PCA (the peripheral segment distal to the junction with the posterior communicating artery) were dissected out under a binocular microscope. The outer diameter was similar between these two arteries (range, 250–300 μm). After the arachnoid membrane and connective tissue had been carefully removed, each segment was cut open along its long axis (using small scissors), and circularly cut strips were prepared using a small razor blade, as described previously (Itoh *et al*., 1992). In some preparations, the endothelium was removed by gently rubbing the intimal surface of the strips with small pieces of razor blade (Itoh *et al*., 1992).

Recording of mechanical responses

Circularly cut strips (0.7–0.8 mm long, 0.2 mm wide) were mounted horizontally in a small chamber and attached to a strain gauge (AE801; SensoNor a.s, Horten, Norway), allowing us to record isometric tension. The chamber (capacity, 0.3 ml) was perfused continuously with Krebs solution (37 °C) at a flow rate of 2 ml min^{-1}. 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. The transducer was connected to a carrier amplifier (AS2101; NEC-San-ei Instruments, Tokyo, Japan) and the output signal was fed into an IBM/AT-compatible PC through an analogue-digital converter (PowerLab; ADInstruments Pty Ltd, Bella Vista, Australia). The resting tension was adjusted to obtain maximum contraction in high-K^+ solution (80 mM).

Concentration–response relationships for the histamine (0.1–30 μM)-induced contraction were obtained in endothelium-intact strips of MCA and PCA, the histamine being applied cumulatively from low-to-high concentration. The relationship was first observed in the absence of the cyclooxygenase inhibitor diclofenac, and then in its presence (3 μM). Finally, this relationship was obtained in the presence of diclofenac plus N^{ω}-nitro-L-arginine (L-NNA, an inhibitor of NO synthase, 0.1 mM). Diclofenac, with or without L-NNA, was given as pretreatment for 60 min, and was present thereafter.

Endothelium-dependent relaxation was induced by cumulative application of ACh (0.1–10 μM) during the contraction induced by histamine (routinely 10 μM, but reduced for amplitude matching when necessary; see Results). 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. In some strips, the effects of diclofenac (3 μM) with or without L-NNA (0.1 mM) were examined on the ACh-induced relaxation. In previous experiments, we found that in the presence of L-NNA, the small-conductance Ca^{2+}-activated K^+ (K_{Ca})-channel inhibitor apamin blocks ACh-induced endothelium-dependent smooth muscle cell hyperpolarization and relaxation in rabbit MCA (Yamakawa *et al*., 1997). In the present experiments, when the effect of apamin on the ACh-induced relaxation was to be examined, the toxin (0.1 μM) was applied concomitantly with histamine for 3 min in the presence of diclofenac with or without

L-NNA, and then ACh was applied in the presence of apamin + diclofenac with or without L-NNA. In a separate series of experiments, the effects of L-arginine (the substrate for NO synthases) or D-arginine on ACh-induced relaxation were examined. To this end, following the recording of control responses, L- or D-arginine (1 mM) was applied as pretreatment for 60 min, and then the ACh-induced relaxation was observed in the presence of the amino acid.

The concentration–response relationship for the NO-donor compound, 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7, 1–300 nM) was obtained by its cumulative application during the steady-state contraction induced by histamine (1 μM) in the presence of L-NNA + diclofenac.

Recording of membrane potential

An endothelium-intact strip of MCA or PCA was placed in a chamber of 0.5 ml volume, and both ends of the strip were pinned down to the bottom of the chamber. The strip was superfused with Krebs solution (37 °C) at a flow rate of 2 ml min^{-1}. Glass microelectrodes were made from borosilicate glass tube (outer diameter = 1.2 mm with a glass filament inside; Hilgenberg, Malsfeld, Germany) and filled with 1 M KCl. The resistance of the electrodes was 120–180 MΩ. The electrode was inserted into smooth muscle cells from the luminal side. Membrane potentials were recorded using an Axoclamp-2B amplifier (Axon Instruments, Foster, CA, USA) and the data were digitalized at an acquisition rate of 20 Hz using an Axoscope 9.0/Digidata 1200 data-acquisition system (Axon Instruments).

To observe the concentration-dependent effects of ACh on the membrane potential, ACh (0.1–10 μM) was applied for 2 min at 25 min intervals in an ascending order in the presence of L-NNA + diclofenac. When effects of apamin or charybdotoxin (an inhibitor of large- and intermediate-conductance K_{Ca} channels) on the ACh-induced membrane potential changes were to be examined, apamin (0.1 μM) or charybdotoxin (0.1 μM) was given as pretreatment for 3 min and ACh was then applied in the presence of the toxin.

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^- and 11.5 glucose. High-K^+ solution (80 mM) was prepared by replacing sodium chloride with potassium chloride isosmotically. The solutions were bubbled with 95% oxygen and 5% carbon dioxide.

Statistics

Values are expressed as mean ± s.e.mean, with the *n* value representing the number of animals used (each animal providing one strip for a given experiment). The pD_2 values [−log (EC_{50})] for the concentration–response curves were obtained by fitting the data points for each strip by a non-linear least-squares method using commercial software (OriginPro; OriginLab Co., Northampton, MA, USA). Statistical analysis was conducted using either a 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, USA). Probabilities less than 5% ($P<0.05$) were considered statistically significant.

Drugs

The drugs used in the current experiments were as follows: ACh chloride (Daiichi Pharmaceutical Co. Ltd, Tokyo, Japan), L-NNA and apamin (Peptide Institute Inc., Osaka, Japan), diclofenac sodium (Sigma Chemical Co., St Louis, MO, USA), guanethidine sulphate (Tokyo Kasei, Tokyo, Japan), histamine dihydrochloride, L- and D-arginine (Wako Pure Chemical, Tokyo, Japan) and NOC-7 (Dojindo Laboratories, Kumamoto, Japan). Famotidine was kindly provided 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 being diluted in Krebs solution to the required final concentration immediately before use. All drug solutions were made using ultra-pure Milli-Q water (Japan Millipore Corp., Tokyo, Japan).

Results

Effects of chronic nitroglycerine administration on ACh-induced changes in membrane potential of smooth muscle cells

In the presence of the NO-synthase inhibitor L-NNA and the cyclooxygenase inhibitor diclofenac, the resting membrane potential of smooth muscle cells in endothelium-intact strips of MCA from nitroglycerine-untreated control rabbits was −47.5 ± 0.5 mV ($n=5$ from five animals). In the presence of L-NNA plus diclofenac, ACh (3 μM) induced a hyperpolarization followed by a rebound depolarization observed after washing-out of ACh in MCA from control rabbits (Figures 1a and b). The hyperpolarizing action of ACh was concentration dependent (0.1–10 μM) (Figure 1c). The ACh-induced smooth muscle cell hyperpolarization was not observed in endothelium-denuded strips of MCA ($n=3$). In the presence of L-NNA + diclofenac, in control rabbits, the hyperpolarization induced by 3 μM ACh (27.9 ± 1.9 mV) was blocked by the small-conductance K_{Ca}-channel inhibitor apamin (0.1 μM; 2.8 ± 1.3 mV, $n=4$; $P<0.001$), but this was not modified by the large- and intermediate-conductance K_{Ca}-channel inhibitor charybdotoxin (0.1 μM; 22.2 ± 1.7 versus 23.5 ± 1.7 mV, $n=4$; $P>0.1$) (Figures 1a and b). The similar inhibitory action of apamin on ACh-induced hyperpolarization was also observed in the presence of diclofenac only (that is in the absence of L-NNA; 21.5 ± 1.2 versus 0.3 ± 0.2 mV, $n=3$; $P<0.01$). The resting membrane potential of smooth muscle cells in endothelium-intact MCA from nitroglycerine-treated rabbits was −47.0 ± 1.0 mV in the presence of L-NNA + diclofenac ($n=5$ from five animals), a value not significantly different from that obtained for control rabbits ($P>0.05$). ACh (0.1–10 μM) induced a smooth muscle cell hyperpolarization in nitroglycerine-treated rabbits that was of similar magnitude to that seen in control rabbits (Figure 1c; $P>0.05$). The hyperpolarization induced by 3 μM

ACh (22.2 ± 1.8 mV) was blocked by 0.1 μM apamin (1.7 ± 0.9 mV, $n = 3$; $P < 0.05$), but this was not modified by 0.1 μM charybdotoxin (20.7 ± 1.2 versus 22 ± 1.9 mV, $n = 3$; $P > 0.1$) in nitroglycerine-treated rabbits.

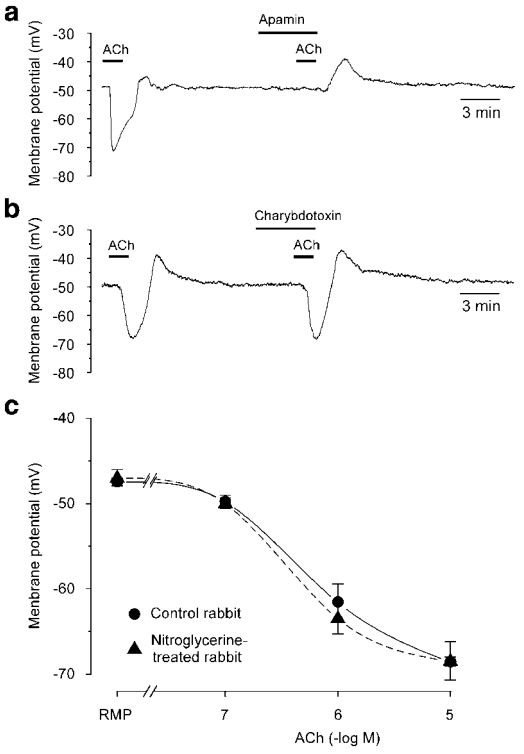

Figure 1 Effects of ACh on membrane potential of smooth muscle cells in endothelium-intact strips of middle cerebral arteries. (**a**) and (**b**) Original recordings of membrane potential changes induced by ACh (3 μM) obtained from a nitroglycerine-untreated control rabbit. Applications of ACh, apamin (0.1 μM) and charybdotoxin (0.1 μM) are indicated by horizontal bars. (**c**) Concentration-dependent effects of ACh (0.1–10 μM) on membrane potentials in nitroglycerine-untreated control and nitroglycerine-treated rabbits. Data are shown as means (± s.e.mean) from five animals. Diclofenac (3 μM) and N^{ω}-nitro-L-arginine (0.1 mM) were present throughout the experiments. RMP, resting membrane potential.

The resting membrane potential of smooth muscle cells in endothelium-intact strips of PCA from nitroglycerine-untreated control rabbits was −47.4 ± 1.6 mV in the presence of L-NNA + diclofenac ($n = 4$ from four animals), a value similar to that obtained for MCA ($P > 0.05$). In the presence of L-NNA + diclofenac, ACh (> 1 μM) induced a hyperpolarization in PCA from control rabbits (1.0 ± 0.5 mV at 1 μM, 5.6 ± 0.6 mV at 3 μM and 10.4 ± 0.5 mV at 10 μM; $n = 4$). The magnitude of the hyperpolarization at a given concentration of ACh was significantly smaller in PCA than in MCA ($P < 0.01$). The resting membrane potential of smooth muscle cells in endothelium-intact PCA from nitroglycerine-treated rabbits was −47.3 ± 1.0 mV in the presence of L-NNA + diclofenac ($n = 4$ from four animals), a value not significantly different from that obtained for control rabbits ($P > 0.05$). ACh (10 μM) induced a smooth muscle cell hyperpolarization (11.0 ± 1.6 mV, $n = 4$) in nitroglycerine-treated rabbits that was of similar magnitude to that seen in control rabbits ($P > 0.1$).

Effects of chronic nitroglycerine administration on ACh-induced endothelium-dependent relaxation

In endothelium-intact MCA and PCA from nitroglycerine-untreated control rabbits, histamine (0.1–30 μM) induced a concentration-dependent contraction. Neither the maximum amplitude of contraction nor the pD_2 value differed significantly between the two arteries (Table 1). Diclofenac (3 μM) did not modify the histamine-induced contraction in either artery from control rabbits (Table 1). In the presence of diclofenac, L-NNA (0.1 mM) enhanced the histamine-induced maximum contraction, with an increase in the pD_2 value, in both arteries, the effect being similar between the two vessels (Table 1). In the presence and absence of diclofenac, the absolute tension induced by histamine in both MCA and PCA was not significantly different between nitroglycerine-untreated control and nitroglycerine-treated rabbits (Table 1). In the presence of diclofenac, L-NNA (0.1 mM) enhanced the histamine-induced maximum contraction, with an increase in the pD_2 value, in both arteries

Table 1 Effects of diclofenac (3 μM) with or without L-NNA (0.1 mM) on histamine-induced contraction in cerebral arteries from nitroglycerine-untreated control and nitroglycerine-treated rabbits

	Control rabbits			*Nitroglycerine-treated rabbits*		
	pD_2	*Maximum tension*		pD_2	*Maximum tension*	
		Absolute (μN)	*Relative*		*Absolute (μN)*	*Relative*
MCA						
None	5.32 ± 0.10	178 ± 36	1.00	5.28 ± 0.12	162 ± 14	1.00
Diclofenac	5.35 ± 0.08	230 ± 43	1.30 ± 0.04	5.37 ± 0.09	217 ± 16	1.35 ± 0.05
Diclofenac + L-NNA	5.72 ± 0.09**,††	316 ± 46**,†	1.88 ± 0.24**,†	5.71 ± 0.08**,†	276 ± 23**,††	1.72 ± 0.10**,††
PCA						
None	5.25 ± 0.07	188 ± 15	1.00	5.32 ± 0.16	214 ± 33	1.00
Diclofenac	5.44 ± 0.04	230 ± 14	1.23 ± 0.05	5.38 ± 0.08	260 ± 31	1.23 ± 0.05
Diclofenac + L-NNA	5.79 ± 0.05**,††	315 ± 18**,††	1.69 ± 0.09**,††	5.77 ± 0.14*,†	331 ± 42**,††	1.56 ± 0.05**,††

Abbreviations: L-NNA, N^{ω}-nitro-L-arginine; MCA, middle cerebral artery; PCA, posterior cerebral artery.
Mean ± s.e.mean from four animals. *$P < 0.05$, **$P < 0.01$ versus '*None*' and †$P < 0.05$, ††$P < 0.01$ versus '*Diclofenac*' in the same artery (one-way repeated-measures ANOVA followed by Scheffe's *post hoc* analysis).

from nitroglycerine-treated rabbits, with these effects of L-NNA being similar to those seen in control rabbits (Table 1).

ACh (0.1–10 μM) induced a concentration-dependent relaxation during the contraction induced by 10 μM histamine in endothelium-intact MCA and PCA from nitroglycerine-untreated control rabbits (Figure 2). In endothelium-denuded preparations of both arteries from control rabbits, ACh (0.1–10 μM) did not induce relaxation during the contraction induced by histamine (1 μM; $n=3$). In control rabbits, diclofenac (3 μM) did not modify the ACh-induced endothelium-dependent relaxation either in MCA (pD_2 value: 5.83 ± 0.12 and 5.80 ± 0.19; maximum relaxation (%): 81.5 ± 5.9 and 83.7 ± 9.5 in the absence and presence of diclofenac, respectively; $n=3$; $P>0.05$) or in PCA (pD_2 value: 5.79 ± 0.16 and 5.88 ± 0.12; maximum relaxation (%): 68.5 ± 8.2 and 73.8 ± 10.9 in the absence and presence of diclofenac, respectively; $n=3$; $P>0.05$). Since L-NNA (0.1 mM) enhanced the amplitude of contraction induced by 10 μM histamine in both arteries from control rabbits in the presence of diclofenac, the concentration of histamine was reduced to 1–2 μM when L-NNA was used (for amplitude matching between the presence and absence of L-NNA). In the presence of diclofenac, absolute tensions induced by histamine (10 μM in the absence of L-NNA and 1–2 μM in its presence) in MCA were 171 ± 21 and 185 ± 37 μN in the absence and presence of L-NNA, respectively ($n=6$; $P>0.05$), while the corresponding values for PCA were 175 ± 35 and 198 ± 23 μN, respectively ($n=6$; $P>0.05$). Under these conditions, L-NNA (0.1 mM) abolished the ACh-induced relaxation in PCA (Figure 2b), but had no significant effect on the same relaxation in MCA ($n=6$; $P>0.05$) (Figure 2a). In the presence of L-NNA + diclofenac, apamin (0.1 μM) did not modify the contraction induced by histamine (0.99 ± 0.01 times that recorded before apamin application, $n=4$; $P>0.05$), but it blocked the ACh-induced relaxation in MCA from control rabbits (Figure 2a). The similar inhibitory action of apamin was also observed in the presence of diclofenac only (that is in the absence of L-NNA): amplitudes of the maximum relaxation induced by 10 μM ACh in the absence and presence of apamin were 71.2 ± 4.2 and 5.0 ± 3.5%, respectively ($n=3$; $P<0.01$).

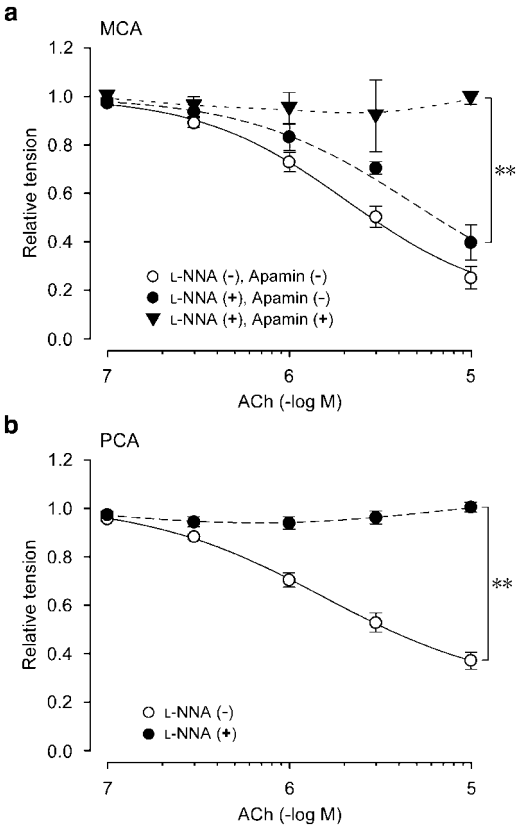

Figure 2 ACh-induced relaxation during the contraction produced by histamine in endothelium-intact strips of middle cerebral artery (MCA, **a**) and posterior cerebral artery (PCA, **b**) in nitroglycerine-untreated control rabbits. L-NNA (−) and (+), in the absence and presence of N^{ω}-nitro-L-arginine (L-NNA), respectively. Apamin (−) and (+), in the absence and presence of apamin, respectively. L-NNA (0.1 mM) was given as pretreatment for 60 min and was present thereafter. Apamin (0.1 μM) was given as pretreatment for 3 min and was present during the cumulative application of ACh in the presence of L-NNA. Diclofenac (3 μM) was present throughout the experiments. The concentration of histamine used to produce contraction was 10 μM in the absence of L-NNA, but for amplitude matching this was reduced to 1–2 μM in the presence of L-NNA. The amplitude of contraction induced by histamine before application of ACh was normalized as a relative tension of 1.0 for each curve. Data are shown as means (± s.e.mean) from four to six animals. In MCA, the pD_2 values for ACh were: for 'L-NNA (−), Apamin (−),' 5.80 ± 0.07 and for 'L-NNA (+), Apamin (−),' 5.72 ± 0.12. For 'L-NNA (−)' in PCA, the corresponding value was 5.85 ± 0.08. **$P<0.01$ by a two-way repeated-measures ANOVA.

In the presence of diclofenac, the relaxation induced by ACh in PCA was significantly weaker in nitroglycerine-treated rabbits than in control rabbits (Figure 3b). In contrast, chronic *in vivo* administration of nitroglycerine did not modify the ACh-induced relaxation in MCA in the presence of diclofenac (Figure 3a). In nitroglycerine-treated rabbits, the concentration of histamine used to induce contraction was reduced to 1–2 μM in the presence of L-NNA + diclofenac (for amplitude matching before and after application of L-NNA). The histamine-induced absolute tensions in MCA in the presence of diclofenac were 189 ± 36 and 160 ± 23 μN in the absence and presence of L-NNA, respectively ($n=5$; $P>0.05$), and the corresponding values in PCA were 194 ± 13 and 233 ± 21 μN, respectively ($n=5$; $P>0.05$). Under these conditions, L-NNA blocked the ACh-induced relaxation in PCA (Figure 3b), but did not significantly modify it in MCA ($n=5$; $P>0.05$) (Figure 3a). In the presence of L-NNA + diclofenac, apamin (0.1 μM) did not modify the contraction induced by histamine (1.01 ± 0.01 times that recorded before apamin application, $n=4$; $P>0.05$), but it blocked the ACh-induced relaxation (Figure 3a).

The effect of an *in vitro* application of L-arginine (1 mM) was examined on ACh-induced relaxation in PCA in the presence of diclofenac. L-Arginine did not change the amplitude of contraction induced by histamine (10 μM) in either control or nitroglycerine-treated rabbits (1.18 ± 0.07 and 0.98 ± 0.07 times before application of L-arginine, respectively; $n=4$; $P>0.05$). L-Arginine did not significantly modify the ACh-induced relaxation in control rabbits ($n=4$; $P>0.1$), but enhanced it in nitroglycerine-treated rabbits (Figure 4). In contrast, D-arginine (1 mM) had no significant effect on the ACh-induced relaxation in nitroglycerine-treated rabbits (pD_2

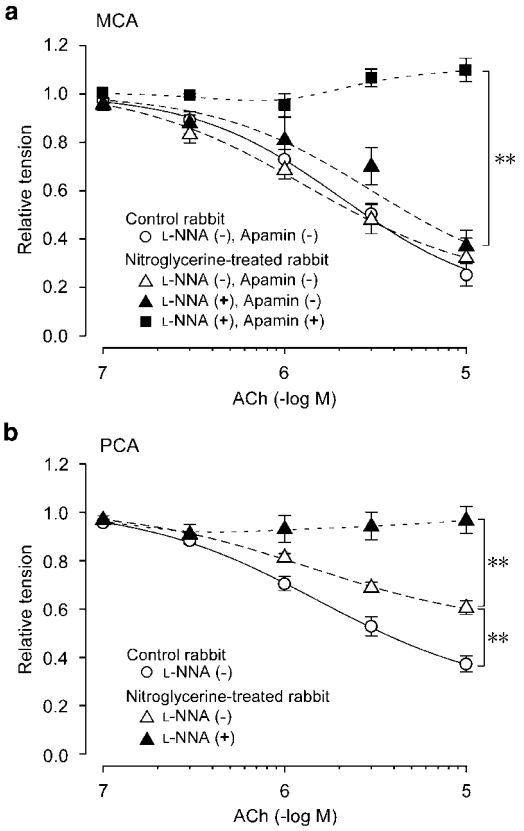

Figure 3 Effects of chronic *in vivo* administration of nitroglycerine on ACh-induced relaxation in endothelium-intact strips of middle cerebral artery (MCA, **a**) and posterior cerebral artery (PCA, **b**). L-NNA (−) and (+), in the absence and presence of N^{ω}-nitro-L-arginine (L-NNA), respectively. Apamin (−) and (+), in the absence and presence of apamin, respectively. Data for 'Control rabbit' are replotted from Figure 1. L-NNA (0.1 mM) was given as pretreatment for 60 min and was present thereafter. Apamin (0.1 μM) was given as pretreatment for 3 min and was present during the cumulative application of ACh in the presence of L-NNA. Diclofenac (3 μM) was present throughout the experiments. The concentration of histamine used to produce contraction was 10 μM in the absence of L-NNA, but for amplitude matching this was reduced to 1–2 μM in the presence of L-NNA. The amplitude of contraction induced by histamine before application of ACh was normalized as a relative tension of 1.0 for each curve. Data are shown as means (± s.e.mean) from four to six animals. In MCA from nitroglycerine-treated rabbits, the pD_2 values for ACh were: for 'L-NNA (−), Apamin (−),' 6.02 ± 0.06 and for 'L-NNA (+), Apamin (−),' 5.86 ± 0.08. For 'L-NNA (−)' in PCA from nitroglycerine-treated rabbits, the corresponding value was 5.96 ± 0.01. $^{**}P<0.01$ by a two-way repeated-measures ANOVA.

value: 5.56 ± 0.02 and 5.59 ± 0.01; maximum relaxation (%): 53.5 ± 4.3 and 55.8 ± 7.5 in the absence and presence of D-arginine, respectively; $n=3$; $P>0.1$).

In the presence of L-NNA + diclofenac, the relaxation induced in PCA by the NO donor NOC-7 (1–300 nM) during the contraction induced by 1 μM histamine did not differ between nitroglycerine-treated rabbits and control rabbits (Figure 5).

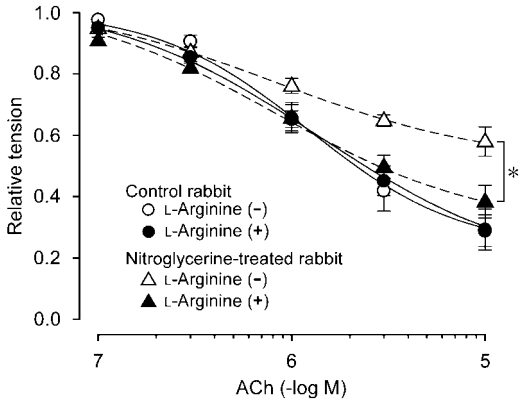

Figure 4 Effect of *in vitro* application of L-arginine (1 mM) on ACh-induced relaxation in posterior cerebral arteries from nitroglycerine-untreated control and nitroglycerine-treated rabbits. L-Arginine (−), in the absence of L-arginine; L-Arginine (+), in the presence of L-arginine, which was given as pretreatment for 60 min. The amplitude of contraction induced by histamine (10 μM) before application of ACh was normalized as a relative tension of 1.0 for each curve. Diclofenac (3 μM) was present throughout the experiments. Data are shown as means (± s.e.mean) from four animals. In control rabbits, the pD_2 values for ACh were: for 'L-Arginine (−),' 6.10 ± 0.17 and for 'L-Arginine (+),' 6.00 ± 0.14, with the corresponding values in nitroglycerine-treated rabbits being 6.05 ± 0.10 and 6.01 ± 0.11, respectively. $^{*}P<0.05$ by two-way repeated-measures ANOVA.

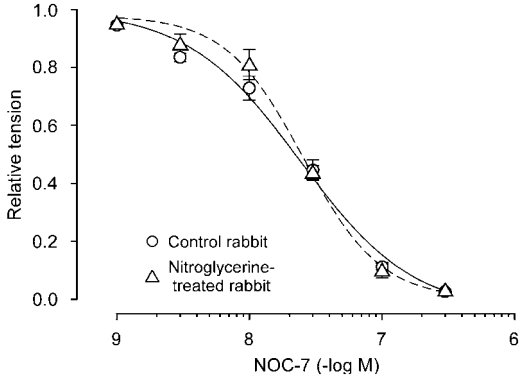

Figure 5 Effect of the NO donor, NOC-7 (1–300 nM), on histamine-induced contraction in endothelium-intact strips of posterior cerebral artery from nitroglycerine-untreated control and nitroglycerine-treated rabbits. N^{ω}-nitro-L-arginine (0.1 mM) and diclofenac (3 μM) were present throughout the experiments. The amplitude of contraction induced by histamine (1 μM) before application of NOC-7 was normalized as a relative tension of 1.0 for each curve. Data are shown as means (± s.e.mean) from four animals.

Discussion and conclusions

In the present study, we found evidence (a) that the endothelium-derived relaxing factors contributing to ACh-induced relaxation are not the same in rabbit MCA as they are in rabbit PCA, and (b) that chronic *in vivo* administration of nitroglycerine modulates the ACh-induced endothelium-dependent relaxation differently between these two arteries.

Characteristics of ACh-induced endothelium-dependent relaxations

ACh induced a concentration-dependent relaxation in endothelium-intact MCA and PCA, and the effects were lost

when the endothelium was removed, indicating that ACh induces an endothelium-dependent relaxation in these cerebral arteries. The cyclooxygenase inhibitor diclofenac did not modify the ACh-induced endothelium-dependent relaxation in either artery, suggesting that endothelium-derived vasodilator prostanoids may not be crucial for such relaxations.

It has been suggested that both endothelium-derived NO and EDHF play significant roles in determining cerebral arterial tone (Faraci and Heistad, 1998; Golding *et al*., 2002). In the present experiments, in the presence of diclofenac, the NO-synthase inhibitor L-NNA abolished the ACh-induced endothelium-dependent relaxation in PCA. In contrast, in MCA, ACh induced a relaxation in the presence of L-NNA + diclofenac and this was blocked by the small-conductance K_{Ca}-channel inhibitor apamin. Moreover, in findings partly consistent with previous data (Yamakawa *et al*., 1997; You *et al*., 1999; McNeish *et al*., 2006), our study revealed that in the presence of L-NNA + diclofenac, ACh (0.1–10 μM) induced endothelium-dependent smooth muscle cell hyperpolarization concentration dependently in MCA. Although at very high concentrations (3 and 10 μM) ACh induced a smaller hyperpolarization in PCA (compared with that in MCA), this agonist-induced relaxation at concentrations ranging 0.1–10 μM, suggesting that in PCA, the hyperpolarization may not play significant roles in ACh-induced endothelium-dependent relaxation. Taken together, these results suggest that endothelium-derived NO is crucial for ACh-induced relaxation in rabbit PCA, whereas EDHF plays an essential role in the same relaxation in rabbit MCA.

Effect of chronic in vivo *administration of nitroglycerine on ACh-induced endothelium-dependent relaxations*

It has been shown that both in large peripheral conduit vessels (such as aorta) and in small peripheral vessels (such as intrapulmonary veins), endothelium-dependent NO-mediated relaxations are weaker following long-term *in vivo* administration of nitroglycerine (Münzel *et al*., 1995; Kusama *et al*., 2005a). We found that chronic *in vivo* administration of nitroglycerine reduced ACh-induced relaxation in PCA (response mediated by endothelium-derived NO), but had no effect on such relaxation in MCA (EDHF-mediated response). Furthermore, *in vitro* application of the NO-synthase substrate L-arginine, but not of D-arginine, enhanced the ACh-induced relaxation in PCA from nitroglycerine-treated rabbits, although it had no effect on this relaxation in PCA from control rabbits. Moreover, the relaxations induced in PCA by the endothelium-independent NO donor NOC-7 were not different between control and nitroglycerine-treated rabbits. It has been shown that prolonged *in vitro* application of nitroglycerine inhibits the uptake of L-arginine into cultured bovine aortic endothelial cells (Abou-Mohamed *et al*., 2000), and it has been suggested that chronic *in vivo* administration of nitroglycerine reduces ACh-induced endothelial-cell NO production via an inhibition of L-arginine bioavailability in rabbit mesenteric arteries (Yamamoto *et al*., 2005). It may also be possible that long-term *in vivo* administration of nitroglycerine increases the production of endogenous NO-synthase inhibitors (such as asymmetric dimethylarginine), thus inhibiting NO production in endothelial cells. Taken together, these results suggest that chronic *in vivo* administration of nitroglycerine may downregulate ACh-induced NO-mediated relaxation in rabbit PCA through a reduction in cellular L-arginine bioavailability.

The peripheral and cerebral vascular endothelium releases NO not only in the presence of agonists but also under basal conditions (that is in the absence of agonists), although the NO production rate is much less in the latter condition than in the former. It was found that in smooth muscle cells of endothelium-intact rabbit MCA, histamine (3 μM) induced a depolarization (rather than a hyperpolarization) in the presence of an H_2-receptor blocker (Yamakawa *et al*., 1997), suggesting that the H_1 receptors activated by histamine may not stimulate endothelial cells in rabbit cerebral arteries. Here, we found that the NO-synthase inhibitor L-NNA caused an almost 1.7-fold increase in the histamine-induced contraction in endothelium-intact rabbit MCA and PCA. These results suggest that NO released from the endothelium under basal conditions may substantially attenuate the histamine-induced contraction in these cerebral arteries. Alternatively, isometric contraction induced by histamine in MCA and PCA may activate endothelial NO synthase, and thus the net result of contraction could be reduced by the increased NO release (Fleming *et al*., 1999). These assumptions raise the possibility that chronic *in vivo* administration of nitroglycerine might also downregulate the function of NO released basally or by an activation of isometric contraction in rabbit MCA and PCA. However, this is unlikely to be so since the effect of L-NNA on histamine-induced contraction in either artery was not modified by chronic *in vivo* administration of nitroglycerine.

It has been found that endothelial-cell hyperpolarization plays a pivotal role in the action of EDHF (Busse *et al*., 2002; Griffith, 2004; von der Weid and Coleman, 2005). We recently found that chronic *in vivo* administration of nitroglycerine reduces ACh-induced endothelial-cell hyperpolarization in rabbit aortic valve (a response that is largely inhibited by the large- and intermediate-conductance K_{Ca}-channel inhibitor charybdotoxin) (Kusama *et al*., 2005b). Furthermore, *in vivo* administration of nitroglycerine downregulates ACh-induced endothelium-dependent smooth muscle cell hyperpolarization and relaxation in rabbit coronary artery (responses that are also sensitive to charybdotoxin) (Kusama *et al*., 2006), suggesting that long-term *in vivo* administration of nitroglycerine downregulates the function of EDHF in some rabbit peripheral arteries. In the present experiments, ACh induced endothelium-dependent smooth muscle cell hyperpolarization and relaxation in MCA (responses that were found to be sensitive to the small-conductance K_{Ca}-channel inhibitor apamin but not to the large- and intermediate-conductance K_{Ca}-channel inhibitor charybdotoxin). The results are in contrast with the findings in rat MCA, in which the EDHF-mediated response appears to involve the sole activation of intermediate-conductance K_{Ca} channels (McNeish and Garland, 2007), suggesting that the species differences in the K_{Ca} channels involved may be relevant to EDHF-mediated responses. Furthermore, we found that long-term *in vivo* administration of nitroglycerine

had no effect on the ACh-induced endothelium-dependent smooth muscle cell hyperpolarization and relaxation in MCA. These results indicate that the function of EDHF in some cerebral arteries may be retained in the setting of nitrate tolerance, a situation different from that encountered in some peripheral arteries. At present, the mechanistic explanation for this difference is unknown, but it is possible that it might involve a difference in the K_{Ca} channels responsible for endothelial-cell hyperpolarization. This needs to be clarified in future studies.

In conclusion, EDHF and endothelium-derived NO play major roles in ACh-induced relaxation in rabbit MCA and PCA, respectively. Chronic *in vivo* administration of nitroglycerine downregulates the function of endothelium-derived NO but not that of EDHF in these major cerebral arteries. Thus, it is suggested that whether or not the function of the endothelium in cerebral arteries is modulated by long-term *in vivo* administration of nitroglycerine depends upon the type of endothelium-derived relaxing factor that contributes to the relaxation.

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Conflicts of interest

The authors state no conflict of interest.

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