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Characteristics of heterogeneity in the expression of vasoconstriction in response to N^G-monomethyl-L-arginine in isolated canine arteries

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Abstract

We characterized the contractile effect of a nitric oxide (NO) synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA), in isolated canine arteries. L-NMMA induced a heterogenous response: potent vasoconstriction in the cerebral arteries, and weak or no vasoconstrictor responses in different peripheral arteries. The vasoconstriction of the cerebral artery was inhibited by L-arginine but not D-arginine. L-NMMA (10^{-4} M) caused a 53% decrease in guanosine 3′5′-cyclic monophosphate (cGMP) levels in the cerebral artery, but it was not significant compared with that in peripheral arteries. The L-NMMA-induced vasoconstriction was inhibited by diltiazem and nicardipine, and the heterogeneity was mimicked by treatment with charybdotoxin, a Ca^{2+}-activated K^+ (BK_{Ca}) channel blocker, channels which are regulated by NO/cGMP. Both L-NMMA and charybdotoxin caused a potent vasoconstriction in the mesenteric artery precontracted with 20 mM KCl. 1*H*-[1,2,4]oxadiazolo[4,3-*a*] quinoxalin-1-one (ODQ) (10^{-5} M), a selective guanylate cyclase inhibitor, caused vasoconstriction in the presence of nitroprusside in the endothelium-denuded basilar artery, but not in the endothelium-denuded mesenteric artery. In conclusion, LNMMA-induced heterogenous vasoconstriction was due to the different sensitivities of vascular smooth muscles to NO/cGMP. The heterogeneity may result from a difference in the basal state of ion channels such as the voltage-dependent Ca^{2+} channel and the BK_{Ca} channel in vascular smooth muscles.

Keywords: Nitric oxide (NO) synthase inhibitor; N^G-Monomethyl-L-arginine (L-NMMA); cGMP; Vasoconstriction, heterogenous; Ion channels

1. Introduction

Recent studies have shown that endothelium plays an important role in the regulation of vascular tone by releasing many factors (Moncada et al., 1991; Vane, 1993; Cohen and Vanhoutte, 1995; Brian et al., 1996). Among these factors, nitric oxide (NO) is now known to hold a central position as a vasodilator. NO can stimulate the soluble guanylate cyclase in smooth muscle cells and cause an increase in the second messenger, guanosine 3′5′-cyclic monophosphate (cGMP), which is thought to lead to vasorelaxation. NO is produced from L-arginine by a family of NO-synthase enzymes (Palmer et al., 1988; Lopez-Jaramill et al., 1990; Sessa, 1994). The production of NO is inhibited by L-arginine analogues such as N^G-monomethyl-L-arginine (L-NMMA) and *N*-nitro-L-arginine (Palmer and Moncada, 1989; Mayer et al., 1989; Rees et al., 1990; Mülsch and Busse, 1990). In isolated arteries, treatment with a NO synthase inhibitor decreases the basal NO release from the endothelium (Gold et al., 1990; Cosentino et al., 1993). However, changes in tension appear to vary among arteries tested. NO synthase inhibitors cause constriction in isolated cerebral arteries (Katusic, 1991; Brain and Kennedy, 1993) but not in other arteries (Topouzis et al., 1991; Busse et al., 1993; Toda et al., 1993). To date, no precise comparative study of the constriction has been carried out in various arteries obtained from the same species. In addition, the mechanism underlying the heterogenous vasoconstriction has remained unclear. In this study, we first confirmed the L-NMMA-induced heterogenous vasoconstriction, then characterized the vasoconstriction in isolated canine arteries.

2. Materials and methods

2.1. Animal and general procedures

This study was approved by the Animal Research Committee of Tanabe Seiyaku. Fifty-nine mongrel dogs weigh-

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ing 12 to 23 kg were anaesthetized with an intravenous injection of sodium pentobarbital (30 mg/kg), and killed by exsanguination. The brain, heart, and arteries (renal, mesenteric, and femoral arteries) were removed immediately, and stored in physiological salt solution (PSS). The basilar artery (o.d. 1.0 to 2.0 mm) and the middle cerebral artery (0.9 to 1.2 mm) were isolated from the brain. The circumflex branch of the left coronary artery (1.1 to 1.8 mm) was isolated from the heart. The distal portion of the mesenteric artery (1.0 to 1.3 mm), the renal artery (1.8 to 2.3 mm) and the femoral artery (2.1 to 3.0 mm) were cleaned of surrounding connective tissue. These arteries were cut into ring segments 5 mm in length, and were used in experiments for the measurement of isometric tension and cGMP levels. The composition of PSS was as follows (mM):NaCl 147.2, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 2.2, $NaHCO_3$ 14.5, glucose 5.4 (pH 7.3 to 7.4).

2.2. Isometric tension

Ring segments were suspended in a 10-ml organ bath containing PSS, which was maintained at 37 ± 0.5°C and gassed with 95% O_2 and 5% CO_2. Resting tension in each artery was adjusted to 1.3 to 3.5 g according to the optimal point on the length-tension curve (Toda et al., 1978). The optimal tension was as follows: middle cerebral artery 1.3 g, basilar artery 1.8 g, renal artery: 2.4 g, mesenteric artery: 2.4 g, coronary artery 2.4 g, femoral artery: 3.5 g. Care was taken not to touch the luminal surface. Isometric tension was measured by means of a strain gauge transducer (UL-10, Minebea, Tokyo, Japan), and recorded on a recticorder (MC6621, Graphtec, Tokyo, Japan). In some preparations, the endothelium was removed by gently rubbing the intimal surface with 22G stainless steel needle. The preservation of endothelial function or the effectiveness of endothelial removal was tested routinely by the checking of responsiveness to acetylcholine (10^{-7} M) or substance P (10^{-8} M), which elicited relaxation in the preparation contracted with prostaglandin F2α (2×10^{-6} M).

All preparations were allowed to equilibrate for 90 to 120 min. After the contractile response to 40 mM KCl was checked, L-NMMA (3×10^{-8} to 3×10^{-4} M), U46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin F2α) (10^{-7} M), or charybdotoxin (10^{-10} to 10^{-7} M) was added to the preparation. When the influence of L-arginine (10^{-3} M), D-arginine (10^{-3} M), diltiazem (10^{-7} to 10^{-5} M) or nicardipine (10^{-9} to 10^{-7} M) on L-NMMA-induced constriction was to be examined, these agents were added to the preparation 10 min before L-NMMA. L-NMMA (10^{-4} M) or charybdotoxin (10^{-7} M) was also added to the preparation (mesenteric artery) which was precontracted with 20 mM KCl. When the influence of ODQ on the basal tension was examined in endothelium-denuded basilar or mesenteric arteries, ODQ (10^{-5} M) was administered to the preparation 5 min after applying sodium nitroprusside (10^{-7} M). At the end of the experiment, papaverine (3×10^{-4} M) was added to the preparation in order to characterize the amount of myogenic tone.

2.3. cGMP levels

Arterial ring segments with endothelium were preincubated for 90 to 120 min in PSS, which was maintained at 37 ± 0.5°C and gassed with 95% O_2 and 5% CO_2. After incubation in the presence and absence of L-NMMA (10^{-4} M) or charybdotoxin (10^{-7} M) for 10 min, which was a long enough period to obtain the maximum change in tension, the ring was frozen immediately with liquid N_2. When the influence of diltiazem on the change in cGMP levels was to be examined, ditiazem (10^{-5} M) was administered to the preparation 10 min before L-NMMA (10^{-4} M).

The frozen tissue was homogenized with a microhomogenizer in 1 ml of 6% trichloroacetic acid containing 1 mM EDTA. After centrifugation (5000 rpm, for 20 min, 4°C), the supernatant was extracted with water-saturated diethyl ether, and aliquots of the aqueous phase were lyophilized, then reconstituted in 100 μl of 50 mM sodium acetate buffer (pH 6.2). The cGMP and protein contents in the solution were measured with a Sakuma cGMP radioimmunoassay kit (YSI-7702, Yamasa, Tokyo, Japan) and BCA protein assay kit (Pierce, Rockford, IL, USA), respectively. The cGMP levels were expressed in picomoles per milligram protein.

2.4. Statistical analysis

Quantitative data are expressed as means $\pm$ S.E.M. Statistical evaluation of the data was performed by two-way analysis of variance (ANOVA), followed by Fisher's post hoc test. A value $P < 0.05$ was considered statistically significant.

2.5. Chemicals

L-NMMA, diltiazem hydrochloride and nicardipine hydrochloride were synthesized by Discovery Research Laboratory, Tanabe Seiyaku (Saitama, Japan). L-arginine hydrochloride, D-arginine and charybdotoxin were purchased from Peptide Institute (Osaka, Japan). Prostaglandin $F_{2\alpha}$, U46619, and sodium nitroprusside were purchased from Sigma (St. Lous, MO, USA). ODQ was purchased from Tocris Cookson (Ballwin, MO, USA). All other chemicals were of analytical grade.

3. Results

Fig. 1A shows typical tracings indicating the difference in responsiveness to L-NMMA between the basilar and mesenteric artery obtained from the same dog. Treatment

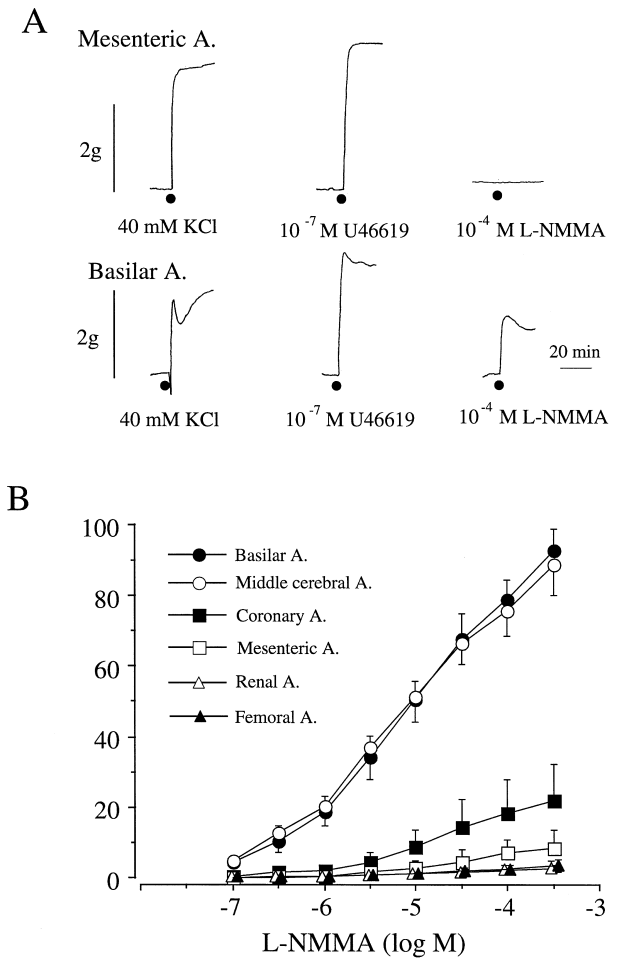

Fig. 1. Heterogenous response to L-NMMA in isolated canine arteries. (A) Typical tracings of response to KCl, U46619 and L-NMMA in isolated mesenteric and basilar arteries, both of which were obtained from the same dog. (B) Concentration–response curves for L-NMMA in isolated canine basilar, middle cerebral, coronary, mesenteric, renal and femoral arteries. After the contractile response to 40 mM KCl was checked, L-NMMA (10^{-7} to 3×10^{-4} M) was added to preparations cumulatively. KCl-induced contractions were as follows; basilar:2.28 ± 0.27 g, middle cerebral:1.24 ± 0.09 g, coronary:3.28 ± 0.49 g, mesenteric:3.20 ± 0.42 g, renal:4.81 ± 0.09 g, femoral arterial rings:3.94 ± 0.12 g. Data are shown as means ± S.E.M. for six preparations, and are expressed as percents of maximal contractions induced by 40 mM KCl.

with 40 mM KCl or U46619 (10^{-7} M), a thromboxane A_2/prostaglandin H_2 receptor agonist, caused a clear vasoconstriction in both arteries. In contrast, L-NMMA caused a constriction only in the basilar artery, but not in the mesenteric artery. Fig. 1B shows concentration–response curves to L-NMMA in the isolated basilar, middle cerebral, coronary, mesenteric, renal and femoral arteries. L-NMMA (10^{-7} to 3×10^{-4} M) caused a potent constriction in the isolated basilar and middle cerebral arteries in a concentration-dependent manner. The constriction at 3×10^{-4} M L-NMMA was comparable to the 40 mM KCl-induced contraction. In other peripheral arteries, the L-NMMA-induced constriction was very weak (in coronary artery) or almost not seen (in mesenteric, renal and femoral artery).

Pretreatment with L-arginine (10^{-3} M) suppressed the L-NMMA-induced constriction of the basilar artery (non-treated vs. L-arginine-treated, $P<0.01$ at 3×10^{-4} M L-NMMA, $n=6$). However, pretreatment with D-arginine (10^{-3} M) did not suppress the L-NMMA-induced constriction (data not shown).

Fig. 2 shows the influence of pretreatment with diltiazem and nicardipine on concentration–response curves to L-NMMA in the isolated basilar artery. Both diltiazem (10^{-7} to 10^{-5} M) and nicardipine (10^{-9} to 10^{-7} M) caused a decrease in basal tension and suppressed the L-NMMA-induced constriction in a concentration-dependent manner.

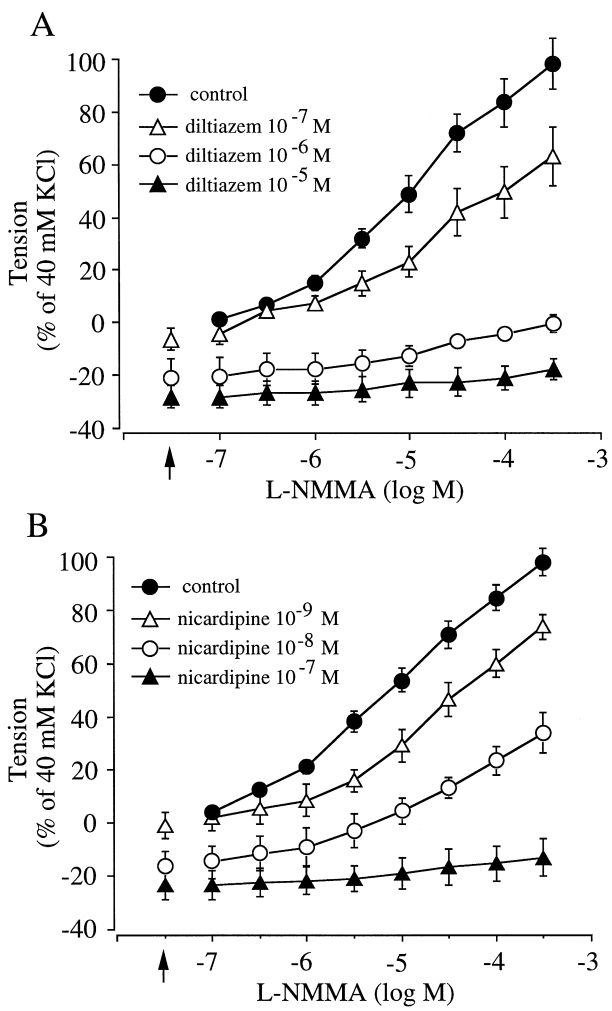

Fig. 2. Concentration–response curves for L-NMMA in isolated basilar arteries in the presence and absence of diltiazem or nicardipine. After the contractile response to 40 mM KCl was checked, L-NMMA (10^{-7} to 3×10^{-4} M) was added to the preparations cumulatively in the presence and absence of diltiazem (A, 10^{-7} to 10^{-5} M) or nicardipine (B, 10^{-9} to 10^{-7} M). Data are shown as means ± S.E.M. for six preparations, and are expressed as percents of maximal contraction induced by 40 mM KCl. Arrows indicate changes in basal tension after treatment with diltiazem or nicardipine.

Fig. 3 shows the change in cGMP levels on treatment with L-NMMA in ring segments of basilar, coronary, mesenteric, renal and femoral arteries. The treatment with L-NMMA (10^{-4} M, for 10 min) caused a 53% decrease in cGMP levels in the basilar artery. The decrease was, however, not significant as compared with that in other arteries (basilar:52.9 ± 10.3%, coronary:56.7 ± 10.3%, mesentery:46.2 ± 12.8%, renal:50.9 ± 20.7%, femoral:75.9 ± 10.3% of that in the respective non-treated preparations, $n = 4$). Treatment with diltiazem (10^{-5} M, for 20 min) did not influence the cGMP levels of the basilar artery in the presence or absence of L-NMMA (10^{-4} M) (data not shown). Treatment with charybdotoxin (10^{-7} M, for 10 min) did not change the cGMP levels of the basilar artery (non-treated:4.6 ± 0.7%, charybdotoxin-treated: 4.9 ± 0.6%, $n = 5$).

Fig. 4 shows concentration–response curves for charybdotoxin in the isolated basilar, middle cerebral, coronary, mesenteric, renal, and femoral arteries. Charybdotoxin (10^{-10} to 10^{-7} M) caused a similar heterogenous vasoconstriction in response to L-NMMA: potent vasoconstriction in basilar and middle cerebral arteries, and weak or no vasoconstrictor responses in the other peripheral arteries.

Fig. 5 shows typical tracings of changes in tension induced by treatment with L-NMMA and charybdotoxin in the isolated mesenteric artery precontracted with 20 mM KCl. Neither charybdotoxin (10^{-7} M) nor L-NMMA (10^{-4} M) influenced basal tension. However, both agents caused an additional constriction in these polarized mesenteric artery.

Fig. 6 shows a typical tracing indicating the vasoconstrictor response to ODQ, a selective guanylate cyclase inhibitor, in the endothelium-denuded basilar artery in the presence of a NO donor (sodium nitroprusside). The treatment with sodium nitroprusside (10^{-7} M) caused a slight decrease in basal tension, and ODQ (10^{-5} M) caused a vasoconstriction (0.41 ± 0.08 g, $n = 5$) in the presence of the NO donor. Such changes in tension were not seen in

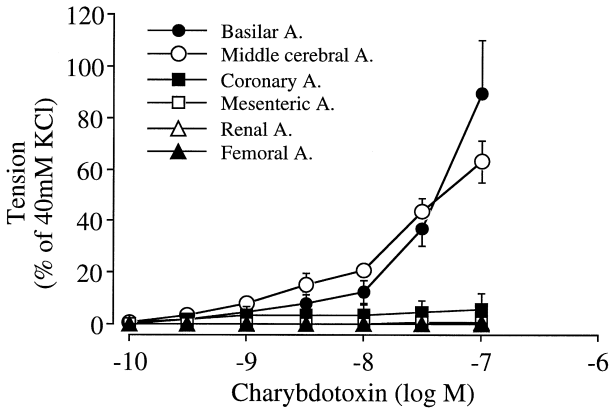

Fig. 4. Concentration–response curves for charybdotoxin in isolated basilar, middle cerebral, coronary, mesenteric, renal and femoral arteries. After the contractile response to 40 mM KCl was checked, charybdotoxin (10^{-10} to 3×10^{-7} M) was added to preparations cumulatively. KCl-induced contractions were as follows; basilar:2.14±0.45 g, middle cerebral:1.08±0.37 g, coronary:3.11±0.68 g, mesenteric:3.36±0.67 g, renal:4.64±0.62 g, femoral: 3.79±0.59 g. Data are shown as means± S.E.M. for five preparations, and are expressed as percents of maximal contractions induced by 40 mM KCl.

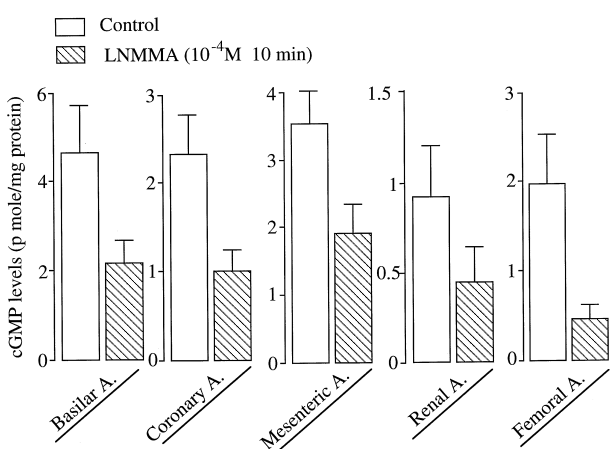

Fig. 3. Bar graphs showing cGMP levels in isolated arterial rings of basilar, coronary, mesenteric, renal and femoral arterial rings in the presence and absence of L-NMMA. After a 90-min equilibration, L-NMMA (10^{-4} M) was added to preparations and incubated for 10 min, then the preparation was frozen immediately with aqueous N_2. Cyclic GMP was measured with a commercially available assay kit (see Section 2). Data are shown as means±S.E.M. for four preparations.

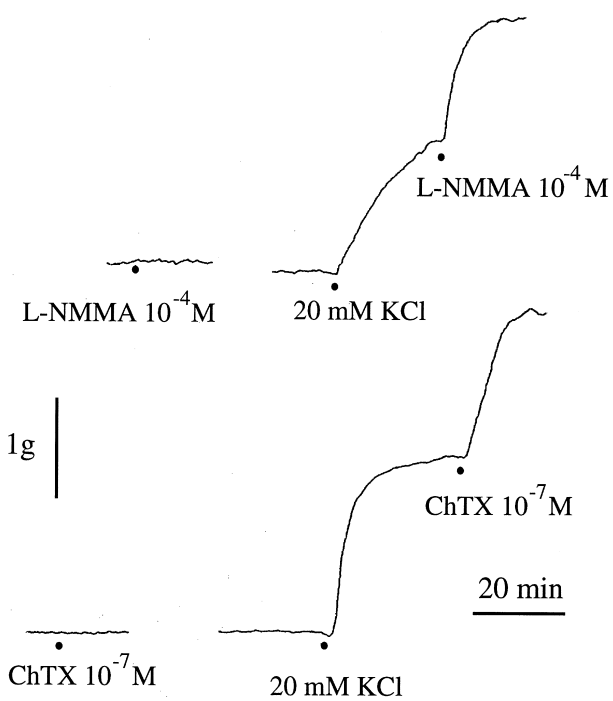

Fig. 5. Typical tracings showing the appearance of vasoconstriction induced by L-MNNA and charybdotoxin in moderately depolarized mesenteric arteries. During a stable contraction with 20 mM KCl, L-NMMA (10^{-4} M) or charybdotoxin (10^{-7} M) was added to preparations.

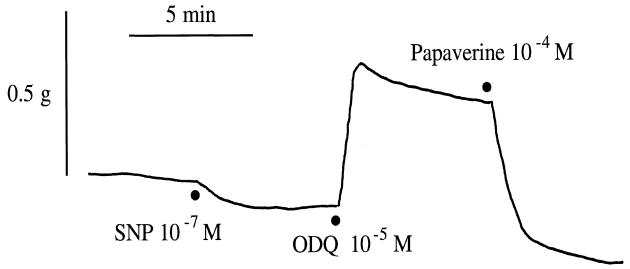

Fig. 6. A typical tracing showing vasoconstriction with 1*H*-[1,2,4]-oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a selective guanylate cyclase inhibitor, in the isolated canine endothelium-denuded basilar artery in the presence of sodium nitroprusside (SNP). ODQ (10^{-5} M) was added to the preparation 5 min after SNP (10^{-7} M).

the endothelium-denuded mesenteric artery (data not shown).

4. Discussion

In this study, we found that L-NMMA induced heterogenous vasoconstriction in isolated canine arteries, and the vasoconstriction appeared to result from blockade of the conversion from L-arginine to NO. To our knowledge, the mechanism of the L-NMMA-induced heterogenous vasoconstriction has not been elucidated. As for the cause of the heterogeneity, two possibilities were considered: (1) L-NMMA selectively inhibited basal NO release in cerebral artery, or (2) cerebrovascular smooth muscles were sensitive to changes in NO/cGMP levels. In order to study the former possibility, we measured cGMP levels in the vasculature as an index of NO release (Ignarro, 1990; Knowles and Moncada, 1994) The result showed that the decrease in cGMP levels caused by L-NMMA was not prominent in the basilar artery compared with that in other peripheral arteries, implying that the heterogeneous vasoconstriction was not explainable by the difference in NO release. Therefore, the former possibility was eliminated. The latter possibility appears likely, based on our additional experiments.

Intracellular Ca^{2+} is an important determinant for vasoconstriction. In particular, the L-type voltage-dependent Ca^{2+} channel (VDCC) plays a major role for Ca^{2+} influx into vascular smooth muscle cells. Therefore, we examined the influence of dilbiazem and nicardipine, typical L-type VDCC blockers, on this vasoconstriction. Interestingly, both diltiazem and nicardipine inhibited the L-NMMA-induced vasoconstriction at a range from 10^{-7} to 10^{-5} M and 10^{-9} to 10^{-7} M, respectively, concentrations which were enough to inhibit KCl-induced contraction of the cerebral artery (Julou-Schaeffer and Freslon, 1987; Kikkawa et al., 1988). In addition, a high concentration of diltiazem (10^{-5} M) did not show a significant effect on cGMP levels in the cerebral artery. These results suggested that diltiazem inhibited the L-NMMA-induced vasoconstriction as a L-type VDCC blocker, and that this vasoconstriction was accompanied by the activation of L-type VDCC as well as a decrease in NO/cGMP levels. Thus, we thought that the Ca^{2+}-activated K^+ (BK_{Ca}) channel was a plausible candidate to combine the decrease in NO/cGMP levels with the activation of L-type VDCC. BK_{Ca} channels on vascular smooth muscle cells have been reported to play a key role in maintaining resting vascular tone (Brayden and Nelson, 1992; Nelson, 1993; Nelson and Quayle, 1995) and their activity is potentiated by NO/cGMP (Robertson and Schubert, 1993; Bolotina et al., 1994; Archer et al., 1994) as well as Ca^{2+}. Therefore, we assumed that the decrease in NO/cGMP levels inhibited BK_{Ca} channel activity, and that the resulting depolarization caused vasoconstriction by the activation of L-type VDCC. Why L-NMMA caused a potent vasoconstriction only in the cerebral artery can be explained as follows. The cerebrovascular smooth muscles are reported to be more depolarized than peripheral arteries (Fujiwara et al., 1982; Suzuki and Fujiwara, 1982), and the BK_{Ca} channel is likely to be more activated as counteraction for the myogenic tone increased by a Ca^{2+} influx (Asano et al., 1993). Under these conditions, the decrease in NO/cGMP levels would cause vasoconstriction through the inhibition of already-activated BK_{Ca} channels. In order to confirm this notion, we carried out three sets of experiments. First, we examined the effect of charybdotoxin, a selective BK_{Ca} channel blocker, on resting tension, and we observed that charybdotoxin induced a heterogenous vasoconstriction similar to that with L-NMMA. The charybdotoxin-induced heterogenous response has been reported for isolated canine basilar and mesenteric arteries (Asano et al., 1993), and it is concluded that L-type VDCC is more active in the resting state of the basilar artery and that the myogenic tone was maintained by charybdotoxin-sensitive K_{Ca} channels in the experiment on transmembrane ^{86}Rb efflux. The conclusion is consistent with our notion. Second, we examined whether both L-NMMA and charybdotoxin caused vasoconstriction in the mesenteric artery when depolarized with 20 mM KCl because the moderate depolarization could produce in mesenteric artery a condition similar to that in cerebral artery: an increase in the transmembrane Ca^{2+} influx and more activated BK_{Ca} channel. As speculated, both charybdotoxin and L-NMMA caused additional vasoconstriction in the moderately depolarized mesenteric artery. Third, we checked the vasoconstriction response to ODQ, a selective guanylate cyclase inhibitor, in the endothelium-denuded vessels in the presence of a NO donor (sodium nitroprusside), because these conditions could mimic the influence of basal NO release from the endothelium on the responsiveness of the vascular smooth muscle to the removal of NO. In this experiment, ODQ induced vasoconstriction in the endothelium-denuded basilar artery, but not in the endothelium-denuded mesenteric artery. Thus, results of these three experiments supported our notion that L-NMMA-induced heterogenous vasoconstric-

tion was due to the different sensitivities of vascular smooth muscles to NO/cGMP levels, and the heterogeneity would contribute to the difference in the basal state of ion channels, including the L-type VDCC and BK_{Ca} channel.

In this study, we did not consider any factors other than NO/cGMP, BK_{Ca} channel and L-type VDCC to explain the L-NMMA-induced vasoconstriction. Recently, it has been reported that some vessels can produce the vasoconstrictor, 20-hydroeicosatetraenoic acid (20-HETE), production of which is inhibited by NO (Harder et al., 1994). More recent studies indicated that 20-HETE not only inhibits BK_{Ca} channel activity (Zou et al., 1996; Harder et al., 1997) but also enhanced L-type VDCC (Harder et al., 1997; Gebremedhin et al., 1998) in vascular smooth muscles. As 17-octadecynoic acid (17-ODYA) is an inhibitor of the formation of 20-HETE (Harder et al., 1994; Sun et al., 1998), we tested the effect of 17-ODYA on L-NMMA-induced vasoconstriction in a preliminary experiment. The treatment with 17-ODYA did not affect the L-NMMA-induced vasoconstriction of basilar artery (personal communication). Therefore, 20-HETE would not be related to the L-NMMA-induced heterogenous vasoconstriction.

As to the other possible mechanisms underlying the heterogeneity, it may be necessary to consider involvement of nitrergic nerves, which are predominant in cerebral arteries compared to peripheral vessels (Nozaki et al., 1993; Iadecola et al., 1993; Toda and Okamura, 1996). Since L-NMMA is a non-selective NOS inhibitor (Cosentino et al., 1993; Faraci et al., 1996), the treatment with L-NMMA may suppress the basal release of NO from the nitrergic nerves. Further study of nitrergic nerves will be required for characterization of the heterogeneity.

Our hypothesis is based on evidence that there are BK_{Ca} channels on vascular smooth muscles and that this channel activity is blocked by charybdotoxin (Vazquez et al., 1989; Asano et al., 1993). Recent studies have shown that BK_{Ca} channels are present in some endothelial cells (Baron et al., 1997; Haburcak et al., 1997; Kestler et al., 1998; Wiecha et al., 1998; Jow et al., 1999) and may be coupled to NO synthesis. In the present study, we measured cGMP levels in the basilar artery in the presence and absence of charybdotoxin. The treatment with charybdotoxin did not change cGMP levels. This result suggested that the effect of charybdotoxin was not attributable to blockade of endothelial BK_{ca} channels in the isolated basilar artery. Further study of the mimicking by charybdotoxin of the L-NMMA effect will be also required in order to validate our hypothesis.

In summary, we confirmed that L-NMMA induced heterogenous vasoconstriction in isolated canine cerebral arteries. We proposed that the heterogenous vasoconstriction was due to the different sensitivities of vascular smooth muscles to changes in NO/cGMP levels, and may result from the difference in the basal state of ion channels such as L-type VDCC and BK_{Ca} channel in vascular smooth muscles.

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