Endothelins: vasoconstrictor effects and localization in canine cerebral arteries

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- 1 The vascular effects of endothelin and localization of endothelin-like immunoreactivity were characterized in isolated cerebral arteries of dogs.
- 2 Endothelin-like immunoreactivity was detected in a few populations of endothelial cells of dog basilar artery.
- 3 Endothelin-1, endothelin-2 and endothelin-3 contracted isolated ring preparations of cerebral arteries in a dose-dependent manner independently of the presence of endothelium. The ED₅₀ values (and 95% confidence intervals) for the contraction were 411 pM (242–697 pM) and 478 pM (295–776 pM) for endothelin-1 and endothelin-2, respectively. Endothelin-3 induced vascular contraction at a higher concentration (ED₅₀ = 26.5 nM, 95% confidence interval = 15.7–45.7 nM).
- 4 The increases in tone induced by endothelin-1 and endothelin-2 did not return to the resting level after repeated washings, while a rinse with Krebs solution reversed the vasoconstrictor response to endothelin-3. The endothelins did not cause any vasodilator response in arteries precontracted with uridine 5'-triphosphate even in the presence of intact endothelial cells.
- 5 NiCl₂ (1 mM) attenuated the contractions induced by endothelin-3 (10–300 nM) and those to relatively low doses (1 nM) but not higher doses (10–100 nM) of endothelin-1 and endothelin-2. The contractions in response to endothelin-1, endothelin-2 and endothelin-3 were greatly attenuated in Ca²⁺-free solutions although high concentrations of endothelin-1 and endothelin-2 still evoked contractions.
- 6 These results suggest that the vasoconstriction induced by endothelin-3 and lower doses of endothelin-1 and endothelin-2, largely depends on the influx of Ca²⁺ ions. The apparent insensitivity to Ni²⁺ shows that additional distinct mechanisms also operate in the vasoconstrictor responses to high concentrations of endothelin-1 and endothelin-2.
- 7 The presence of endothelin-like immunoreactivity in endothelial cells suggests that endothelin is a potential endogenous spasmogen.

Keywords: Dog cerebral artery; endothelin; immunohistochemistry; nicardipine; nickel; vasoconstriction

Introduction

Cultured vascular endothelial cells produce endothelin, a vasoconstrictor peptide with 21 amino acid residues (Yanagisawa *et al.*, 1988). Analysis of genomic DNA has shown the presence of three members of an endothelin family, endothelin-1, endothelin-2 and endothelin-3, in mammals including man (Inoue *et al.*, 1989a; Yanagisawa & Masaki, 1989). Endothelin-3 is a less potent constrictor of porcine coronary arteries *in vitro*, while endothelin-2 is equally or more potent than endothelin-1 (Inoue *et al.*, 1989a). Although quantitative differences in the vasoconstrictor effects of these endothelins have been shown in porcine coronary arteries, it is not known whether or not there is a qualitative difference in the vasoconstrictor mechanism of these peptides.

Endothelin-1 causes a direct and sustained vasoconstriction of cerebral arteries from cats and dogs *in vitro* and *in vivo* (Saito *et al.*, 1989b; Mima *et al.*, 1989; Asano *et al.*, 1989). Although endothelin-1 constricts various vascular tissues, there seems to be a considerable difference in the pharmacological effects of endothelin-1 among the tissues. For example, the vasoconstrictor responses induced by endothelin-1 are reportedly sensitive to nicardipine, a Ca²⁺-channel blocker, in porcine coronary and cat cerebral arteries but not in the aortae of rats and rabbits (Kasuya *et al.*, 1989a; Saito *et al.*, 1989b; Sakata *et al.*, 1989; Ohlstein *et al.*, 1989). Endothelin-1 also evokes an endothelium-dependent vasodilator response

in rat aorta and mesenteric arteries (Sakata *et al.*, 1989; De Nucci *et al.*, 1988) but not in cat cerebral arteries.

Recently the presence of endothelin-1 in both plasma and cerebrospinal fluid was demonstrated in patients with subarachnoid haemorrhage (Masaoka *et al.*, 1989; Fujimori *et al.*, 1990). In the present study, we have characterized the cerebrovascular effects of the endothelin family and investigated the localization of endothelin by an immunohistochemical method in dog cerebral arteries.

Methods

Mongrel dogs of either sex (11–19 kg) were anaesthetized with sodium pentobarbitone (Nembutal; Abbot, North Chicago, U.S.A.; 60 mg kg⁻¹, i.v.) and exsanguinated. The brains were removed, then the middle cerebral and basilar arteries were dissected out and placed in a Krebs solution. The data obtained from these arteries were pooled since there is no difference in the responses of these arteries to agonists (Toda, 1990). The composition of the Krebs solution was (mM): NaCl 113, KCl 4.8, CaCl₂ 2.2, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25 and glucose 5.5. A high K⁺ (100 mM) Krebs solution was prepared by replacing NaCl with equimolar amounts of KCl. A Ca²⁺-free Krebs solution was prepared by omitting CaCl₂ from the Krebs solution. An 0.1 M stock solution of ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetra-acetic acid (EGTA) was prepared with distilled water and the pH of this solution was adjusted to 7.4; in some experiments, EGTA was

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added to the Ca^{2+} -free Krebs solution to give a concentration of $200\ \mu\text{M}$.

A stainless rod and a short piece of tungsten wire were carefully passed through the lumen of ring segments (4 mm long) of the arteries which were then mounted in tissue baths containing 30 ml Krebs solution. The tungsten wire was anchored to a plastic gate and the steel rod was connected to a transducer (Nihon Kohden TB 612T) for isometric recordings of changes in the contractile force. The organ baths were siliconized before the experiments to prevent nonspecific adhesion of the peptides. The Krebs solutions were maintained at 37°C and bubbled with a gas mixture of 95% O_2 and 5% CO_2 throughout the experiment. The resting tension of the tissue was adjusted to 1.0 g. At the beginning of the experiment, the vasoconstrictor response to 100 mM KCl was obtained in each tissue so that the vasoconstrictor responses to the endothelins could be expressed as a % of K-induced contractions. In some tissues uridine 5'-triphosphate (UTP; $30\text{--}100\ \mu\text{M}$) was applied to gain an active tone of 0.5 to 1.0 g.

Full dose-response curves were constructed by cumulative addition of the endothelin peptides. In some preparations the endothelium was removed by rubbing the intimal surface with a scored needle (Saito *et al.*, 1989a). The functional absence of endothelium was confirmed by the lack of an endothelium-dependent vasodilator response to substance P. From the ED_{50} values of the preparations the geometric mean and the 95% confidence interval were obtained (Fleming *et al.*, 1972).

For immunohistochemical experiments, basilar arteries were fixed with formaldehyde and picric acid, dehydrated with ethanol, cleared with xylene, rehydrated and then frozen (Saito *et al.*, 1989a). Transverse sections ($10\ \mu\text{m}$) of the arteries were obtained by cutting with a cryostat. Monoclonal antibodies (KM 565; Goto *et al.*, 1990) were applied (1 : 2000) and then the avidin-biotin peroxidase complex (ABC) method (Hsu *et al.*, 1981) was used to demonstrate the localization of endothelin-like immunoreactivity. The immunoreaction was observed under a microscope (Axioskop; Carl Zeiss; Oberkochen, Germany). In the same sections, the endothelium was identified as a monolayer of cells covering the inner surface of the internal elastic lamina of the artery (Lüscher, 1988) by use of a differential interference contrast. The monoclonal antibodies used in the present study cross-react with endothelin-1, endothelin-2, endothelin-3 and porcine big endothelin-1. Since

the antibodies might have cross-reacted with unknown substances, the immunoreactive material was termed "endothelin-like" in the present study.

The drugs used were endothelin-1, endothelin-2 and endothelin-3 (Peptide Institute; Osaka, Japan), EGTA (Dojin; Kumamoto, Japan), nicardipine (Sigma; St Louis, U.S.A.), nickel chloride (Wako; Osaka, Japan), substance P (Peptide Institute) and UTP (Sigma). The endothelins were prepared in a phosphate buffered saline (pH 7.4) containing 0.05% bovine serum albumin. The statistical analysis was carried out by Dunnett's test using a computer package (YUKUMUS Statistic Library; Yukumus; Kawasaki, Japan).

Results

Endothelin-1 and endothelin-2 caused slowly developing and dose-dependent vasoconstrictor responses at doses from $20\ \text{pM}$ – $20\ \text{nM}$. Repeated washings of the tissue with fresh Krebs solution did not reduce the tone induced in the arteries by these endothelins (Figure 1). In the presence of tone induced by UTP, additional vasoconstrictor responses occurred in response to relatively high concentrations of endothelins. In these precontracted tissues, substance P induced a vasodilator response (Figure 2).

Endothelin-3 also caused a vasoconstrictor response. In contrast to the responses caused by endothelin-1 and endothelin-2, the endothelin-3-induced vasoconstriction was not sustained after rinsing the tissue with fresh Krebs solution (Figure 1). Endothelin-3 did not cause a vasodilator response in the presence of UTP-induced tone (Figure 2).

Figure 3 shows the cumulative dose-response curves in endothelium-intact and endothelium-free preparations; the ED_{50} values and the maximum responses induced by the endothelins are shown in Table 1. The vasoconstrictor effects did not differ between endothelium-intact and endothelium-free preparations. There was no difference in the ED_{50} values between endothelin-1 and endothelin-2. The ED_{50} value for endothelin-3 was about 60 times greater than those for endothelin-1 and endothelin-2. Endothelin-1 and endothelin-3 induced similar maximum responses, but endothelin-2 evoked a significantly larger maximum response than those given by endothelin-1 and endothelin-3.

Figure 1 Typical vasoconstrictor responses to endothelin-1 (a), endothelin-2 (b) and endothelin-3 (c). Washing with fresh Krebs solution (W) did not reduce the increased tone by endothelin-1 or endothelin-2. The increased tone induced by endothelin-3 returned to a resting level by repeated washing with Krebs solution. The numbers in the figure indicate the negative logarithm of the peptide concentration. Each trace was obtained from separate preparations.

Figure 2 The effect of endothelins on precontracted dog cerebral arteries. Endothelin-1 (a), endothelin-2 (b) and endothelin-3 (c) did not cause any vasodilator responses in tissues precontracted with uridine 5'-triphosphate (UTP). In all these tissues substance P (SP) caused a vasodilator response. The numbers in the figure indicate the negative logarithm of the concentration of the peptides.

Addition of 1 mM NiCl_2 caused a slight vasodilatation and then a slight vasoconstriction in some tissues (Figure 4). NiCl_2 suppressed the vasoconstrictions induced by 1 nM but not by 10 or 100 nM of either endothelin-1 or endothelin-2 (Figure 4 and Figure 5). The vasoconstrictor response to endothelin-3

Figure 3 Dose-response curves to the cumulative addition of endothelin-1 (○), endothelin-2 (●) and endothelin-3 (▲) in endothelium-intact (a) and endothelium-free (b) preparations. The vasoconstrictor response was expressed as a percentage of the contraction induced by 100 mM KCl. Each point and bar represents the mean and s.e.mean. At some points the bars are within the symbol. In the endothelium-intact preparations the number of tissues (*n*) and number of animals (*N*) were 9 and 6, respectively, for all the peptides. In endothelium-denuded preparations, (*n*, *N*) were (7, 4), (8, 5) and (5, 4) for endothelin-1, endothelin-2 and endothelin-3, respectively.

(10–300 nM) was profoundly attenuated by 1 mM NiCl_2 . Nicardipine (10 nM) also caused a vasodilator response in some arterial preparations. The vasoconstrictor responses induced by the three endothelins were similarly reduced by nicardipine (Figure 5).

Removal of Ca^{2+} ions also caused a vasodilatation from

Table 1 ED_{50} values and the maximum responses for endothelins in dog isolated cerebral arteries

	ED_{50} (nM) (geometric mean and 95% confidence interval)	Maximum response (% of K-induced contraction) mean $\pm$ s.e.mean	<i>n</i> , <i>N</i>
<i>Endothelium-intact</i>			
Endothelin-1	0.411 0.242–0.697	74.4 $\pm$ 2.8	9, 6
Endothelin-2	0.478 0.295–0.776	95.5 $\pm$ 4.2 ^c	9, 6
Endothelin-3	26.5 ^{ab} 15.7–45.7	62.6 $\pm$ 8.7 ^d	9, 6
<i>Endothelium-removed</i>			
Endothelin-1	0.741 0.468–1.17	88.5 $\pm$ 9.5	7, 4
Endothelin-2	0.513 0.331–0.812	117.7 $\pm$ 4.4 ^c	8, 5
Endothelin-3	35.8 22.9–56.2	75.6 $\pm$ 5.7 ^d	5, 4

n, *N*: number of tissues and number of animals.

^a Significant difference between the ED_{50} for endothelin-1 and endothelin-3 ($P < 0.01$).

^b Significant difference between the ED_{50} for endothelin-2 and endothelin-3 ($P < 0.01$).

^c Significant difference between the maximum response to endothelin-1 and endothelin-2 ($P < 0.05$).

^d Significant difference between the maximum response to endothelin-2 and endothelin-3 ($P < 0.01$).

There were no significant differences ($P > 0.05$) between the ED_{50} s for endothelin-1 and endothelin-2 and the maximum responses to endothelin-1 and endothelin-3.

Figure 4 Representative tracings of the effects of 1 mM NiCl_2 , 10 nM nicardipine and Ca^{2+} -removal (no EGTA) on the vasoconstrictor effects of endothelin peptides. Three doses (1, 10 and 100 nM for both endothelin-1 and endothelin-2, and 10, 100 and 300 nM for endothelin-3) were examined. The numbers in the figure indicate the negative logarithm of the concentration of the peptide.

Figure 5 Effects of various treatments on the vasoconstrictor responses induced by endothelin-1, endothelin-2 and endothelin-3. Each point and bar represents the mean and the s.e.mean. Number of tissues (*n*) and number of animals (*N*) for control (○), 1 mM NiCl_2 (●), 10 nM nicardipine (□), Ca^{2+} -removal without EGTA (△) and Ca^{2+} -removal with 0.2 mM EGTA (▲) were as follows (*n*, *N*): endothelin-1 (7, 4), (7, 4), (14, 8), (6, 4), (8, 5); endothelin-2 (7, 4), (8, 5), (6, 3), (6, 4), (6, 3) and endothelin-3 (9, 6), (7, 4), (8, 5), (6, 4), (6, 3). *Significantly different from the control ($P < 0.05$). **Significantly different from control ($P < 0.01$).

the resting state (Figure 4). In Ca^{2+} -free solutions, the contraction to endothelin-3 was abolished whereas the endothelin-1- and endothelin-2-induced contractions were reduced, but not abolished. Addition of EGTA (0.2 mM) to Ca^{2+} -free solutions further suppressed the endothelin-1- and endothelin-2-induced vasoconstrictions (Figure 5).

In 8 out of 9 tissues (from 6 animals), endothelin-like immunoreactivity was found to be present in endothelial cells of the basilar arteries (Figure 6). Even in these positive tissues only a few cells in each section exhibited an endothelin-like immunoreactivity. The immunoreaction was not observed when the antibodies were preincubated with 10 μM endothelin-1 overnight.

Discussion

Endothelin-1 causes potent and long-lasting vasoconstriction of cerebral arteries of cats and dogs *in vitro* (Saito *et al.*, 1989b; Asano *et al.*, 1989). The present study showed that not only endothelin-1 but also endothelin-2 and endothelin-3 were potent vasoconstrictors of dog cerebral arteries. Judging from the ED_{50} values, endothelin-2 was as potent as endothelin-1; however, endothelin-3 was 60 times less potent than endothelin-1. Although the maximum contractile responses induced by endothelin-1 and endothelin-3 were not significantly different, endothelin-2 caused a larger maximum response than endothelin-1. The orders of the potency of, and the maximum responses to, the endothelins were similar to those observed in the porcine coronary artery (Inoue *et al.*, 1989a). The vasoconstrictions induced by endothelin-1 and endothelin-2 were not easily reversed by removing the peptide from the bathing solution. The long-lasting effect of endothelin-1 may be due to its low dissociation rate from the binding sites (Hirata *et al.*, 1988). In contrast, the elevated tone of the artery induced by endothelin-3 returned to the

Figure 6 Endothelin-like immunoreactivity (arrow) in endothelial cells of the basilar artery of the dog. Bar equals 10 μ m. IEL: internal elastic lamina; SM: smooth muscle.

resting level upon washing with a fresh solution. Since endothelin-3 is less hydrophobic than endothelin-1 and endothelin-2, endothelin-3 may dissociate from its receptor more easily than endothelin-1 and endothelin-2.

Although endothelins evoke endothelium-dependent vasodilatation in some peripheral vascular tissues (De Nucci *et al.*, 1988; Sakata *et al.*, 1989; Warner *et al.*, 1989; Douglas & Hiley, 1990), a vasodilator response was not evoked by the endothelins in dog cerebral arteries. Acetylcholine, arachidonic acid and angiotensins induce vasoconstriction by releasing a vasoconstrictor substance from endothelial cells in dog cerebral arteries (Shirahase *et al.*, 1987; Manabe *et al.*, 1989). However, the vasoconstrictor actions of the endothelin were independent of the presence of endothelial cells in the present study. Thus, in dog cerebral arteries all these endothelins cause strong vasoconstriction by acting directly on the smooth muscle rather than indirectly by acting on endothelial cells. Further, it is suggested that endothelins do not release endothelium-derived relaxing factor in cerebral arteries.

Endothelin-1 seems to cause vasoconstriction by both increasing the influx of Ca^{2+} and also releasing Ca^{2+} from intracellular store sites in coronary arteries of pigs (Kasuya *et al.*, 1989a,b). Although the vasoconstrictor responses induced by the endothelins were profoundly attenuated, small but consistent vasoconstrictor responses were evoked by endothelin-1 and endothelin-2 in Ca^{2+} -free solutions in dog cerebral arteries. This suggests that the endothelins cause vasoconstrictor responses by an extracellular Ca^{2+} -dependent mechanism and that endothelin-1 and endothelin-2 may act also by a mechanism which is independent of extracellular Ca^{2+} . Since the removal of Ca^{2+} from the medium, especially in the presence of EGTA, may reduce the vasoconstriction induced by not only Ca^{2+} -influx but also mobilization of intracellular Ca^{2+} (Ohlstein *et al.*, 1989), the effects of the blockade of Ca^{2+} -influx by Ni^{2+} and of nicardipine, an organic Ca^{2+} -channel blocker, were further examined in the present study.

The vasoconstrictor responses induced by endothelin-3 and those caused by low (1 nM), but not those by high (10–100 nM), doses of endothelin-1 and endothelin-2 were sensitive to Ni^{2+} . The pharmacological effects of Ni^{2+} on membrane Ca^{2+} -channels are not known in dog cerebral arteries. However, Ni^{2+} suppresses the Ca^{2+} -influx through channels which are distinct from L-type Ca^{2+} -channels in the smooth muscle of the aorta (Lawson & Caverio, 1989). Accordingly, it appears that the vasoconstrictor responses induced by either endothelin-3 or low doses of endothelin-1 and endothelin-2 are mediated by the influx of Ca^{2+} into the smooth muscle cells through the Ni^{2+} -sensitive pathway.

Organic Ca^{2+} -channel blockers inhibit the endothelin-1-induced vasoconstrictor responses of porcine coronary and cat cerebral arteries but not of aorta or mesenteric arteries of rats and rabbits (Kasuya *et al.*, 1989a; Saito *et al.*, 1989b; Ohlstein *et al.*, 1989; D'Orleans-Juste *et al.*, 1989). In the present study, nicardipine attenuated the vasoconstrictor responses to all three endothelins to a similar extent. It has been shown that endothelin-1 causes its vasoconstrictor effects by the activation of L-type Ca^{2+} -channels in porcine coronary arteries (Yanagisawa *et al.*, 1988; Goto *et al.*, 1989). However, Ca^{2+} -channel blockers inhibit the vasoconstrictor responses induced by various vasoconstrictors in dog cerebral arteries by not only suppressing the influx of Ca^{2+} through Ca^{2+} -channels but also by depleting Ca^{2+} from intracellular stores (Asano *et al.*, 1987; Yamamoto *et al.*, 1983; Fujiwara *et al.*, 1982). In either case, the results suggest that Ca^{2+} -channel blockers may be useful in the relief of any cerebral vasoconstriction induced by endogenous endothelins.

The vasoconstrictor responses induced by high doses of endothelin-1 and endothelin-2 were entirely resistant to Ni^{2+} . Thus it may be suggested that the mode of Ca^{2+} -influx induced by high doses of endothelin-1 and endothelin-2 is different from that by low doses in dog cerebral arteries. However, endothelin-1, at doses above 1 nM induces contraction of vascular smooth muscles by mobilization of Ca^{2+} from intracellular stores both in porcine coronary arteries (Kasuya *et al.*, 1989b) and in the aortae of rats and rabbits (August *et al.*, 1988; Sakata *et al.*, 1989; Huang *et al.*, 1990; Ohlstein *et al.*, 1989). Therefore, it is likely that in dog cerebral arteries, endothelin-1 and endothelin-2 at high doses evoke vasoconstriction by an additional extracellular Ca^{2+} -independent mechanism.

The attenuation by Ni^{2+} of the vasoconstriction induced by endothelin-3, but not that caused by high doses of endothelin-1 or endothelin-2, indicates that the vasoconstrictor effect of endothelin-3 was not only quantitatively but also qualitatively distinct from those of endothelin-1 and endothelin-2. Recently, the presence of multiple binding sites for the endothelins has been shown in chicken cardiac membranes; a binding site for endothelin-1 and endothelin-2, and the other for endothelin-3 (Watanabe *et al.*, 1989). It is conceivable that these receptor subtypes couple to different intracellular messenger systems or channels. Therefore, high doses of endothelin-1 and endothelin-2 may activate distinct receptor subtypes from that for endothelin-3, although the vascular endothelin-binding sites are yet to be characterized.

The present study revealed the presence of endothelin-like immunoreactivity in a few endothelial cells of the cerebral arteries of dogs. Similar endothelin-like immunoreactivity is also present in neurones of the porcine spinal cord (Yoshizawa *et al.*, 1989). Unlike neuronal cells, secretory granules are not present in endothelial cells. Thus it has been supposed that endothelial cells release endothelin synthesized *de novo* (Yanagisawa & Masaki, 1989). Nevertheless, the demonstration of endothelin-like immunoreactivity suggests that some cells do retain a certain amount of an endothelin-like substance.

The antibodies used in the present study cross-reacted with endothelin-1, endothelin-2 and endothelin-3. Cultured endothelial cells from human umbilical vein produce mRNA for preproendothelin-1 but not preproendothelin-2 or

preproendothelin-3 (Inoue *et al.*, 1989b). Furthermore, cerebral microvessels of pigs also produce mRNA for preproendothelin-1 (Yoshimoto *et al.*, 1990). Although this observed endothelin-like substance is likely to be endothelin-1, it cannot be determined exactly which form of endothelin is present in endothelial cells of cerebral arteries. As shown in the present study, endothelin-1, as well as endothelin-2 and endothelin-3, causes a sustained vasoconstriction of cerebral arteries. Accordingly, endogenous endothelin produced in

endothelial cells, even if it is endothelin-2 or endothelin-3, may affect the tone of the underlying vascular smooth muscles.

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