Pharmacological Effect of Endothelin, an Endothelium-derived Vasoconstrictive Peptide, on Canine Basilar Arteries

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Abstract

The purpose of this study was to evaluate the constrictive effect of endothelin, a peptide vasoconstrictor derived from endothelium, on canine basilar artery. Constriction was measured by an isometric tension recording method. Endothelin induced prolonged contraction of canine basilar artery in a dose-dependent fashion, the estimated concentration at 50% contraction being $(2.1 \pm 0.5) \times 10^{-9}$ M (mean $\pm$ SEM). Removal of endothelium significantly augmented the arterial response to endothelin. In Ca^{++} -free medium no contractile response was elicited at 10^{-8} M endothelin. Papaverine (10^{-4} M) and nicardipine (10^{-8} M), a calcium channel blocker, almost completely inhibited the contraction induced by 10^{-8} M endothelin. Pretreatment with nicardipine (10^{-8} – 10^{-6} M) also significantly suppressed the response to endothelin. Acetylcholine (10^{-7} – 10^{-4} M), adenosine triphosphate (10^{-7} – 10^{-5} M), and thrombin (0.1 and 1.0 U/ml) dose-dependently inhibited contraction of canine basilar artery in response to 3×10^{-9} M endothelin. These results show that endothelin has a potent constrictive effect on canine basilar artery and suggest that this substance may play an important role in the pathogenesis of vasospasm following subarachnoid hemorrhage.

Key words: endothelin, subarachnoid hemorrhage, cerebral vasospasm, canine basilar artery

Introduction

In 1980, Furchgott and Zawadzki⁽¹⁴⁾ demonstrated the role of endothelium in the relaxation of isolated rabbit aorta in response to acetylcholine. The substance released from the endothelium to elicit relaxation was later termed "endothelium-derived relaxing factor" (EDRF).⁽⁵⁾ Since then, other investigators^(13,43) have confirmed endothelium-dependent vasodilatory responses to various agents in different preparations. One recently-identified EDRF is nitric oxide or a closely related substance.⁽³³⁾

In certain blood vessels, the presence of endothelium is also required for the contractile response to stimulation by various chemicals, such as norepinephrine,⁽⁹⁾ thrombin,⁽⁹⁾ acetylcholine,^(3,22,25) arachidonic acid,⁽²²⁾ serotonin,⁽²⁴⁾ neuropeptide Y,⁽⁸⁾ and calcium ionophore A23187.⁽²²⁾ Mechanical stretch-

ing,⁽²¹⁾ increased transmural pressure,⁽¹⁵⁾ and anoxia^(10,37) can also cause endothelium-dependent contractions. These findings suggest the existence of a diffusible vasoconstrictor substance, specifically, "endothelium-derived constricting factor" (EDCF). The EDCF candidates include prostanoids^(29,42) and peptides.^(16,32) Recently, Yanagisawa *et al.*⁽⁴⁵⁾ isolated a potent vasoconstrictive peptide, endothelin, from the culture supernatant of porcine aortic endothelium, determined its amino acid sequence, and showed that it is one of the most potent vasoconstrictors in the blood vessels of many mammals.

Subarachnoid hemorrhage (SAH) results in various degrees of endothelial damage to the cerebral arteries, including vacuolation and denudation of the endothelial cells,^(1,12,38,41) and it has been suggested that impairment of vasodilation due to endothelial damage may play an important role in the pathogenesis of vasospasm following SAH.^(23,26,30,34,39,44) Arterial wall prostacyclin, a powerful vasodilator, decreases progressively following SAH,^(26,39,44) and en-

endothelium-dependent vasodilation is also impaired following SAH.^{18,23,30,34)}

It is possible that endothelial damage caused by SAH also affects endothelium-dependent vasoconstriction. Production or release of endothelin might be stimulated in slightly injured endothelial cells following SAH. Permeation of endothelin into the smooth muscle layer is facilitated by disruption of the blood-arterial wall barrier following SAH,³⁹⁾ which could induce prolonged contraction of the major cerebral arteries. Therefore, investigation of the pathogenesis of vasospasm requires evaluation of the effect of endothelin on the major cerebral arteries. The aim of this investigation was to evaluate the vasoconstricting activity of endothelin in canine basilar arteries *in vitro*.

Materials and Methods

I. Preparation of arteries

Adult mongrel dogs of both sexes, weighing 9–14 kg, were anesthetized with sodium pentobarbital (30 mg/kg) and sacrificed by exsanguination from the femoral artery. These procedures were conducted in accordance with National Institute of Health guidelines for animal care and handling. The brain, with the basilar artery *in situ*, was removed and placed in a dissecting chamber filled with a modified Krebs bicarbonate solution (NaCl 120 mM, KCl 4.5 mM, MgSO₄ 1.0 mM, NaHCO₃ 27.0 mM, KH₂PO₄ 1.0 mM, CaCl₂ 2.5 mM, and dextrose 10.0 mM). The basilar artery was dissected free under magnification and ring segments 4 mm in length were prepared. The outer diameters of the basilar arteries were 0.8–1.2 mm. Each specimen was suspended between L-shaped stainless steel rods in a siliconized organ bath with a 10 ml working volume, which was bubbled with 95% O₂/5% CO₂. The pH of the solution ranged from 7.4 to 7.5. The preparations were allowed to equilibrate at 37°C for 90 minutes before use. The resting tension was adjusted to 1.0 gm. Contractile force was recorded isometrically with a force-displacement transducer (model WT-611T; Nihon Koden, Tokyo, Japan) and displayed on a chart recorder (model CDR-12A; Toa Electronics Ltd., Tokyo, Japan).

Each arterial ring was exposed to 40 mM KCl and, after confirmation of a consistent contractile response to KCl followed by repeated washings, experiments with endothelin were begun. The contractile response to endothelin was expressed as a percentage of the contraction elicited by 40 mM KCl. First, the concentration-response curve for endothelin was obtained for canine basilar arteries by cumulative

addition. For comparison, canine mesenteric and femoral arteries were also prepared for this experiment. Ring segments 4 mm in length were cut from both, the outer diameters being 1.3–1.8 mm for the mesenteric and 3.4–4.0 mm for the femoral arteries. The basal tension was adjusted to 2.5 gm for the mesenteric and 4.0 gm for the femoral arteries.

The second experiment was designed to determine whether or not the contractile response to endothelin is altered by the removal of endothelial cells. The basilar artery attached to the brainstem was cut in cross section, and one half was used for control studies with intact endothelium. The other half was de-endothelialized by the following procedure. A no. 30 needle connected to a gas supply delivering 95% O₂/5% CO₂ was introduced into one end of the basilar artery. A gentle stream of gas was passed through the lumen of the vessel for 10 minutes to produce a drying injury of the endothelium. The vascular lumen was then reperfused with Krebs solution containing 10⁻⁴ M papaverine to reverse the contraction induced by the above procedure. The specimen was placed in normal Krebs solution, and endothelial cells were removed by gentle rubbing with a polyethylene tube (PE20). The integrity of the endothelium was confirmed by relaxation in response to thrombin during contraction with 10⁻⁶ M prostaglandin F_{2α} (PGF_{2α}).

The third experiment was designed to evaluate the influence of extracellular calcium on endothelin-induced contraction of canine basilar artery. The preparations were exposed to a Ca⁺⁺-free medium containing 1 mM ethylene glycol-bis(β-aminoethyl ether)-N,N'-tetra-acetic acid (EGTA) for 15 minutes. Next, 10⁻⁸ M endothelin was added, and 10 minutes later 2.5 mM CaCl₂ was added. For comparison, the same experiment was conducted without endothelin, under Ca⁺⁺-free conditions.

In a fourth experiment, the effects of papaverine (10⁻⁵ and 10⁻⁴ M) and nicardipine (10⁻⁸ M), a calcium channel blocker, were evaluated with respect to the contraction induced by 10⁻⁸ M endothelin. Preparations were also exposed to nicardipine (10⁻⁸–10⁻⁶ M) for 15 minutes before the cumulative addition of endothelin.

Finally, the effects of vasoactive agents that induce endothelium-dependent relaxation were evaluated with respect to the contraction already induced by 3 × 10⁻⁹ M endothelin. Acetylcholine (10⁻⁷–10⁻⁴ M), adenosine triphosphate (ATP) (10⁻⁷–10⁻⁵ M), and thrombin (0.1 and 1.0 U/ml) were then added in a cumulative fashion. In the relaxation studies with ATP, the arterial rings were pretreated with 10⁻⁵ M 8-phenyltheophylline, an adenosine antago-

nist, 5 minutes before application of endothelin to exclude the effect of endothelium-independent relaxation by adenosine. The relaxation induced by these agents was expressed as a percentage of the contraction induced by 3×10^{-9} M endothelin.

II. Drugs

Synthetic human and porcine endothelin was generously provided by the Peptide Institute (Osaka, Japan). Acetylcholine, ATP, bovine thrombin, $\text{PGF}_{2\alpha}$, papaverine, EGTA, and 8-phenyltheophylline were obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Nicardipine was provided by Yamanouchi Pharmaceutical Co. (Tokyo, Japan). For preparation of stock solutions, endothelin was dissolved in Krebs solution, and all other drugs except nicardipine were dissolved in distilled water, then diluted in Krebs solution before use. Nicardipine was dissolved in Krebs solution each day before use.

III. Statistical analysis

The data are expressed as means $\pm$ SEM. The contractile responses at each concentration of endothelin was statistically compared with Student's *t*-test. A *p* value of <0.05 was considered statistically significant.

Results

I. Effects of endothelin on canine basilar, mesenteric, and femoral arteries

The contractile responses of the canine basilar, mesenteric, and femoral arteries to the standard KCl dose of 40 mM were 3.5 ± 0.3 , 3.9 ± 0.3 , and 5.7 ± 0.6 gm, respectively. Endothelin induced contractions of these three arteries in a dose-dependent fashion (Figs. 1 and 2). The contraction of the basilar artery induced by 10^{-8} M endothelin was long-lasting, as shown in Figs. 1 and 4A. After contracting maximally, the arterial ring gradually returned to the initial contractile level. The contractile response of the canine basilar artery to 10^{-8} M endothelin lasted for more than 1 hour ($n = 6$). The estimated concentration at 50% contraction (EC_{50}) for the basilar, mesenteric, and femoral arteries were $(2.1 \pm 0.5) \times 10^{-9}$, $(1.4 \pm 0.3) \times 10^{-9}$, and $(3.0 \pm 0.5) \times 10^{-9}$ M, respectively. There was no significant difference between these three EC_{50} values. The minimal concentration of endothelin that resulted in contraction of the basilar artery was 10^{-12} M; it was higher for the mesenteric and femoral arteries. The maximal contractile response of the basilar artery to endothelin was much larger than that induced by 40

Fig. 1 Representative tracing of the effect of endothelin (ET) on canine basilar artery. Endothelin (10^{-12} – 10^{-8} M) induced strong contraction in a dose-dependent fashion. After contracting maximally, the arterial ring gradually returned to the initial contractile level.

Fig. 2 Concentration-response curves to endothelin for canine basilar, mesenteric, and femoral arteries. The minimal concentration of endothelin that produced contraction of the basilar artery was 10^{-12} M. The maximal contractile response of the basilar artery to endothelin was not only much larger than that induced by 40 mM KCl, but also was larger than that of the mesenteric or femoral arteries. *n*: number of animals studied.

mM KCl. When the maximal endothelin-induced contractions of each of these arteries were compared with the contractions induced by the standard dose of KCl, the basilar artery showed greater contraction in response to endothelin than either the mesenteric or femoral artery. However, the maximal contractile responses of the basilar and mesenteric arteries were not significantly different.

II. Effect of removal of the endothelium on endothelin-induced contraction

Contractions to 40 mM KCl were not significantly

different between the preparations with and without endothelium. Removal of the endothelium significantly ($p < 0.05$) shifted the concentration-response curve to endothelin to the left in canine basilar artery (Fig. 3). In de-endothelialized arterial rings, endothelin at a concentration of 10^{-13} M produced contraction.

III. Effect of Ca^{++} -free medium on endothelin-induced contraction

Endothelin at a dose of 10^{-8} M induced potent contraction of canine basilar artery in normal Krebs solution (Fig. 4A). In a Ca^{++} -free medium containing 1 mM EGTA, no contraction of the basilar artery was

Fig. 3 Concentration-response curves to endothelin for canine basilar artery. The curve shifted significantly to the left after removal of endothelium. n: number of animals studied. * $p < 0.05$, ** $p < 0.01$ vs. with endothelium.

elicited by 10^{-8} M endothelin ($n = 6$) (Fig. 4B). The addition of 2.5 mM CaCl_2 , in the absence of endothelin, induced phasic contraction of the basilar artery (Fig. 4C). The arterial rings exposed to 10^{-8} M endothelin for 10 minutes in a Ca^{++} -free medium showed, upon re-addition of 2.5 mM CaCl_2 , larger contractions than those not exposed to endothelin ($n = 6$) (Fig. 4B).

IV. Effect of papaverine and nicardipine on endothelin-induced contraction

Papaverine (10^{-5} and 10^{-4} M) caused vasodilation of the canine basilar arteries in a dose-dependent fashion during contraction to 10^{-8} M endothelin (Fig. 5A, B). Nicardipine (10^{-8} M) also induced vasodilation of the basilar artery pre-contracted with 10^{-8} M endothelin (Fig. 5C). The inhibition by both papaverine and nicardipine of endothelin-induced contraction was nearly absolute. The inhibitory effect of papaverine was noted just after its addition (Fig. 5A). The action of nicardipine, however, was weaker, and it took more than 1 hour for the basilar artery to relax to the baseline value (Fig. 5C). The results were the same at higher doses of nicardipine (10^{-7} and 10^{-6} M) (data not shown). Pretreatment with nicardipine (10^{-8} – 10^{-6} M) significantly and dose-dependently inhibited endothelin-induced contraction of canine basilar artery (Fig. 6). Moderate contractions of the artery persisted at higher concentrations of endothelin.

V. Effects of acetylcholine, ATP, and thrombin on endothelin-induced contraction

Acetylcholine (10^{-7} – 10^{-4} M) induced only slight relaxation in canine basilar artery pre-contracted by 3×10^{-9} M endothelin, in a dose-dependent fashion

Fig. 4 Representative tracings of the contraction induced by 10^{-8} M endothelin (ET) in normal (A) and in a Ca^{++} -free medium containing 1 mM EGTA (B). No contractile response to endothelin was elicited in Ca^{++} -free medium. Addition of 2.5 mM CaCl_2 caused phasic contraction of the basilar artery without endothelin (C). Re-addition of 2.5 mM CaCl_2 induced larger contractions in the basilar arteries with 10^{-8} M endothelin than in those without endothelin. Pap: papaverine.

Fig. 5 Representative tracings of the effect of papaverine (Pap) (A) and nicardipine (C), a calcium channel blocker, on endothelin (ET)-induced contraction of the canine basilar artery, and concentration-response curve to papaverine (B). Papaverine (10^{-4} M) and nicardipine (10^{-8} M) almost completely reversed the contraction induced by 10^{-8} M endothelin. n: number of animals studied.

Fig. 6 Effect of nicardipine pretreatment on the contractile response of canine basilar artery to endothelin. Nicardipine pretreatment significantly suppressed the response to endothelin. n: number of animals studied.

(Fig. 7A). ATP and thrombin also induced dose-dependent relaxation of the basilar artery pre-contracted by 3×10^{-9} M endothelin (Fig. 7B, C). The maximal relaxations of the basilar artery to 10^{-4} M acetylcholine, 10^{-5} M ATP, and 1.0 U/ml thrombin were $16.1 \pm 4.3\%$, $71.7 \pm 5.1\%$, and $84.9 \pm 5.0\%$, respectively.

Discussion

The results of this study demonstrate that endothelin has a potent, long-lasting constricting effect on canine basilar artery. The EC_{50} was $(2.1 \pm 0.5) \times 10^{-9}$ M. Moreover, the minimal concentration required for contraction of the normal canine basilar

artery was extremely low (10^{-12} M). The maximal contractile response to endothelin was much greater than that induced by 40 mM KCl. Thus, endothelin is one of the most potent vasoconstrictors known.

No contractile response was elicited at 10^{-8} M endothelin in Ca^{++} -free medium, and nicardipine, at a concentration of 10^{-8} M, almost completely reversed the contraction induced by 10^{-8} M endothelin. Nicardipine pretreatment (10^{-8} – 10^{-6} M) also significantly suppressed the contractile response to endothelin. These results suggest that extracellular Ca^{++} plays a major role in the endothelin-mediated contraction of the canine basilar artery. However, moderate contractions were observed at higher concentrations of endothelin, and the inhibitory effect of nicardipine on endothelin-induced contraction was non-competitive. Therefore, one or more mechanisms other than the stimulation of dihydropyridine-sensitive Ca^{++} channel may be involved in the endothelin-induced contractions of the canine basilar artery.

Removal of the arterial endothelium significantly shifted the concentration-response curve to endothelin to the left. This result is consistent with our previous results with serotonin, $PGF_{2\alpha}$, PGE_2 , and PGD_2 in the canine basilar artery.³¹⁾ Enhancement of vasoconstriction following removal of the endothelium appears to be a universal phenomenon, with no differences among species, anatomical site of the artery, or the vasoconstrictor agents used.^{2,4,6,7,11,23,27,31,40)} It has been suggested that endothelium may exert a tonic relaxing influence on the smooth muscle cells through spontaneous release of EDRF.^{15,18,28,31)} Abolition of EDRF release may be responsible for the enhanced vasoconstriction following endothelium removal.

The endothelin-induced contraction of canine basilar artery was almost completely suppressed by

Fig. 7 Representative tracings (left) of the effect of acetylcholine (A), ATP (B), and thrombin (C) on the contraction of canine basilar artery induced by 3×10^{-9} M endothelin (ET), and concentration-response curves (right). Acetylcholine, ATP, and thrombin induced relaxation of the artery in a dose-dependent fashion. Pap: papaverine, n: number of animals studied.

the addition of 10^{-4} M papaverine. Acetylcholine, ATP, and thrombin, which are known to induce endothelium-dependent relaxation, also evoked relaxation of the basilar artery pre-contracted with 3×10^{-9} M endothelin. The relaxation induced by these agents is associated with activation of the enzyme guanylate cyclase and an increase in the intracellular production of cyclic guanosine monophosphate.^{17,35} The relaxation produced by glyceryl trinitrate is also associated with an increase in the cytoplasmic concentration of this cyclic nucleotide,²⁰ and has been reported to inhibit endothelin-induced vasocon-

triction in porcine artery.⁴⁵ These observations suggest that endothelin does not affect the basic mechanism of relaxation in smooth muscle cells.

The present study demonstrated that acetylcholine induced vasodilation in the canine basilar artery, although to a minimal degree. This result is inconsistent with the finding of Katusic *et al.*²² that acetylcholine induces endothelium-dependent vasoconstriction. The reason for this discrepancy is unclear, but might be attributable to differences in experimental conditions or in the experimental dogs used.

Yanagisawa *et al.*⁴⁵ showed that the messenger ribonucleic acid (RNA) encoding prepro-endothelin increased rapidly when the endothelial cells were exposed to thrombin, epinephrine, or the calcium ionophore A23187. In endothelial cells cultured under flowing conditions, prepro-endothelin messenger RNA is substantially reduced.⁴⁵ These chemical and mechanical stimuli can, at the same time, induce the release of EDRF. These authors suggested that both EDRF and endothelin play an important role in the regulation of vascular smooth muscle tone.

Endothelial damage of varying degree often occurs in the major cerebral arteries following SAH, and may include vacuolation, loss of tight junctions, and denudation of the endothelial cells.^{1,12,30,38,41} It is likely that such injury affects endothelium-dependent contraction of the artery. Endothelin is produced constitutively by endothelial cells in culture,⁴⁵ and Ross³⁶ hypothesized that cultured endothelium may more closely approximate an *in vivo* state of injury. According to his theory, production of endothelin may be stimulated in slightly injured cerebral arterial endothelial cells following SAH. In addition, disruption of the barrier of major cerebral arteries occurs following SAH, which may allow endothelin to permeate the smooth muscle layer.³⁸ We recently found that endothelin injected into the cisterna magna caused biphasic contraction of the canine basilar artery, which lasted for more than 24 hours.¹⁹ Therefore, it is reasonable to suspect that endothelin plays an important role, as a possible spasmogenic substance, in the pathogenesis of cerebral vasospasm following SAH. Impairment of endothelium-dependent vasodilation following SAH probably contributes to sustained constriction of the artery induced by endothelin and other vasoconstrictors.

At present, it is still unclear whether or not human cerebral arteries can produce endothelin, or if there is enough endothelin to induce prolonged arterial contraction in the cerebrospinal fluid of patients with SAH. Clarification of the role of endothelin in the

pathogenesis of vasospasm following SAH requires more extensive study.

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