ELSEVIER

European Journal of Pharmacology 277 (1995) 77–87

ejp

The role of endothelin and nitric oxide in modulation of normal and spastic cerebral vascular tone in the dog

Hiroyasu Hirose [a], Katsuhisa Ide [b], Tomio Sasaki [b], Risa Takahashi [a], Masahiko Kobayashi [a], Fumihiko Ikemoto [a], Mitsuo Yano [a], Masaru Nishikibe [a,*]

[a] *Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Tsukuba Techno-Park Oho, Tsukuba 300-33, Japan*
[b] *Department of Neurosurgery, Faculty of Medicine, University of Tokyo, Hongo, Tokyo 113, Japan*

Received 28 July 1994; revised 30 December 1994; accepted 27 January 1995

Abstract

To investigate the roles of endothelin and nitric oxide (NO) in the regulation of cerebral vascular tone under basal conditions and in cerebral vasospasm following subarachnoid hemorrhage in dogs, we used BQ-123 (cyclo(-D-Trp-D-Asp-L-Pro-D-Val-L-Leu-) sodium salt), an endothelin ET_A receptor antagonist, L-arginine, a substrate for the formation of NO, and N^G-nitro-L-arginine methyl ester, an NO synthesis inhibitor, and measured the angiographic diameter of the basilar artery in vivo. In normal dogs, intracisternal (i.c.) injection of BQ-123 (0.6 mg/kg) produced a 29.4 ± 6.11% ($P < 0.01$) increase in the basal diameter 24 h after injection. N^G-nitro-L-arginine methyl ester (0.6 mg/kg i.c.) produced a 19.3 ± 2.93% ($P < 0.05$) decrease in the basal diameter 2 h after injection. This decrease was significantly attenuated by both BQ-123 (0.06–0.6 mg/kg i.c.) and L-arginine (6 mg/kg i.c.), but not by D-arginine. In the two-hemorrhage canine model, BQ-123 significantly inhibited the development of cerebral vasospasm (36.9 ± 4.11% decrease on day 5 and 42.0 ± 4.54% decrease on day 6 in controls vs 21.7 ± 4.75% decrease ($P < 0.05$) on day 5 and 20.8 ± 4.14% decrease ($P < 0.05$) on day 6 for 0.6 mg/kg i.c.). Furthermore, in this model, L-arginine (6 mg/kg i.c.) significantly attenuated the cerebral vasospasm on day 4 from a 30.9 ± 5.78% decrease (before) to a 12.6 ± 5.99% decrease (after). The immunoreactive endothelin-1 levels in the endothelial layer and the adventitia of the basilar artery were much higher on days 3 and 7 after the injection of autologous blood than on day 0 before blood injection. These results suggest that endogenous endothelin and NO both participate in regulating the basal tone of cerebral arteries, and, therefore, the development of cerebral vasospasm following subarachnoid hemorrhage may be at least partially attributed to an impairment of the balanced action of endothelin and NO. Furthermore, endothelin ET_A antagonists or NO products may be useful in the treatment of cerebral vasospasm following subarachnoid hemorrhage.

Keywords: BQ-123; Endothelin; Nitric oxide (NO); Cerebral vasospasm; Subarachnoid hemorrhage

1. Introduction

The endothelium plays an important role in the regulation of vascular tone through the production and release of endothelium-derived vasoconstricting factor (EDCF), relaxing factor (EDRF) and hyperpolarizing factor (EDHF) (Taylor and Weston, 1988; Yanagisawa et al., 1988; Angus and Cocks, 1989; Furchgott and Vanhoutte, 1989). Endothelin-1 has been isolated as a vasoconstrictor from the culture medium of porcine aortic endothelial cells (Yanagisawa et al., 1988). EDRF has been identified as nitric oxide (NO), or a closely related compound (Palmer et al., 1987; Palmer et al., 1988; Sakuma et al., 1988). Several in vitro studies have shown that the NO synthesis inhibitors, N^G-monomethyl-L-arginine, N^G-nitro-L-arginine methyl ester and N^G-nitro-L-arginine, produce endothelium-dependent contraction under basal conditions and inhibit endothelium-dependent relaxation in response to acetylcholine or vasopressin in the isolated cerebral artery (Trezise et al., 1992; Brian and Kennedy, 1993; Katusic et al., 1993) as well as in several other peripheral blood vessels (Rees et al., 1989; Gold et al., 1990;

* Corresponding author. Department of Pharmacology, New Drug Discovery Research Laboratories, Tsukuba Research Institute, Banyu Pharmaceutical Co., Ltd., Tsukuba Techno-Park Oho, Tsukuba 300-33, Japan. Tel. 81.298.77-2000, fax 81.298.77-2028.

SSDI 0014-2999(95)00070-4

Moore et al., 1990). Endothelium-dependent relaxation is inhibited in the isolated basilar arteries after injection of autologous blood in the canine subarachnoid hemorrhage model (Kim et al., 1988). These findings suggest that NO regulates cerebral vascular tone under basal conditions and that the loss of endothelium-dependent relaxation may be one component in the development of cerebral vasospasm.

However, it has been reported that endothelin-1 is present in cerebral vascular beds (Yamaura et al., 1992) and causes persistent and potent vasoconstriction in canine cerebral arteries (Asano et al., 1989; Kamata et al., 1991). Yoshimoto et al. (1991) reported that endothelin messenger RNA was expressed in cultured endothelial cells of canine cerebral microvessels and that endothelin-1 produced by cultured endothelial cells of cerebral microvessels grown on a collagen-coated filter was released mainly to the basal side (corresponding to the basement membrane side), not to the apical side (corresponding to the vascular lumen side). This finding indicates that endothelin-1 constricts arterioles locally at the same place where it is produced by the endothelial cells (Yoshimoto et al., 1991). In in vivo studies, Asano et al. (1989) demonstrated that intracisternal (i.c.) injection of endothelin-1 (10 pmol) into dogs induced an angiographic decrease in the diameter of the basilar artery which lasted for at least 2 days following the injection. It is believed that the basal tone of cerebral vascular beds in vivo can be influenced by the level of endothelin-1 production. In addition, it has recently been reported that, in the canine subarachnoid hemorrhage model, the immunoreactive endothelin-1 content of the basilar arteries increases (Yamaura et al., 1992) and that treatment with endothelin ET_A receptor antagonists prevents cerebral vasospasm (Ide et al., 1993; Itoh et al., 1993; Nirei et al., 1993).

These data suggest that the vascular tone of cerebral vascular beds may be, at least in part, affected by physiological antagonism between the vasoconstriction induced by endothelin and the vasodilation caused by NO. If there is a reciprocal relationship between endothelin and NO with respect to cerebral vascular tone under basal conditions, an impairment of the balanced action of endothelin and NO may contribute to chronic cerebral vasospasm following subarachnoid hemorrhage.

Therefore, in the present study, we investigated angiographically the roles of endothelin and NO in the regulation of the diameter of the basilar artery under basal conditions and in chronic cerebral vasospasm following subarachnoid hemorrhage in the canine two-hemorrhage model, using BQ-123 (cyclo(-D-Trp-D-Asp-L-Pro-D-Val-L-Leu-) sodium salt), an endothelin ET_A receptor antagonist, and L-arginine, a substrate for the formation of NO in vivo.

2. Materials and methods

2.1. Animal preparation and angiography

Adult beagle dogs of both sexes weighing from 9 to 15 kg were anesthetized with sodium pentobarbital (30 mg/kg i.v. bolus followed by 2 to 5 mg/kg/h i.v. infusion). All surgical procedures (cannulation, intracisternal injection of drug and autologous blood, sampling of cerebrospinal fluid, etc.) were performed under strict aseptic conditions. The animals were intubated and arterial blood gases were maintained in the range of 100–150 mmHg (PO_2), 30–40 mmHg (PCO_2), and 7.35–7.45 (pH) under artificial ventilation (613, Harvard, USA). The left femoral artery was cannulated to monitor mean blood pressure and heart rate (Polygraph RM-6000, Nihonkoden, Japan) and to collect blood samples. Arterial blood gases were determined before angiography with a blood gas analyzer (288 Blood Gas System, CIBA-Corning, USA). Catheters were inserted into the left femoral vein for administration of pentobarbital and the right vertebral artery for angiography.

An angiogram of the basilar artery was taken by vertebral injection of 5 ml of Angiografin® (Schering, Germany) at a rate of 3 ml/s under pentobarbital anesthesia. The diameter of the basilar artery was measured at the midpoints of the upper, lower and middle thirds of its length in a blind fashion. These values were then averaged.

2.2. Changes in the diameter of the basilar artery in normal dogs

The drugs were injected in a volume of 0.1 ml/kg into the cisterna magna through a 21-gauge needle. To avoid a marked increase in intracranial pressure, the same volume of cerebrospinal fluid was removed before injection. Changes in the diameter are expressed as a percentage of the basal diameter measured 30 min before drug treatment in each dog.

The concentration of BQ-123 in the cerebrospinal fluid was measured by high performance liquid chromatography 24 h after drug injection.

2.3. Changes in the diameter of the basilar artery in the two-hemorrhage canine experimental cerebral vasospasm model

Experimental cerebral vasospasm following subarachnoid hemorrhage was produced by two intracisternal (i.c.) injections of autologous blood (the two-hemorrhage canine model), using a method slightly modified from that reported by Varsos et al. (1983). On day 0, after control angiography of the basilar artery, the first aliquot of autologous arterial blood (0.5

ml/kg) was injected into the cisterna magna through a 21-gauge needle. To avoid a marked increase in intracranial pressure, the same volume of cerebrospinal fluid was removed before injection. After the injection, the dogs were restrained in the head-down position for 15 min. On day 2, the animals received the second injection of blood in the same manner as on day 0.

The drugs were injected into the cisterna magna in a volume of 0.1 ml/kg on days 4 and 5 in the BQ-123-treated group, and on day 4 in the L-arginine-treated group, after the presence of angiographic cerebral vasospasm in the basilar artery was observed on day 4. Changes in the diameter are expressed as a percentage of the basal diameter measured 30 min before the first injection of blood on day 0 in each dog.

2.4. Immunohistochemical study

The basilar artery specimens were obtained from the dogs used in the two-hemorrhage experimental cerebral vasospasm model after they had been killed on day 0, 3 or 7. On day 0, the dogs were killed before the injection of autologous blood, while on days 3 and 7, the dogs were killed after they received two injections of blood. The isolated basilar arteries were dissected transversely followed by fixation with poly-L-lysine paraformaldehyde solution at 4° C overnight. The specimens were dehydrated with graded ethyl alcohol at 4° C, then embedded in paraffin wax (melting point 58° C). The sections were cut at 3 μm and stained immunohistochemically using the biotin-streptavidin-horseradish peroxidase method for detection of endothelin-1. Rabbit anti-endothelin-1 polyclonal antibody (Peninsula Lab., USA) was used as the primary antibody. Deparaffinized sections were incubated with rabbit anti-endothelin-1 antibody (dilution 1:400) at room temperature for 3 h. After being washed, the sections were incubated with secondary antibody (biotinylated anti-rabbit immunoglobulin G; IBL, Japan) diluted 1:100 for 30 min, then with horseradish peroxidase-conjugated streptavidin diluted 1:100 for 30 min. Immunoreactive endothelin-1 was visualized with 0.05% 3,3″-diaminobenzidine-0.02% hydroperoxide. The specificity of the immunoreaction was determined by the use of non-immune normal rabbit serum to replace the primary antibodies for endothelin-1, or supernatant of anti-endothelin-1 antibody (dilution 1:100) preabsorbed with synthetic endothelin-1 (20 μM) at 4° C overnight.

Intensity (optical density) of immunoreactive endothelin-1 products in each zone of the endothelial layer, smooth muscle layers and adventitia in the basilar artery were measured by an image analyzer (BIOCOM 200 System, Computor Bilud, Japan). The optical density of each zone as endothelial layer, smooth muscle layers or adventitia was traced on a TV monitor magnified 100- to 200-fold. Calibration was performed by setting up a section-free area for maximum transmission (100%) and one without lighting for a black image (0%).

2.5. Drugs

BQ-123 (cyclo(-D-Trp-D-Asp-L-Pro-D-Val-L-Leu-) sodium salt) was synthesized at Tsukuba Research Institute, Banyu Pharmaceutical. N^G-nitro-L-arginine methyl ester and serotonin hydrochloride (5-HT) were purchased from Sigma Chemical (USA), and L-arginine hydrochloride and D-arginine hydrochloride were purchased from Wako Chemicals (Japan). All of these drugs were dissolved in saline before use.

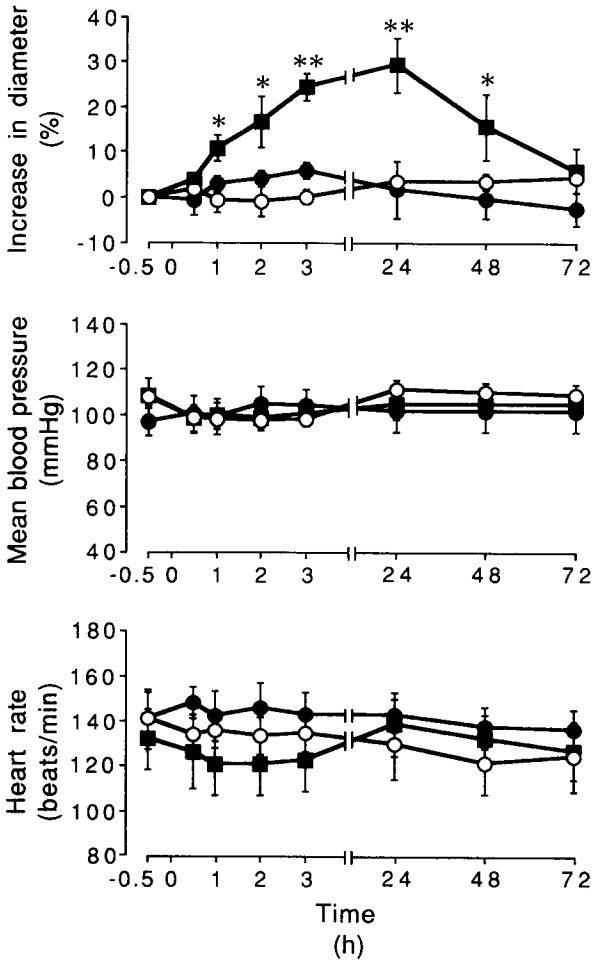

Fig. 1. Effect of intracisternal injection of BQ-123 (control (○), 0.06 mg/kg (●) and 0.6 mg/kg (■)) on the diameter of the basilar artery (upper), mean blood pressure (middle) and heart rate (lower) in normal dogs. Increases in the diameter are expressed as a percentage of the basal diameter measured 30 min before injection in each dog. The basal diameters were 1.09±0.04 mm (control), 1.05±0.04 mm (BQ-123, 0.06 mg/kg) and 1.07±0.01 mm (BQ-123, 0.6 mg/kg). $^*P < 0.05$, and $^{**}P < 0.01$ significantly different from the value in the control (saline-treated) group. The values are mean±S.E. for five animals. The Newman-Keuls test was used.

2.6. Statistical analysis

All results are expressed as means ± S.E. Statistical analyses were performed using the Newman-Keuls test or the Duncan multiple range test after analysis of variance. P values less than 0.05 were considered significant.

3. Results

3.1. Effects of intracisternal (i.c.) injection of BQ-123 on the diameter of the basilar artery, mean blood pressure and heart rate in normal dogs

Intracisternal injection of BQ-123 at a dose of 0.6 mg/kg induced a significant dilation of the angiographic diameter of the basilar artery. Maximal dilation (29.4 ± 6.11% increase in the basal diameter, $N = 5$, $P < 0.01$) was observed 24 h after injection, with a return to the basal level 72 h later (6.0 ± 4.85% increase in the basal diameter, $N = 5$, n.s.) (Fig. 1). In some cases, dilation was nearly maximal at 3 h after injection. BQ-123 at a dose of 0.06 mg/kg i.c. did not affect the diameter of the basilar artery during the 72 h after injection. Saline (as a vehicle of drug) also produced no significant changes in the diameter of the basilar artery during the 72 h after injection (Fig. 1). An angiogram of a representative vasodilation is shown in Fig. 2. Intracisternal injection of BQ-123 did not affect mean blood pressure or heart rate (Fig. 1). The concentration of BQ-123 in the cerebrospinal fluid 24 h after injection of 0.6 mg/kg was 7.7 ± 1.80 μM.

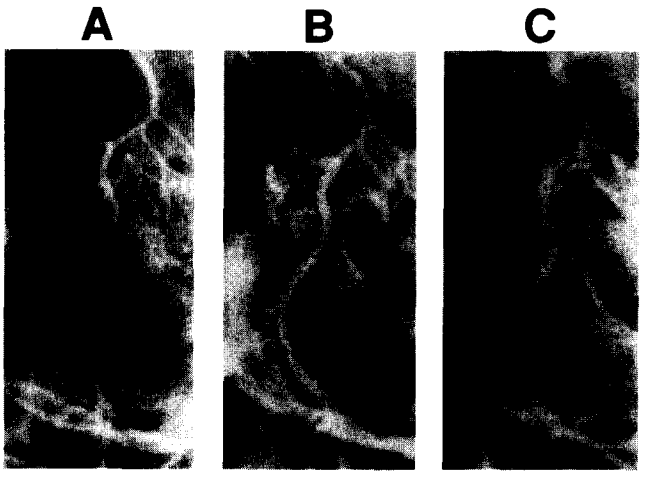

Fig. 2. Angiogram of a canine basilar artery 30 min before (A), and 2 (B) and 24 h (C) after intracisternal injection of BQ-123 (0.6 mg/kg) in a normal dog. Note the increase in the diameter of the basilar artery 24 h after dosing.

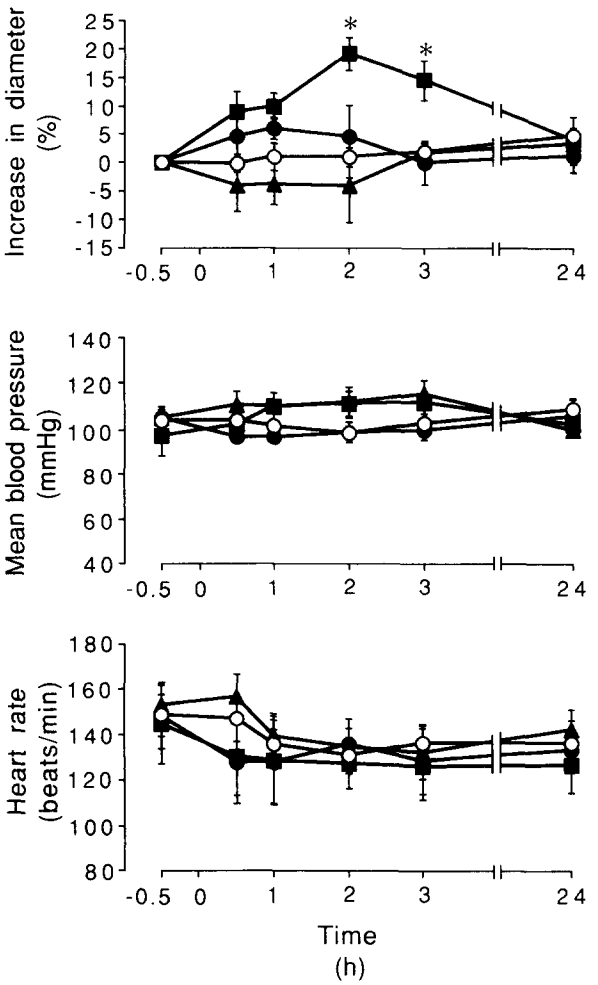

Fig. 3. Effect of intracisternal injection of saline (○), L-arginine (6 mg/kg (▲)) and N^G-nitro-L-arginine methyl ester (0.12 mg/kg (●) and 0.6 mg/kg (■)) on the diameter of the basilar artery (upper), mean blood pressure (middle) and heart rate (lower) in normal dogs. Decreases in the diameter are expressed as a percentage of the basal diameter measured 30 min before injection in each dog. The basal diameters were 1.09 ± 0.03 mm (control), 1.00 ± 0.08 mm (L-arginine), 1.10 ± 0.04 mm (N^G-nitro-L-arginine methyl ester, 0.12 mg/kg) and 1.07 ± 0.04 mm (N^G-nitro-L-arginine methyl ester, 0.6 mg/kg). $^*P <$ 0.05 significantly different from the value in the control (saline-treated) group. The values are mean ± S.E. for five animals. The Newman-Keuls test was used.

3.2. Effect of i.c. injection of L-arginine and N^G-nitro-L-arginine methyl ester on the diameter of the basilar artery, mean blood pressure and heart rate in normal dogs

L-arginine at a dose of 6 mg/kg i.c. produced no significant changes in the angiographic diameter of the basilar artery during the 24 h after injection (Fig. 3). N^G-nitro-L-arginine methyl ester at a dose of 0.6 mg/kg i.c. induced a significant decrease in the diameter of the basilar artery. Maximal constriction (19.3 ± 2.93%

decrease in the basal diameter, $N = 5$) was observed 2 h after the injection of 0.6 mg/kg (Fig. 3). L-arginine and N^G-nitro-L-arginine methyl ester did not affect mean blood pressure or heart rate (Fig. 3).

L-arginine (6 mg/kg i.c.), D-arginine (6 mg/kg i.c.) or saline (0.1 ml/kg i.c.) was injected 1 h after the injection of N^G-nitro-L-arginine methyl ester (0.6 mg/kg i.c.). Post-treatment with L-arginine, but not D-arginine, significantly attenuated the constriction of the basilar artery 2 h after injection of N^G-nitro-L-arginine methyl ester ($9.1 \pm 3.18\%$ decrease in the basal diameter, L-arginine-treated group, vs $20.7 \pm 2.44\%$, saline-treated group, $N = 5$, $P < 0.05$) (Fig. 4).

3.3. Effects of i.c. injection of BQ-123 on the cerebral vasospastic responses to N^G-nitro-L-arginine methyl ester and 5-HT

N^G-nitro-L-arginine methyl ester at a dose of 0.6 mg/kg i.c. induced a significant decrease in the angiographic diameter of the basilar artery 2 h after injection ($23.9 \pm 5.58\%$ decrease in the basal diameter, N^G-nitro-L-arginine methyl ester-treated group, vs $2.0 \pm 2.95\%$, saline-treated group, $N = 5$, $P < 0.05$) (Fig. 5 upper). 5-HT at a dose of 5 ng/kg i.c. induced a significant decrease in the angiographic diameter of the basilar artery 2 h after injection ($12.7 \pm 2.38\%$ decrease in the basal diameter, 5-HT-treated group, vs $2.0 \pm 2.95\%$, saline-treated group, $N = 5$, $P < 0.05$) (Fig. 5 upper). The angiographic diameters 1, 2 and 3 h after injection of 5-HT at a dose of 5 ng/kg were assessed, and the maximal constriction was observed 2 h after the injection (data not shown).

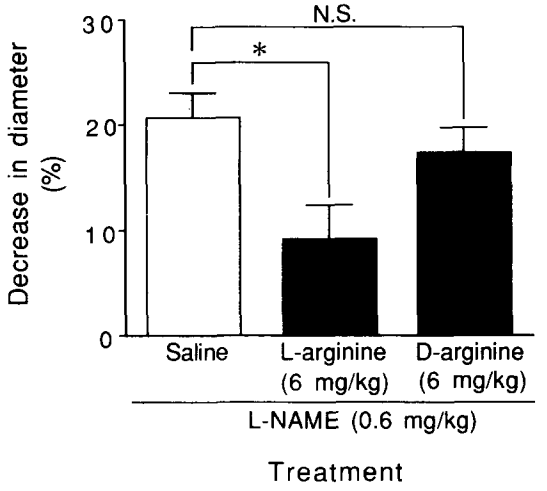

Fig. 4. Effect of intracisternal injection of saline, L-arginine (6 mg/kg) and D-arginine (6 mg/kg) on the angiographic vasospastic response to N^G-nitro-L-arginine methyl ester (L-NAME, 0.6 mg/kg i.c.). L-arginine, D-arginine or saline was injected 1 h after injection of L-NAME. Decreases in the diameter of the basilar artery 2 h after injection of L-NAME are expressed as a percentage of the basal diameter measured 30 min before injection of L-NAME in each dog. The basal diameters were 1.10 ± 0.05 mm (control), 1.08 ± 0.03 mm (L-arginine) and 1.03 ± 0.03 mm (D-arginine). $^*P < 0.05$ significantly different from the value in the control (saline-treated) group. The values are mean ± S.E. for five animals. The Newman-Keuls test was used.

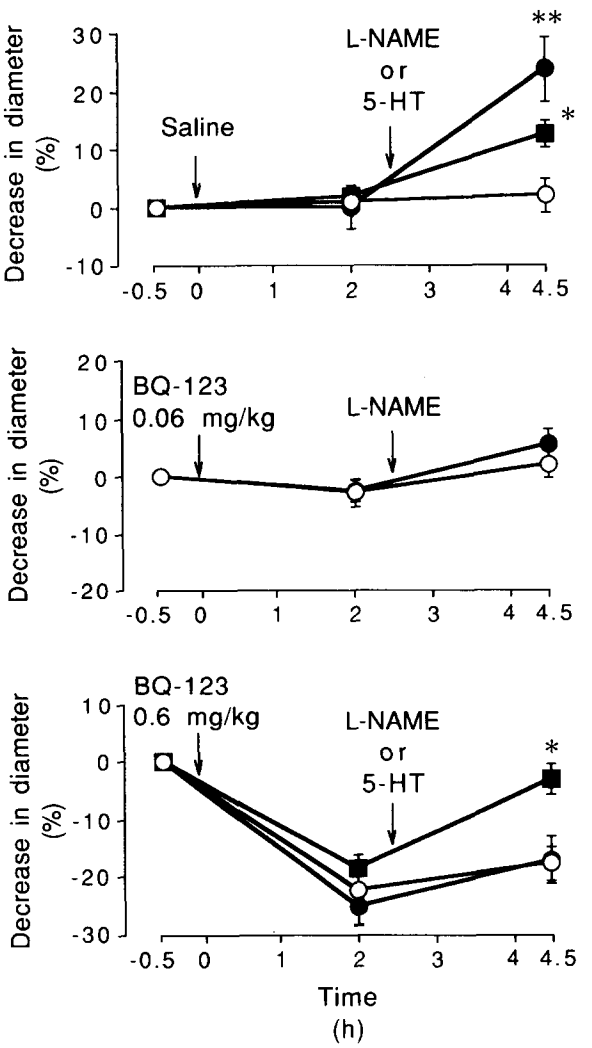

Fig. 5. Effect of intracisternal injection of BQ-123 (0.06 and 0.6 mg/kg) on the angiographic vasospastic response to 5-HT (5 ng/kg i.c.) and N^G-nitro-L-arginine methyl ester (L-NAME, 0.6 mg/kg i.c.). Saline or BQ-123 was injected 2.5 h before injection of saline (○), L-NAME (●) or 5-HT (■). Decreases in the diameter of the basilar artery 2 and 4.5 h after saline or BQ-123 treatment are expressed as a percentage of the basal diameter measured 30 min before saline or BQ-123 treatment in each dog. In the saline-pretreated groups (upper), the basal diameters were 1.03 ± 0.02 mm (control), 1.08 ± 0.03 mm (L-NAME) and 1.08 ± 0.05 mm (5-HT). In the BQ-123 (0.06 mg/kg)-pretreated groups (middle), the basal diameters were 1.08 ± 0.03 mm (control) and 0.96 ± 0.08 mm (L-NAME). In the BQ-123 (0.6 mg/kg)-pretreated groups, the basal diameters were 1.09 ± 0.02 mm (control), 1.02 ± 0.02 mm (L-NAME) and 1.04 ± 0.04 mm (5-HT). $^*P < 0.05$ and $^{**}P < 0.01$ significantly different from the value in the control group. The values are mean ± S.E. for five animals. The Newman-Keuls test was used.

After the injection of BQ-123 at a dose of 0.06 mg/kg i.c., N^G-nitro-L-arginine methyl ester (0.6 mg/kg i.c.) did not produce any significant decrease in the basilar artery diameter (5.8 ± 2.42% decrease in the basal diameter, N^G-nitro-L-arginine methyl ester-treated group, vs 2.1 ± 2.36%, saline-treated group, $N = 5$, n.s.) (Fig. 5 middle). BQ-123 (0.6 mg/kg i.c.) completely inhibited the N^G-nitro-L-arginine methyl ester (0.6 mg/kg i.c.)-induced vasoconstriction, but not the 5-HT (5 ng/kg i.c.)-induced vasoconstriction of the basilar artery (-17.0 ± 4.09% (n.s.) decrease in the basal diameter, N^G-nitro-L-arginine methyl ester-treated group, and −3.0 ± 2.60% ($P < 0.05$), 5-HT-treated group, vs −17.6 ± 2.92%, saline-treated group, $N = 5$) (Fig. 5 lower).

3.4. Effect of i.c. injection of BQ-123 and L-arginine on cerebral vasospasm in the two-hemorrhage canine model

On day 4 before i.c. injection of BQ-123 (0.6 mg/kg), the angiographic diameter of the basilar artery was decreased by 31.6 ± 2.84% ($N = 5$) and 31.4 ± 2.65% ($N = 8$) compared with the basal diameter in the control and BQ-123-treated groups, respectively. The diameter 24 h after injection of saline (as a vehicle of drug) was decreased by 36.9 ± 4.11% and 42.0 ± 4.54% compared with the basal diameter on days 5 and 6, respectively, in the control group. The constricted basilar artery was significantly relaxed by post-treatment with BQ-123 on days 5 and 6 (21.7 ± 4.75% ($P < 0.05$) and 20.8 ± 4.14% ($P < 0.05$) decrease in the basal diameter, respectively) (Fig. 6).

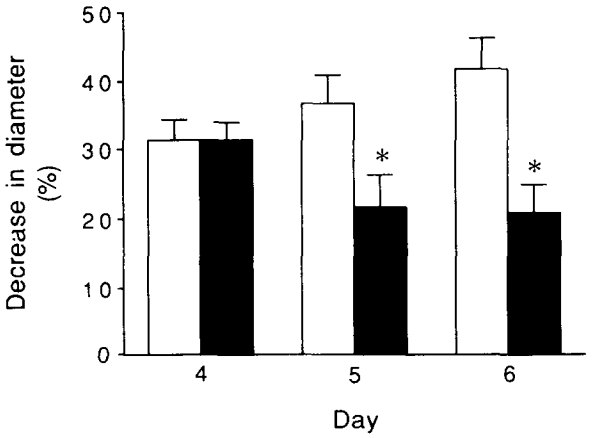

Fig. 6. Effect of intracisternal injection of BQ-123 (0.6 mg/kg) on cerebral vasospasm in the two-hemorrhage canine model. Decreases in the diameter of the basilar artery on days 4, 5 and 6 are expressed as a percentage of the basal diameter on day 0 in each dog. BQ-123 or saline was injected two times on both days 4 and 5. The diameters on day 0 were 1.06 ± 0.06 mm (control) and 1.16 ± 0.08 mm (BQ-123). $^*P < 0.05$ significantly different from the value in the control group. The values are mean ± S.E. for five (the control group) or eight (the BQ-123-treated group) animals. The Newman-Keuls test was used.

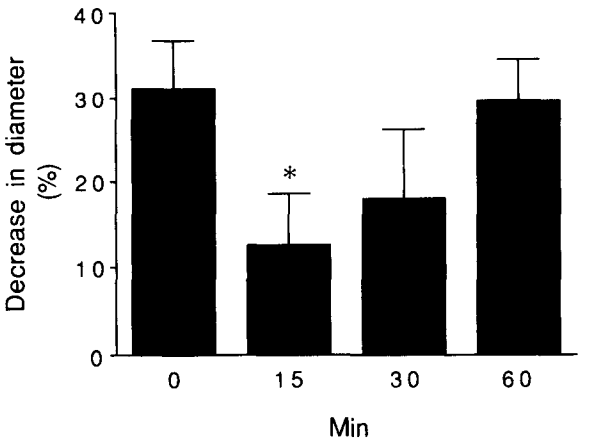

Fig. 7. Effect of intracisternal injection of L-arginine (6 mg/kg) on cerebral vasospasm in the two-hemorrhage canine model. Decreases in the diameter of the basilar artery 30 min before and 15, 30 and 60 min after injection of L-arginine on day 4 are expressed as a percentage of the basal diameter on day 0 in each dog. The diameter on day 0 was 1.11 ± 0.07 mm. $^*P < 0.05$ significantly different from the value 30 min before injection on day 4. The values are mean ± S.E. for five animals. The Duncan multiple range test was used.

On day 4 before i.c. injection of L-arginine (6 mg/kg), the decrease in the angiographic diameter of the basilar artery was 30.9 ± 5.78% ($N = 5$) compared with the basal diameter of the basilar artery. The treatment with L-arginine significantly relaxed the constricted basilar artery 15 min after injection (12.6 ± 5.99% ($P < 0.05$) decrease in the basal diameter) (Fig. 7).

3.5. Immunoreactive endothelin-1 levels in the basilar artery in the two-hemorrhage canine model

On day 0 (non-hemorrhage), immunoreactive endothelin-1 levels were low and irregularly observed in the endothelial layer, smooth muscle layers and adventitia of the basilar artery. Immunoreactive endothelin-1 products in the endothelial layer and adventitia of the basilar artery were at much higher levels on days 3 and 7 than on day 0 (Fig. 8). Optical densities of immuno-

Table 1
Immunoreactive endothelin-1 levels in the endothelial layer, smooth muscle layers and adventitia in the basilar artery of the canine subarachnoid hemorrhage model

Day	N	Intensity of immunoreactive endothelin-1 (optical density)		
		Endothelial layer	Smooth muscle layers	Adventitia
0	6	14.5 ± 0.6	16.4 ± 0.4	12.5 ± 0.9
3	7	23.1 ± 0.9 [a]	18.9 ± 0.9	21.5 ± 1.5 [a]
7	5	18.5 ± 1.5 [b]	17.0 ± 0.9	19.7 ± 2.4 [a]

The values are means ± S.E. for N animals. On day 0, the dogs were killed before the injection of autologous blood, while on days 3 and 7, the dogs were killed after they received two injections of blood. Significant difference from the value on day 0, [a] $P < 0.01$, [b] $P < 0.05$. The Newman-Keuls test was used.

reactive endothelin-1 products in the endothelial layer, smooth muscle layers and adventitia are presented in Table 1.

4. Discussion

We found that immunoreactive endothelin-1 products were localized in the endothelial layer and the adventitia of the basilar artery under normal conditions (on day 0 in the two-hemorrhage canine model), and that i.c. injection of BQ-123 (0.6 mg/kg) produced a marked dilation of the diameter of the basilar artery in dogs without affecting mean blood pressure or heart rate in the present study. BQ-123 has a high affinity for endothelin ET_A receptors of porcine aortic smooth muscle cells ($IC_{50} = 7.3$ nM), antagonizes endothelin-1-induced vasoconstriction of isolated porcine coronary

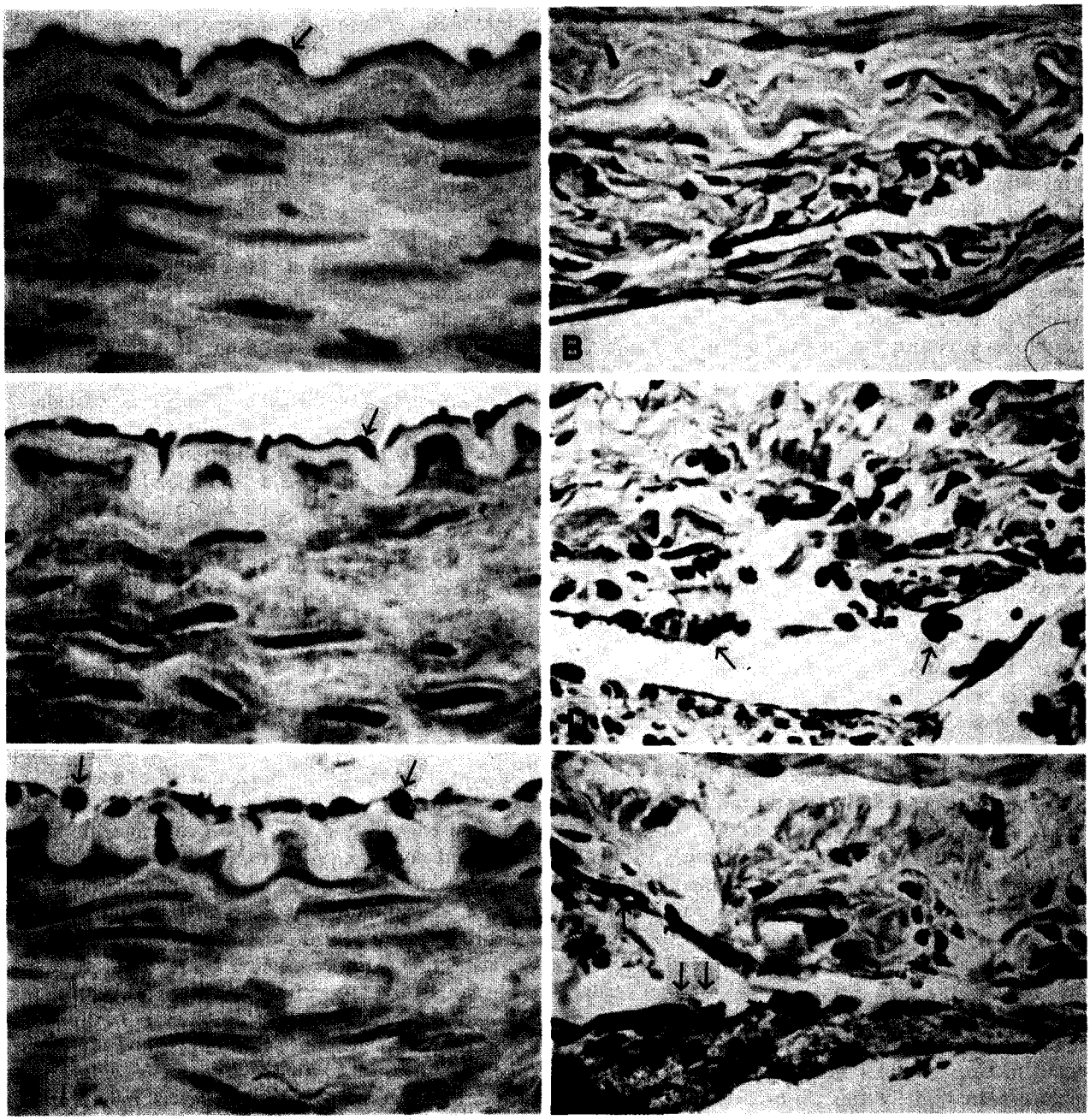

Fig. 8. Photomicrographs with immunohistochemical staining by anti-endothelin-1 antibody of the canine basilar arteries on day 0 (upper; A and B) before the injection of autologous blood, and day 3 (middle; C and D) and day 7 (lower; E and F) after the injection of autologous blood in the two-hemorrhage canine model. The color of stained immunoreactive endothelin-1 is pale brown or brown. Left panels (A, C and E) show sections of the endothelial layer, and right panels (B, D and F) show sections of adventitia. The single arrows in A, C and E indicate nuclei of endothelial cells. The single arrows in D and F indicate infiltration cells. The double arrow in F indicates clots.

artery ($pA_2 = 7.4$) and inhibits endothelin-1-induced pressor responses without affecting the endothelin-1-induced depressor response in conscious rats (Ihara et al., 1992). We confirmed not only in vitro that pretreatment with BQ-123 (0.1–10 μM) dose-dependently inhibited endothelin-1-induced contraction of isolated canine basilar artery and shifted the concentration-response curve for endothelin-1 to the right in a parallel fashion ($pA_2 = 7.35$), although 1 mM and 10 μM of BQ-123 did not inhibit constrictive responses to serotonin (5-HT, 10 nM-1 μM), or prostaglandin F_{2a} (0.1–10 μM) (data not shown), but also in vivo that BQ-123 at a dose of 0.6 mg/kg i.c. significantly prevented only the N^G-nitro-L-arginine methyl ester-induced constriction of the basilar artery without affecting the 5-HT-induced vasoconstriction in normal dogs. Similarly, several investigators have also confirmed the selectivity of BQ-123 for endothelin ET_A receptors in vitro (Sumner et al., 1992; Ide et al., 1993; Salom et al., 1993) and in vivo (Douglas et al., 1992; Mosquedagarcia et al., 1993). In addition, in this study, higher doses of BQ-123 did not affect behavior, body weight, body temperature, mean blood pressure or heart rate in any animal throughout the expermental period. Taken together, these results suggest that, in in vitro and in vivo studies, BQ-123 even at high concentrations has little effect except for its endothelin receptor antagonistic action. Therefore, it is likely that the vasodilator effect of BQ-123 at the dose of 0.6 mg/kg is due not to nonselective inhibition, but to a selective endothelin ET_A receptor blocking action. Our data strongly suggest that endogenous endothelin plays, at least in part, an important role in the regulation of the basal vascular tone of the basilar artery through endothelin ET_A receptors. However, it is unknown whether BQ-123 has an identical selectivity profile in cerebral arteries with developed vasospasm following subarachnoid hemorrhage, because there are no data available concerning the selectivity of BQ-123 in spastic arteries.

Recently, Nirei et al. (1993) reported that i.c. injection of an endothelin ET_A receptor antagonist (FR139317) produced no significant changes in mean blood pressure or heart rate, nor in the diameter of the basilar artery in dogs. However, these authors measured diameter for only 30 min following drug injection, whereas we monitored it for 72 h in the present study: we found a significant dilation of the basilar artery from 2 to 3 h after injection, and observed maximal dilation after 24 h, at which point BQ-123 was detected in cerebrospinal fluid (7.7 ± 1.80 μM). One possible explanation for this difference is that inhibition of endothelin-1-induced vasoconstriction by post-treatment with endothelin ET_A receptor blockade is time-consuming, because endothelin-1 barely dissociates from its receptors in binding studies (Hirata et al., 1988) and endothelin-1-induced vasoconstriction in several types of isolated vessels is characteristically difficult to stop (Yanagisawa et al., 1988; Asano et al., 1989).

In the present study, i.c. injection of N^G-nitro-L-arginine methyl ester produced a significant angiographic constriction of the basilar artery in normal dogs and this constriction was significantly attenuated by L-arginine, but not by D-arginine. In the isolated cerebral artery, N^G-monomethyl-L-arginine, N^G-nitro-L-arginine methyl ester or N^G-nitro-L-arginine not only inhibited endothelium-dependent relaxation in response to a vasoactive substance such as vasopressin (Katusic et al., 1993), but also elicited endothelium-dependent contraction under basal conditions (Trezise et al., 1992; Brian and Kennedy, 1993). In an in vivo study, Rosenblum et al. (1990) reported that the topical application of N^G-monomethyl-L-arginine inhibited endothelium-dependent dilation of the pial arteriole in response to acetylcholine and produced endothelium-dependent constriction of the pial arteriole under basal conditions. Furthermore, Faraci (1990) reported the possibility that NO synthesized from L-arginine influenced basal tone in the basilar artery. Our results and these reports suggest that the production and release of L-arginine-derived NO in cerebral vascular beds plays, at least in part, a role in the regulation of basal cerebrovascular tone. In addition to endothelium- dependent NO, endothelium-independent NO is liberated after nerve stimulation in the isolated cerebral artery, resulting in cerebroarterial relaxation (Toda and Okamura, 1991). Therefore, it is possible that NO as a neuronal messenger molecule paticipates in N^G-nitro-L-arginine methyl ester-induced angiographic constriction of the basilar artery. Based on these data, we are convinced that N^G-nitro-L-arginine methyl ester-induced vasoconstriction is due to the inhibition of L-arginine-derived NO synthesis and that L-arginine-derived NO plays an important role in the regulation of vascular tone of the normal basilar arteries.

BQ-123 at a dose of 0.6 mg/kg i.c. significantly prevented only the N^G-nitro-L-arginine methyl ester-induced constriction of the basilar artery without affecting the 5-HT-induced vasoconstriction. Furthermore, the low dose of BQ-123 (0.06 mg/kg i.c.) prevented the N^G-nitro-L-arginine methyl ester-induced constriction without affecting the basal tone of the basilar artery. In preliminary in vivo experiments, we found that low doses of BQ-123 partially inhibited constriction in response to endothelin-1 (100 pmol/kg i.c.) (data not shown). However, it is widely assumed that basal cerebrovascular tone is maintained at a constant level by autoregulation in the cerebral vascular system. Therefore, we suggest that the vasodilation due to low-dose BQ-123 in normal preparations may be masked by autoregulation in the cerebral vascular system. These results suggest that endothelin-1 is one

of the most important vasoconstrictive factors in N^G-nitro-L-arginine methyl ester-induced constriction and that physiological antagonism between NO (vasodilative factor) and endothelin-1 (vasoconstrictive factor) is important in the regulation of basal cerebral vascular tone.

In the canine subarachnoid hemorrhage model, on the other hand, immunoreactive-endothelin-1 levels in the endothelial cells and adventitia of the basilar artery were higher on days 3 and 7 than on day 0, and the post-treatment period with BQ- 123 (0.6 mg/kg i.c. on days 4 and 5) showed a significant reversal in cerebral vasospasm. Our results suggest that, even in the late phase (day 5-7), endothelin-1, via endothelin ET_A receptors, may participate in the development of cerebral vasospasm following subarachnoid hemorrhage. However, Shigeno et al. (1991) reported that overexpression of immunoreactive endothelin-1 was observed in the endothelium of the spastic basilar artery 3 days after subarachnoid hemorrhage in a canine experimental model and was no longer present 7 days after subarachnoid hemorrhage. Furthermore, Yamaura et al. (1992) reported that the immunoreactive endothelin-1 level in the wall of the basilar artery was significantly increased on day 2, but not significantly raised on day 7 in the canine subarachnoid hemorrhage model. In addition, they reported that endothelin-1 could act as a trigger in the early stages of cerebral vasospasm, because vasospasm was moderately reversed by the topical application of monoclonal antibody aganist endothelin-1 on day 2 but was rather resistant to the same antibody on day 7. In our study, the arterial wall was divided into 3 zones consisting of the endothelial layer, the smooth muscle layers and the adventitia for measurment of the immunoreactive endothelin-1 levels. Immunoreactive endothelin-1 levels in the endothelial layer and the adventitia on days 3 and 7 were much higher than those on day 0. The production and release of endothelin-1 has been reported in macrophages (Ehrenreich et al., 1990) as well as vascular endothelial cells (Yanagisawa et al., 1988). Macrophages are frequently observed in the adventitia of cerebral arteries following subarachnoid hemorrhage (Hughes and Schianchi, 1987) and large quantities of intensive immunoreactive endothelin-1 products are localized on macrophages (Kobayashi et al., submitted for publication). Furthermore, large amounts of thrombin and oxyhemoglobin, which stimulate the synthesis of endothelin-1 (Yanagisawa et al., 1988; Ohlstein and Storer, 1992), are present in blood clots surrounding cerebral arteries following subarachnoid hemorrhage. These substances may stimulate the production of endothelin-1 in the cerebral arteries and macrophages, and some elements of the adventitia may have some advantage over the smooth muscle layers and the endothelial layer in the production of endothelin-1. Recently, Nirei et al. (1993) reported that FR139317, an endothelin ET_A receptor antagonist, ameliorated the constriction of the basilar artery in a canine two-hemorrhage model. It is not clear whether FR139317 prevented or attenuated the development of cerebral vasospasm, because the treatment was applied on days 0 and 2, just before injection of autologous blood, and on day 4. Therefore, it is of interest that post-treatment with BQ-123 showed the ability to attenuate vasospasm in this study. However, the vasodilator effect of BQ-123 on spastic basilar artery in our subarachnoid hemorrhage model was less than that on normal basilar artery. One reason for this difference may be that it is difficult for an adequate concentration of BQ-123 to penetrate into the vasospastic artery covered with blood clots. In addition, it has been reported that other vasoconstrictors (prostaglandin $F_{2\alpha}$, thromboxane A_2, 5-HT, etc) may participate in the development of cerebral vasospasm following subarachnoid hemorrhage (Chehrazi et al., 1989; Sasaki et al., 1982). Therefore, we cannot rule out the possibility that other vasoconstrictors also participate in the genesis of cerebral vasospasm following subarachnoid hemorrhage. Taken together, these findings suggest that the difference between the vasodilator effects of BQ-123 on normal versus spastic basilar arteries may be explained by blood clots around the artery and the presence of other vasoconstrictors.

L-arginine significantly reversed both the N^G-nitro-L-arginine methyl ester-induced vasoconstriction in normal dogs and cerebral vasospasm in the canine subarachnoid hemorrhage model, although L-arginine did not produce any significant vasodilation under basal conditions. A high concentration of L-arginine, by acting as a substrate for the formation of NO, can reverse the inhibition of NO synthetase. Our results suggest that the ability to synthesize NO is impaired, but not abolished, in the basilar artery following subarachnoid hemorrhage. In the present study, the vasodilator effect of L-arginine was of short duration in the canine subarachnoid hemorrhage model (< 1 h). Possible reasons for the short duration of L-arginine activity are as follows: (1) the half-life of NO is less than 10 seconds; and (2) oxyhemoglobin, which is present in blood clots surrounding cerebral arteries following subarachnoid hemorrhage, is a potent inactivator of NO (Griffith et al., 1984; Martin et al., 1985). Interestingly, Kim et al. (1988) reported that, in isolated canine basilar artery following subarachnoid hemorrhage, the endothelium-dependent relaxation in response to vasopressin or thrombin was significantly abolished, with no effect on the endothelium-dependent contraction, in response to acetylcholine, arachidonic acid, adenosine diphosphate, the calcium ionophore A-23187, mechanical stretching, or hypoxia. Therefore, endothelin-predominant vasoconstriction may occur in cerebral arteries

following subarachnoid hemorrhage as a result of an imbalance between endothelin and NO. However, it is not clear whether NO synthesis is 'substrate limited' during vasospasm but not under basal conditions, because we did not measure the activity of NO synthase and the content of L-arginine in the endothelium of the basilar artery in the canine subarachnoid hemorrhage model. Nevertheless, two interesting results of the present study are the following: (1) the physiological role of endogenous endothelin-1 as a vasoconstrictor of the basilar artery under basal conditions was supported by the dilator effect of BQ-123 on the diameter of the normal basilar artery; and (2) endothelin-1-predominant vasoconstriction as a result of the imbalance between endothelin and NO due to the inhibition of NO synthesis was supported by the preventive effect of BQ-123 on N^G-nitro-L-arginine methyl ester-induced vasoconstriction. Thus, it is likely that an imbalance between endothelin and NO plays an important role in the pathogenesis of cerebral vasospasm even during the late phase.

In conclusion, our results strongly suggest that endogenous endothelin and NO may counteract one another's effects, and that they play an important role in the regulation of the cerebral vascular tone under basal conditions. Thus the genesis of cerebral vasospasm following subarachnoid hemorrhage may be, at least in part, due to an impairment of the balanced action of endothelin and NO. In addition, i.c. injection of either BQ-123 or L-arginine produced significant dilation of the basilar artery during cerebral vasospasm following subarachnoid hemorrhage in the two-hemorrhage canine model, although the effective duration of L-arginine activity was short. Thus, an endothelin ET_A receptor antagonist or NO products may be useful in the treatment of cerebral vasospasm following subarachnoid hemorrhage.

References

Angus, J.A. and T.M. Cocks, 1989, Endothelium-derived relaxing factor, Pharmacol. Ther. 41, 303.

Asano, T., I. Ikegaki, Y. Suzuki, S. Satoh and M. Shibuya, 1989, Endothelin and the production of cerebral vasospasm in dogs, Biochem. Biophys. Res. Commun. 159, 1345.

Brian, J.E., Jr. and R.H. Kennedy, 1993, Modulation of cerebral arterial tone by endothelium- derived relaxing factor, Am. J. Physiol. 264, H1245.

Chehrazi, B.B., S. Giri and R.M. Joy, 1989, Prostaglandins and vasoactive amines in cerebral vasospasm after aneurysmal subarachnoid hemorrhage, Stroke 20, 217.

Douglas, S.A., J.D. Elliott and E.H. Ohlstein, 1992, Regional vasodilation to endothelin-1 is mediated by a non-ETA receptor subtype in the anaesthetized rat-effect of BQ-123 on systemic haemodynamic responses, Eur. J. Pharmacol. 221, 315.

Ehrenreich, H., R.W. Anderson, C.H. Fox, P. Rieckmann, G.S. Hoffman, W.D. Travis and J.E. Coligan, 1990, Endothelins, peptides with potent vasoactive properties, are produced by human macrophages, J. Exp. Med. 172, 1741.

Faraci, F.M., 1990, Role of nitric oxide in regulation of basilar artery tone in vivo, Am. J. Physiol. 259, H1216.

Furchgott, R.F. and P.M. Vanhoutte, 1989, Endothelium-derived relaxing and contracting factors, FASEB J. 3, 2007.

Gold, M.E., J.M. Fukoto and L.J. Ignarro, 1990, Structural analogs of L-arginine cause endothelium-dependent and endothelium-independent, nitric oxide-mediated cyclic GMP formation and vasodilation, and cause vasoconstriction associated with decreased cyclic GMP formation, Eur. J. Pharmacol. 183, 1610.

Griffith, T.M., D.H. Edwards, M.J. Lewis, A.C. Newby and A.H. Henderson, 1984, The nature of endothelium-derived vascular relaxant factor, Nature 308, 645.

Hirata, Y., H. Yoshimi, S. Takata, T.X. Watanabe, S. Kumagai, K. Nakajima and S. Sakakibara, 1988, Cellular mechanism of action by a novel vasoconstrictor endothelin in cultured rat vascular smooth muscle cells, Biochem. Biophys. Res. Commun. 154, 868.

Hughes, J.T. and P.M. Schianchi, 1987, Cerebral artery spasm. A histological study at necropsy of the blood vessels in cases of subarachnoid hemorrhage, J. Neurosurg. 48, 515.

Ide, K., S. Ito, T. Sasaki, M. Yano and T. Kirino, 1993, Effects of ET_A receptor antagonist on normal and spastic canine basilar arteries, in: Cerebral Vasospasm, ed. J.M. Findlay (Elsevier, Amsterdam) p. 213.

Ihara, M., K. Noguchi, T. Saeki, T. Fukuroda, S. Tsuchida, S. Kimura, T. Fukami, K. Ishikawa, M. Nishikibe and M. Yano, 1992, Biological profiles of highly potent novel endothelin antagonists selective for the ET_A receptor, Life Sci. 50, 247.

Itoh, S., T. Sasaki, K. Ide, K. Ishikawa, M. Nishikibe and M. Yano, 1993, A novel endothelin ET_A receptor antagonist, BQ-485, and its preventive effect on experimental cerebral vasospasm in dogs, Biochem. Biophys. Res. Commun. 195, 969.

Kamata, K., H. Nishiyama, N. Miyata and Y. Kasuya, 1991, Changes in responsiveness of the canine basilar artery to endothelin-1 after subarachniod hemorrhage, Life Sci. 49, 217.

Katusic, Z.S., J.H. Milde, F. Consentino and B.S. Mitrovic, 1993, Subarachnoid hemorrhage and endothelial L-arginine pathway in small brain stem arteries in dogs, Stroke 24, 392.

Kim, P., T.M. Jr. Sundt and P.M. Vanhoutte, 1988, Alterations in endothelium-dependent responsiveness of the canine basilar artery after subarachnoid hemorrhage, J. Neurosurg. 69, 239.

Martin, W., G.M. Villani, D. Jothianandan and R.F. Furchgott, 1985, Selective blockade of endothelium-dependent and glyceryl trinitrate-induced relaxation by hemoglobin and by methylene blue in the rabbit aorta, J. Pharmacol. Exp. Ther. 232, 708.

Moore, P.K., O.A. Al-Swayeh, N.W.S. Chong, R.A. Evans and A. Gibson, 1990, L-N^G-nitroarginine (L-NOARG), a novel, L-arginine-reversible inhibitor of endothelium dependent vasodilatation in vitro, Br. J. Pharmacol. 99, 408.

Mosquedagarcia, R., T. Inagami, M. Appalsamy, M. Sugiura and R.M. Robertson, 1993, Endothelin as a neuropeptide-cardiovascular effects in the brainstem of normotensive rats, Circ. Res. 72, 20.

Nirei, H., K. Hamada, M. Shoubo, K. Sogabe, Y. Notsu and T. Ono, 1993, An endothelin ET_A receptor antagonist, FR139317, ameliorates cerebral vasospasm in dogs, Life Sci. 52, 1869.

Ohlstein, E.H. and B.L. Storer, 1992, Oxyhemoglobin stimulation of endothelin production on cultured endothelial cells, J. Neurosurg. 77, 274.

Palmer, R.M.J., A.G. Ferrige and S. Moncada, 1987, Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor, Nature 327, 524.

Palmer, R.M.J., D.S. Ashton and S. Moncada, 1988, Vascular endothelial cells synthesize nitric oxide from L-arginine, Nature 333, 664.

Rees, D.D., R.M.J. Palmer, H.F. Hodson and S. Moncada, 1989, A specific inhibitor of nitric oxide formation from L-arginine attenuates endothelium-dependent relaxation, Br. J. Pharmacol. 96, 418.

Rosenblum, W.I., H. Nishimura and G.H. Nelson, 1990, Endothelium-dependent L-Arg and L-NAME-sensitive mechanisms regulate tone of brain microvessels, Am. J. Physiol. 259, H1396.

Sakuma, I., D.J. Stuehr, S.S. Gross, C. Nathan and R. Levi, 1988, Identification of arginine as a precursor of endothelium-derived relaxing factor, Proc. Natl. Acad. Sci. USA 85, 8664.

Salom, J.B., G. Torregrosa, M.D. Barbera, T. Jover and E. Alborch, 1993, Endothelin receptors mediating contraction in goat cerebral arteries, Br. J. Pharmacol. 109, 826.

Sasaki, T., S. Wakai, T. Asano, K. Takakura and K. Sano, 1982, Prevention of cerebral vasospasm after SAH with a thromboxane synthetase inhibitor, OKY-1581, J. Nurosurg. 57, 74.

Shigeno, T., T. Mima, M. Yanagisawa, A. Saitoh, A. Fujimori, R. Shiba and K. Goto, 1991, Possible role of endothelin in pathogenesis of cerebral vasospasm, J. Cardiovasc. Pharmacol. 17, (Suppl. 7), S480.

Sumner, M.J., T.R. Cannon, J.W. Mundin, D.G. White and I.S. Watts, 1992, Endothelin ET_A and ET_B receptors mediate vascular smooth muscle contraction, Br. J. Pharmacol. 107, 858.

Taylor, S.G. and A.H. Weston, 1988, Endothelium-derived hyperpolarising factor: a new endogenous inhibitor from the vascular endothelium, Trends Pharmacol. Sci. 9, 272.

Toda, N. and T. Okamura, 1991, Role of nitric oxide in neurally induced cerebroarterial relaxation, J. Pharmacol. Exp. Ther. 258, 1027.

Trezise, D.J., G.M. Drew and A.H. Weston, 1992, Analysis of the depressant effect of the endothelium on contractions of rabbit isolated basilar artery to 5-hydroxytryptamine, Br. J. Pharmacol. 106, 587.

Varsos, V.G., T.M. Liszczak, D.H. Han, J.P. Kistler, J. Vielma, P.M. Black, R.C. Heros and N.T. Zervas, 1983, Delayed cerebral vasospasm is not reversible by aminophylline, nifedipine, or papaverine in a 'two-hemorrhage' canine model, J. Neurosurg. 58, 11.

Yamaura, I., E. Tani, Y. Maeda, N. Minami and H. Shindo, 1992, Endothelin-1 of canine basilar arteries in vasospasm, J. Neurosurg. 76, 99.

Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto and T. Masaki, 1988, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, Nature 332, 411.

Yoshimoto, S., Y. Ishizaki, A. Mori, T. Sasaki, K. Takakura and S. Murota, 1991, The role of cerebral microvessel endothelium in regulation of cerebral blood flow through production of endothelin-1, J. Cardiovasc. Pharmacol. 17 (Suppl. 7), S260.