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EFFECT OF ENDOTHELIN-1 RECEPTOR ANTAGONIST BQ-123 ON BASILAR ARTERY DIAMETER AFTER SUBARACHNOID HEMORRHAGE (SAH) IN RATS.

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Aim of the study was to quantify cerebral vasospasm in rats after subarachnoid hemorrhage (SAH) by morphometric examination of basilar artery and to evaluate the influence of endothelin receptor blocker BQ-123 on basilar artery constriction. The rat cisterna magna (CM) was cannulated and after 7 days SAH was developed by administration of 100µl autologic, non-heparinized blood to the CM. The sham subarachnoid hemorrhage was developed by intracisternal administration of 100µl of artificial cerebrospinal fluid. Endothelin receptor blocker BQ-123 was injected into the CM in a dose of 40nmol diluted in 50µl of cerebrospinal fluid 20 min. before SAH, and 24h and 48h after SAH. After perfusion fixation the brains were removed from the skull and histological preparations of basilar artery were done. The internal diameter and wall thickness of basilar arteries was measured by interactive morphometric method. The most severe vasospasm was found in rats after SAH. The presence of numerous infiltrations composed of neutrophils and macrophages correlated with advanced vasospasm (index of constriction 5 times lower than in normal), suggesting the role of other factors participating in the late phase of vasospasms after SAH. Administration of BQ-123 in the late phase after SAH caused the dilatation of basilar artery. Following the administration of BQ-123 in the late phase (48h after SAH) the basilar artery dilated, its wall became thinner, and the number of leukocyte infiltrations in the subarachnoid space decreased compared to the values after SAH alone.

K e y   w o r d s: *subarachnoid hemorrhage, cerebral vasospasm, endothelin-1, antagonist ET_A receptors — BQ-123, basilar artery diameter.*

INTRODUCTION

Factors involved in the development of prolonged cerebral vasospasm after subarachnoid hemorrhage (SAH) include a number of spasmogenic substances generated as a result of endothelial damage or hypoxia.

One of the major vasoconstrictors after SAH is endothelin — 1 (ET_1) (1, 2).

Under normal conditions, it is known to regulate vascular smooth muscle cell tone by increasing myocyte contractility (3). In vessels with damaged endothelium, regardless of the cause, the action of endothelin is potentiated due to reduced amounts of vasodilating substances, e.g. NO, whereas inflammatory and immunologic reactions cause enhancement of endothelin production. As reported previously (2, 4, 5), the levels of ET_1 in the blood and cerebrospinal fluid (CSF) after SAH are markedly elevated and vasospasm persists longer compared to the transient action of other vasoconstricting agents such as serotonin, thromboxane and leukotrien C_4 (6, 7, 8).

Of the three endothelin isoforms, ET_1, ET_2 and ET_3, endothelin — 1 has the most potent constrictor effect on cerebral vessels. Endothelins are mainly formed in vascular endothelial cells and act through three receptor subtypes, ET_A, ET_{B1} and ET_{B2}, localized in vascular smooth muscle cells and ganglionic neurons (9,10). The receptors differ in molecular weight, ET_1 having the highest weight (50,000—70,000). Endothelin — 1, when bound to ET_A receptor subtype present in smooth muscle myocytes, shows the highest affinity for NH_2 groups and the most potent constrictor effect on both large cerebral arteries and microvessels. Interaction of ET_1 and ET_3 with ET_{B1} produces only a mild, transient vasodilation, while ET_1 binding to ET_{B2} causes constriction of the vessels. Both ET_B receptor subtypes have lower affinities for endothelins than ET_A (11).

It seems that prolonged vasospasm due to an increase in blood endothelin level after SAH produces endothelial cell hypoxia which induces a secondary release of endothelin, and the resultant positive feedback further aggravates cerebral ischemia (1)

The present study was designed to examine basilar artery contractility in subarachnoid hemorrhage and the effect of intraventricular administration of a selective ET_A receptor antagonist, cyclic pentapeptide BQ-123.

MATERIALS AND METHODS

The study was performed on male Wistar rats weighing 220 and 250g.

The procedures were approved by the Bioethics Committee of the Silesian Medical University (NN—5-089/98). The animals were housed in couples in cages under controlled standard conditions (temperature $20°$—$22°C$, humidity 50—60%, lights on from 06:00 to 18:00) and had free access to standard food (obtained from „Murigran", Motycz Factory) and tap water.

All the experiments were performed between 14:00 and 16:00 on animals anesthetized with intraperitoneally injected Ketamine in a dose of 100 $mg \times kg^{-1}$.

Cannulation of the brain cisterna magna (CM) was performed according to the technique by Solomon *et al.* (12), with a slight own modification.

Subarachnoid hemorrhage (SAH) was induced by administering nonheparinized fresh autologous arterial blood (100µl) to CM through a previously inserted cannula. Arterial blood was collected with a 0.6mm Neoflon cannula from the axillary artery prepared under the operating microscope.

Sham SAH was induced by artificial CSF (aCSF) administered to CM.

CM cannulation was performed 7 days before establishing SAH and sham SAH by aCSF to ensure healing of lesion from cannulation.

The animals were divided into 7 groups of 8—10 rats each : 1) control group, 2) non SAH, 3) aSAH — sham SAH by aCSF, 4) SAH, 5)non SAH + BQ-123, 6) aSAH + BQ-123, 7)SAH + BQ-123.

BQ-123 — ET_A receptor antagonist (40 nmol in 50µl aCSF) was given to CSF through a cannula inserted to CM after aspirating the same amount of animal CSF from CM. BQ-123 was administered three times : 20 minutes before SAH, 24 hours after SAH and 48 hours after SAH. The same BQ-123 dose had also been used by other investigators (13).

Forty eight hours after completion of the above phase of experiment, the animals were anesthetized with Ketamine and the brains were removed for histologic examination.

Brain removal

After thoracotomy and exposure of the heart, a catheter was inserted into the left ventricle to which, under the pressure of 200 cm H_2O, 100ml of phosphatic buffer (PBS, pH 7.4) and then 200ml of fixative solution (1% glutaraldehyde and 4% paraformaldehyde dissolved in PBS) were administered. In such a way the vessels were washed and fixed and the excess fluids escaped through an incision in the right atrium.

Then, after excising the skin in the craniocerebral area and exposing the bones, the brain was removed and immersed in the fixative solution at 4°C. After 24 hours it was placed in cold PBS solution and subjected to histologic evaluation.

Histologic examination

The brainstem was dissected from the cerebral hemispheres, cut transversely at the middle of the pons and processed through alcohols and xylens to paraffin (Paraplast Xtra, Sigma). 5µm paraffin sections were stained with hematoxyllin and eosin. The morphometric examination was performed with image analysis system from Leica with original software (Qwin). The measurements were done at the magnification of $100\times$. In each case three transverse sections of the basilar artery from its middle third portion were evaluated. The arterial wall thickness and internal vessel diameter were measured interactively and the index of constriction defined as the ratio of the mean diameter of the lumen to the mean thickness of the arterial wall exclusive of the adventitia. The index of constriction was calculated according to Duff *et al.* (14).

Statistical analysis

The values were expressed as a group mean $\pm$ SEM. The statistical analysis of differences was performed using Student's t-test for unpaired variables. Significance was accepted at $p < 0.05$.

RESULTS

Results of basilar artery measurements in all animal groups, expressed as indexes of constriction, are given in *Fig. 1*. The index value calculated from the ratio of the mean diameter of the lumen to the mean thickness of the arterial wall, correlates with the change in vessel diameter. The reduced diameter indicates an increase in vascular contractility.
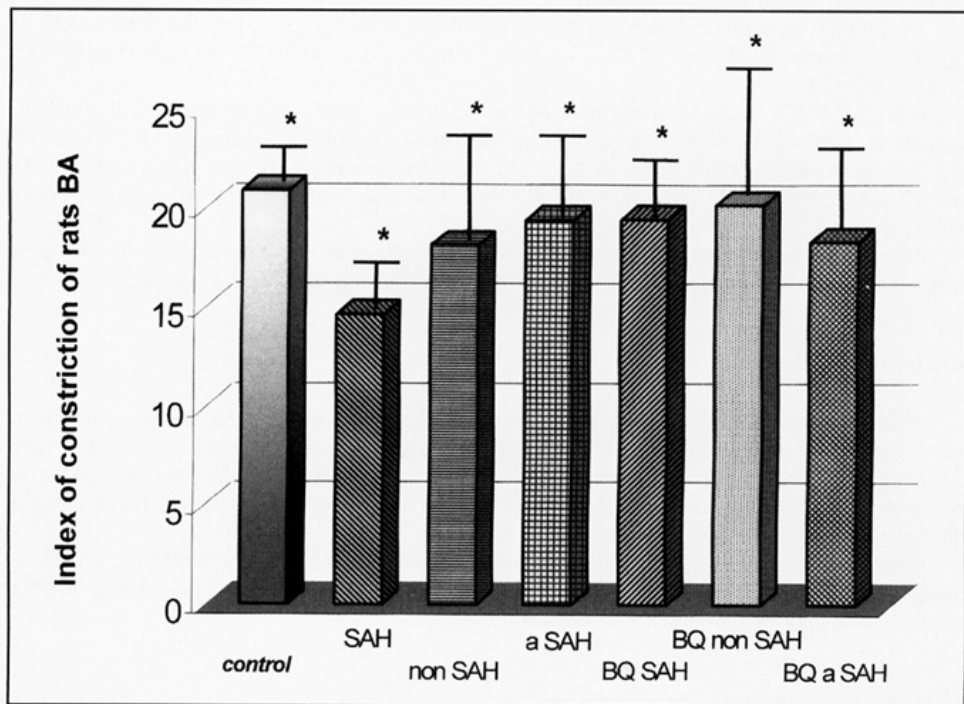

Fig. 1. Effect of endothelin — 1 receptor antagonist BQ-123 on basilar artery diameter after SAH. Asterisk indicates significant difference at $p < 0.05$ (all in relation to SAH).

Microscopic sections of cerebral basilar artery in controls, animals with chronic phase of vasospasm (48h after SAH) and those administered BQ-123 are shown in *Fig. 2*$_{A,B,C}$.

In the control group, the index of constriction was 20.88 ± 1.68, the wall thickness was 12.29 and the internal diameter was 256.06 (*Fig. 1 and Fig. 2*$_A$)

In animals with SAH, the index value was found to be 14.66 ± 1.96, the wall thickness was 16.01 and the internal diameter was 235.02 (*Fig. 1*), the difference being highly statistically significant ($p < 0.0001$). *Fig. 2*$_B$ shows a marked decrease in the vessel diameter as well as thickening of the arterial wall with endothelial cell protrusion and corrugation of the internal layer. Monocyte infiltrations and scarce neutrophils can be seen in the subarachnoid space.
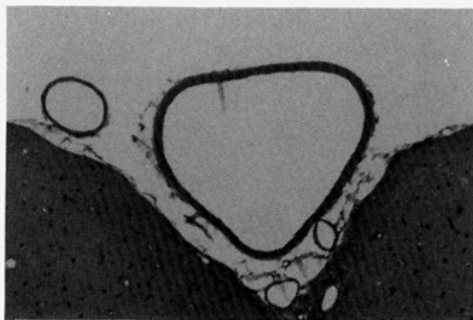
Fig. 2_A. **Control group**
Basilar artery of the rat. Index of constriction = 20.88, the wall thickness was 12.29 and the internal diameter was 256.06. Staining HE, 200×.
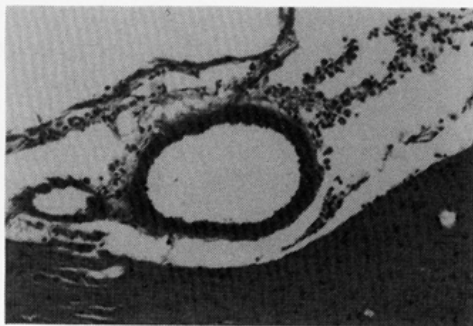

Fig. 2_B **SAH**
The wall of basilar artery is markedly thickened with narrowing of the lumen. Numerous macrophages in subarachnoid space. Index of constriction = 14.66, the wall thickness was 16.01 and the internal diameter was 235.02. Staining HE, 200x.
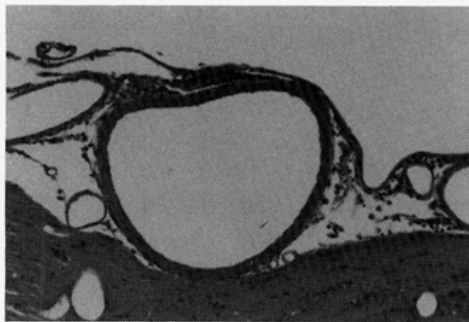

Fig. 2_C **SAH + BQ-123** — ET_A receptors antagonist
Slighty thickened wall of the basilar artery, and the number of leukocyte infiltrations in the subarachnoid space decreased compared to the values after SAH alone. Index of constriction = 19.45, the wall thickness was 13.86 and the internal diameter was 269.2. Staining HE, 200×.

In animals subjected to intracisternal administration of BQ-123 48h after SAH the index value was 19.45 ± 2.12, the wall thickness was 13.86 and the internal diameter was 269.2 (*Fig. 1 and Fig. 2*_C), which was similar to the control value but statistically significantly different ($p < 0.05$) from that in the SAH group, thus indicating relaxation of the artery. *Fig.* 2_C demonstrates the return of the vessel to its normal diameter and cessation of the vasoconstrictor effect. The arterial wall is slightly thickened and there are only scarce monocytes in the subarachnoid space.

Cannulation of the cisterna magna (nonSAH group) and cisternal application of artificial cerebrospinal fluid (aSAH group) appeared to have little effect on arterial diameter, the index values being 18.14 ± 4.74 and 19.40 ± 3.20, respectively (*Fig. 1*).

Similarly, no significant differences from the control value were observed in the nonSAH and aSAH animals after cisternal administration of BQ-123. The respective indexes of constriction for the two groups were 20.16 ± 5.70 and 18.31 ± 3.49 (*Fig. 1*).

It was found that animals with SAH had the highest increase in basilar artery contractility and the constriction could by reversed by blocking ET_A receptors.

DISCUSSION

The present study has demonstrated that the chronic phase of SAH — induced vasospasm causes pronounced narrowing of cerebral basilar artery, vascular wall thickening and a decrease in luminal diameter.

The decrease in the index of constriction compared to the control value is significant ($p < 0.0001$) and indicates enhanced myocyte contractility.

Prolonged vasospasm may be due to endothelial damage and a secondary release of endothelin -1 which acts directly on the muscular layer.There is considerable evidence that endothelin -1 has a long-lasting constrictor effect on both large arterial trunks and arterioles (15, 16, 17, 18).

Arterial middle layer constriction after SAH is accompanied by numerous morphologic changes in the arterial wall, mostly within endothelial and smooth muscle cells. The changes, known collectively as posthemorrhagic arteriopathy, include vacuolization and necrosis of myocytes, middle layer fibrosis, internal layer edema, and proliferation of endothelial cells (19, 20).

However, stereologic examinations have revealed that the decrease in arterial lumen in SAH — induced vasospasm is primarily due to muscle layer constriction, the arterial wall changes playing a minimal role (19).

The present study has shown posthemorrhagic endothelial protrusion and corrugation of internal elastic lamina, as well as monocyte infiltrations and scarce neutrophils in the subarachnoid space. Their presence indicates inflammatory reaction which, even in the absence of erythrocytes, may be responsible for the development of cerebral vasospasm (21).

It is possible that immunological mechanisms play a role in progressive vascular changes after SAH. The mechanisms are activated by factors released from blood clots in the subarachnoid space. As a result, proinflammatory cytokins such as interleukin -1β (IL-1β), interleukin -6 (IL-6) and tumor necrosis factor α (TNFα) are released along cerebral vascular trunks (22). These cytokins also have an effect on inflammatory processes, as shown by an increase in the number and differentiation of leukocytes. Interleukin-1β has a direct stimulatory effect on endothelin synthesis at the mRNA transcription level, while IL-6 inhibits the formation of prostaglandin I_2 (PGI$_2$), thus preventing vasodilation (23, 24).

Experiments in animals have also revealed the mitogenic effect of endothelin-1 on macrophages whose accumulation may be the source of ET_1 synthesis. In this way, ET_1 is involved in positive feedback (25).

More evidence of the role of endothelin in cerebral vasospasm is provided by studies demonstrating increased ET_1 production by endothelial cells exposed to oxyhemoglobin (26,27), a potent spasmogenic agent released from subarachnoid blood clots (28). Oxyhemoglobin is known to induce further production of ET_1 in vascular endothelial and smooth muscle cells (29).

The constrictor effect of ET_1 in prolonged SAH-induced vasospasm is confirmed by measurements of basilar artery diameter after intracisternal administration of ET_A receptor antagonist BQ-123. The receptor blockage reverses vasospasm and causes the return of the artery to its near-normal diameter, as shown by the index of constriction. Moreover, the arterial wall is only slightly thickened after BQ-123 application, the subarachnoid space contains single monocytes and there are no changes in endothelial cells. Thus, BQ-123 seems to prevent indirectly inflammatory changes in prolonged vasospasm.

Intracisternal administration of ET_A receptor antagonist BQ-123 appears to be the treatment of choice in preventing vasospasm and its consequences. Selective action of BQ-123 has been demonstrated by a complete reversal of vasospasm in isolated arteries. In the presented animal model there was a 93% reversal.

Experiments in animals and clinical trials have shown ET_1 to be the major vasoconstrictor, much more potent than ET_2 and ET_3 (30). Numerous attempts to use synthetic endothelin antagonists for ET_A and ET_B receptors, such as Bosentan and RO 46-2005 administered orally, intravenously and intraarterially, have proved less effective since these compounds decrease vasospasm by only 50%, despite their competitively antagonistic action on both receptor subtypes (31).

Vasospasm is abolished almost completely only when BQ-123 is given intraventricularly, whereas intravenous application is not effective (32). A full vasospasm reversal in the basilar artery and microvessels does not appear possible at increased blood levels of endothelin which potentiates the constrictor effects of other agents present in the blood, mainly vasopressin, noradrenaline and serotonin (17, 33). Besides, in damaged endothelium the vasodilator effect of NO is markedly reduced (6, 34). Thus, the vasoconstrictor effect of ET_1 depends not only on its dose but also on the state of vascular endothelium.

In conclusion, in prolonged SAH-induced vasospasm, endothelin-1 demonstrates a very potent constrictor effect on basilar artery smooth muscle cells, and this effect can be reversed by ET_A receptor antagonist BQ-123. Following the administration of BQ-123 in the late phase (48h after SAH) the

basilar artery dilated, its wall became thinner, and the number of leukocyte infiltrations in the subarachnoid space decreased compared to the values after SAH alone.

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