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INFLUENCE ENDOTHELIN ET$_A$ RECEPTOR ANTAGONIST — BQ-123 — ON CHANGES OF ENDOTHELIN-1 LEVEL IN PLASMA OF RATS WITH ACUTE VASOSPASM FOLLOWING SUBARACHNOID HEMORRHAGE

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Endothelin participates in regulating the vascular tone, and it is also involved in the pathogenesis of vasospasm following subarachnoid hemorrhage (SAH). Endothelin-1 (ET-1) induced cerebral vasospasm is inhibited by ET$_A$ receptors specific antagonist-BQ-123; this protects the neurons from ischemic damage. The present study evaluates the dynamics of ET-1 concentration changes in the plasma of rats in the acute phase of vasospasm after SAH, which was induced by administering 100 μl non-heparinized fresh autologous arterial blood into the brain cisterna magna (CM). The study also assesses the effect of blocking ET$_A$ receptors on the changes in ET-1 level. BQ-123, the specific ET$_A$ receptors antagonist, was administered to cerebrospinal fluid (CSF) through a cannula inserted into CM; the antagonist — 40 nmol in 50μl CSF — was given 20 minutes prior to SAH. In the control group, sham SAH was induced by administering 100 μl artificial CSF (aCSF) to CM. ET-1 concentration in the plasma of rats in the acute phase of vasospasm was assessed by radioimmunoassay 30 and 60 minutes after SAH or sham SAH. It has been showed that both SAH and sham SAH cause significant increase in the ET-1 concentration (p < 0,05) in the rat plasma after 30 minutes; the concentration returns to an initial value after following 30 minutes, which may suggest that ET-1 released binds to its receptors in the acute phase of the vasospasm. On the other hand, in the two groups of rats with blocked ET$_A$ receptors there was a significant rise in ET-1 concentration 30 minutes after SAH or sham SAH, and a still further rise was observed 60 minutes after the procedure. The rise was significantly higher in animals with SAH (p < 0,05). The dynamics of the ET-1 concentration changes observed in rats with blocked ET$_A$ receptor suggests that SAH is an ET-1 production stimulator significantly more potent than other factors assessed in the study, such as a rise in the intracranial pressure resulting from administering aCSF to CM. Blocking ET$_A$ receptors makes it impossible for the ET-1 released to bind to the receptors, which may be a factor preventing the occurrence of cerebral vasospasm following SAH

INTRODUCTION

In physiological conditions in healthy people endothelin-1 (ET-1) is secreted in very small concentrations in plasma (0.3 — 3.0 $pg \times ml^{-1}$), and it participates in maintenance of the normal vascular tone (1). In pathological conditions, in numerous disorders leading to vascular endothelium damage, ET-1 concentration increases considerably. Subarachnoid hemorrhage (SAH), for example, is followed by an ET-1 level increased three- to five-fold (2). The reasons for such a rise in the ET-1 concentration are different factors stimulating ET-1 secretion; among these the most significant are oxyhemoglobin (oxyHB) (3), free radicals and peroxide anions (4) which directly damage the cells of the vascular endothelium. ET-1 released in large amounts may be a factor causing cerebral vasospasm following SAH (5—8). Such an ET-1 effect was observed in large cerebral vessels where ET_A receptors are the responsible for the constrictor response (9). There is less information available on the ET-1 effect on cerebral microcirculation. In the studies using the visualisation of the cerebral microcirculation, the focus was on the changes in the vascular diameter following administration of ET-1 and ET-3. ET-1 resulted in an intensive spasm of the cerebral vessels, whereas ET-3 had no such effect (10). Additional evidence confirming ET-1 role in vasospasm after SAH, comes from the studies using ET-1 receptors antagonists (11—15), metalloprotease inhibitors, which suppress big-ET-1 conversion to ET-1 (16), and RNA synthesis inhibitors (17). It fails to be clear whether other factors, an increased intracranial pressure, for instance, contribute to a bigger ET-1 secretion to an equal extent, and what the dynamics of the changes is in comparison with ET-1 secretion in the acute phase of vasospasm after SAH. In order to assess the role of other factors stimulating ET-1 secretion, the present study examines the changes in the ET-1 concentrations in rat plasma after SAH, and after sham SAH induced by administering an equal quantity of aCSF to CM. The changes noted are compared to the values obtained in animals in which, prior to the experiment, ET_A receptors were blocked with its specific antagonist — BQ-123. The aim of the present study is to assess the effect of the two factors, i.e. intracranial pressure rise and SAH, on the ET-1 secretion in rat plasma, and to observe the dynamics of changes in the ET-1 secretion in the acute phase of vasospasm.

MATERIALS AND METHODS

The study was performed on Wistar male rats weighing between 220 and 250g. The Bioethical Committee of the Silesian Medical University (NN-043-395/97) granted the permission for the study. The animals were housed in couples in cages under controlled standard microclimate conditions (temperature 20—22° C, humidity 50—60%) and illumination (light 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 dose of 100 $mg \times kg^{-1}$.

Cannulation of the brain cisterna magna (CM) was performed according to the technique described by Solomon co-workers (1985) (18) with a slight modification added by the authors of the present study, and described previously (19).

Subarachnoid hemorrhage (SAH) was induced in rats by administering non-heparanised fresh autologous arterial blood (100 μl) to CM through a previously inserted cannula. Arterial blood from animals was collected with a 0.6mm Neoflon cannula from axillary artery prepared in the operating microscope. In the control group, sham SAH was induced by aCSF, prepared according to rules given by (20), and administered to CM.

CM cannulation was performed 7 days before establishing SAH and sham SAH by aCSF. During that time all the injury caused by the procedure and CM cannulation subsided.

The animals were divided into 4 groups with 8—10 rats each: 1) — with SAH, 2) — with sham SAH by aCSF, 3) with BQ-123 — ET_A receptors antagonist — given to animals 20 min. before SAH, 4) with BQ-123 — ET_A receptors antagonist — given to animals 20 min. before sham SAH by aCSF.

BQ-123 — ET_A receptors antagonist — (40nmol in 50 μl aCSF) was given to CSF through a cannula inserted to CM after aspirating the same amount of animal's own CSF from CM.

In all groups the venous blood (2ml) was drawn from the orbital vascular plexus, and in the blood plasma the ET-1 concentration was measured. The blood was taken three times: before the experiment, 30 minutes after SAH and sham SAH, and 60 minutes after SAH and sham SAH. The blood was placed in test tubes with EDTA ($1mg \times ml^{-1}$) and Aprotinin ($500 KIU \times ml^{-1}$) added. The specimens were immediately cooled to about $0°C$ and centrifuged (5 min. at 3000 rpm). The plasma obtained was stored for further investigation for 2 weeks at $-25°C$.

ET-1 concentrations in the plasma specimens were measured by radioimmunoassay using Peninsula RIA kits (Endothelin (Rat) Peninsula Laboratories RIK 6901). Peptides were isolated from the plasma by column chromatography on SEP Columns — $C18-200mg$.

Statistical analysis: the results were calculated as a group mean $\pm$ SEM. The statistical analysis of differences was performed using Student's t-test for unpaired variables. A p value of < 0.05 was considered to be significant.

RESULTS

Mean ET-1 concentration assayed in the rat plasma before the experiment in the group without ET-1 antagonist was 8.48 $pg \times ml^{-1} \pm 1.78$ and in the group with BQ-123 — 10.5 $pg \times ml^{-1} \pm 3.85$. In the plasma rats groups without BQ-123, 30 min after SAH induction, and in the control group after injecting aCSF to CM, mean ET-1 concentrations increased and were 19.9 $pg \times ml^{-1}$ ± 11.87 and 25.4 $pg \times ml^{-1} \pm 13.56$, respectively (*Fig. 1*). In both groups the increase in the ET-1 concentrations was statistically significant ($p < 0,05$ and $p < 0,005$; *Fig. 1*). 60 min after SAH and sham SAH there was a considerable decrease in the mean ET-1 concentrations in both groups: 8.9 $pg \times ml^{-1} \pm 2.5$ and 13.58 $pg \times ml^{-1} \pm 3.98$, respectively (*Fig. 1*). In both groups the ET-1 concentrations decrease was not statistically significant, but the differences between the ET-1 concentrations in both groups in that period showed statistical significance ($p < 0.05$; *Fig. 3*).
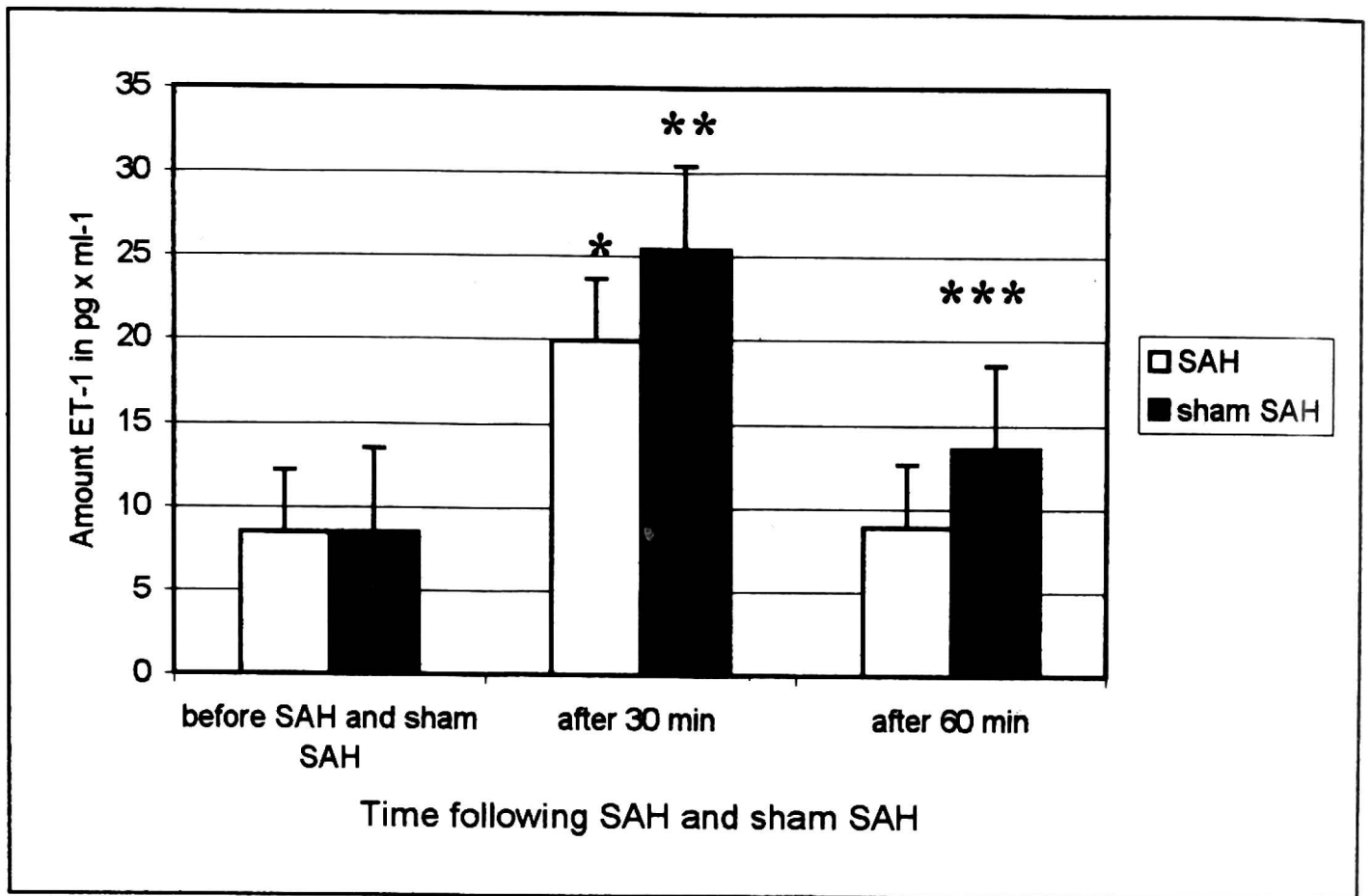
Fig. 1. Bar graphs showing dynamics of changes in the ET-1 concentrations in the rats plasma groups without ET_A antagonist after SAH and sham SAH by aCSF. In both groups there was a statistically significant increase in the ET-1 concentration 30 min after SAH and sham SAH (*-$p < 0.05$ and **-$p < 0.001$ respectively). 60 min after SAH and sham SAH there was a decrease in the ET-1 concentrations in both groups but the decrease was not statistically significant in comparison with the values noted after 30 min. Also the ET-1 concentration noted after 60 min in the SAH group and compared with the concentration observed before the procedure did not differ statistically. In the sham-SAH group, in spite of a decrease noted, the ET-1 concentration remained significantly higher in comparison with the concentration measured before the procedure (***-$p < 0.05$).

The dynamics of changes in ET-1 concentrations in the two groups of rats which were given ET_A antagonist 20 min before SAH and aCSF-induced sham SAH, was considerably different in comparison with groups of rats which were not pretreated with BQ-123. The ET-1 concentrations decrease, which was noted in the groups with no ET_A antagonist, was not observed after the period of a statistically significant increase in ET-1 concentrations present in both groups of animals 30 min after SAH and sham SAH. In fact, ET-1 concentration further increased in the animals with SAH 60 min after the hemorrhage; and the increase noted was statistically significant ($p < 0.05$; *Fig. 2*). In the group with sham SAH, however, there was a slight decrease in the ET-1 level which showed no statistical differences compared with the ET-1 level after 30 min (*Fig. 2*). Mean ET-1 values in the group with SAH were 28.86 $pg \times ml^{-1} \pm 9.87$ in 30 min after SAH, and 40.57 $pg \times ml^{-1} \pm 12.7$ in

60 min. after SAH; and in the group with aCSF the concentrations were $31 \text{ pg} \times \text{ml}^{-1} \pm 13.66$ in 30 min after sham SAH, and $27.17 \text{ pg} \times \text{ml}^{-1} \pm 10.05$ in 60 min after sham SAH; the values noted were significantly higher than ET-1 concentrations measured in groups of rats with no ET_A antagonist (*Fig. 2*).
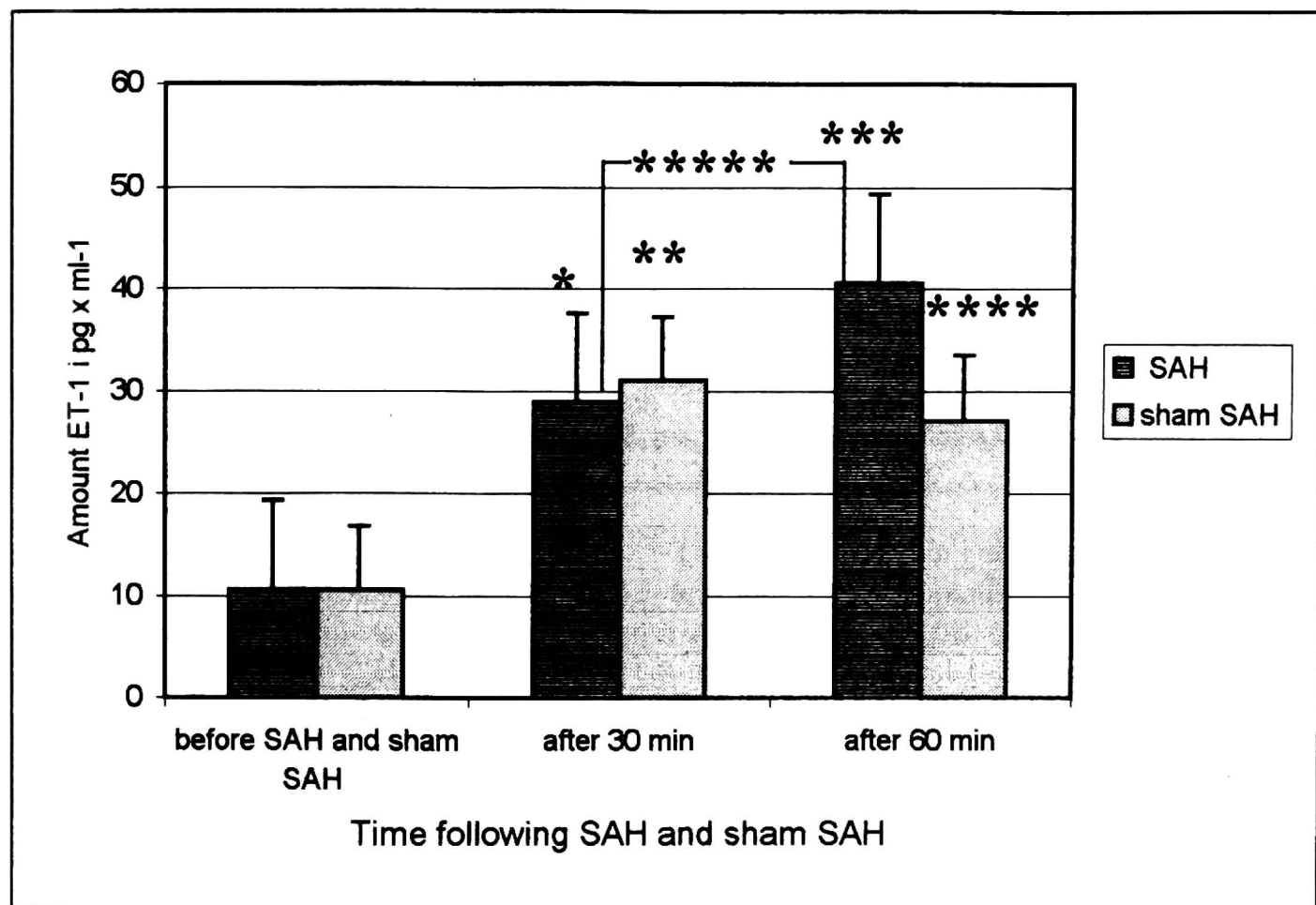

Fig. 2. Bar graphs showing dynamics of changes in the ET-1 concentrations in the plasma of rats with SAH and sham SAH by aCSF, with ET_A antagonist (40 nmol BQ-123 in 50 μl CSF) administered through a cannula inserted into CM 20 min. before the experiment. 30 min after SAH and sham SAH there was a statistically significant increase in the ET-1 concentrations in both groups (*-p$<$0.001 and **-p$<$0.0005- respectively). After 60 min., in the SAH group, there was a further ET-1 level increase, which was statistically significant in comparison with the level after 30 minutes (*****-p$<$0.05), and, in the sham-SAH group, there was a slight, insignificant ET-1 level decrease present. ET-1 concentrations in the rat plasma 60 min after SAH and sham SAH were statistically significantly higher in comparison with the concentrations noted before the experiment (***-p$<$0.005 and ****-p$<$0.005- respectively).

Mean ET-1 values in plasma in the two groups with ET_A antagonist were similar to each other and did not differ statistically 30 min after SAH and sham SAH, whereas 60 min. after the procedures there was a slight decrease in ET-1 level in the sham-SAH group, and a further significant increase in ET-1 level in the SAH group; the comparison of the ET-1 values in both groups revealed a statistically significant difference ($p<0.05$; *Fig. 3*).
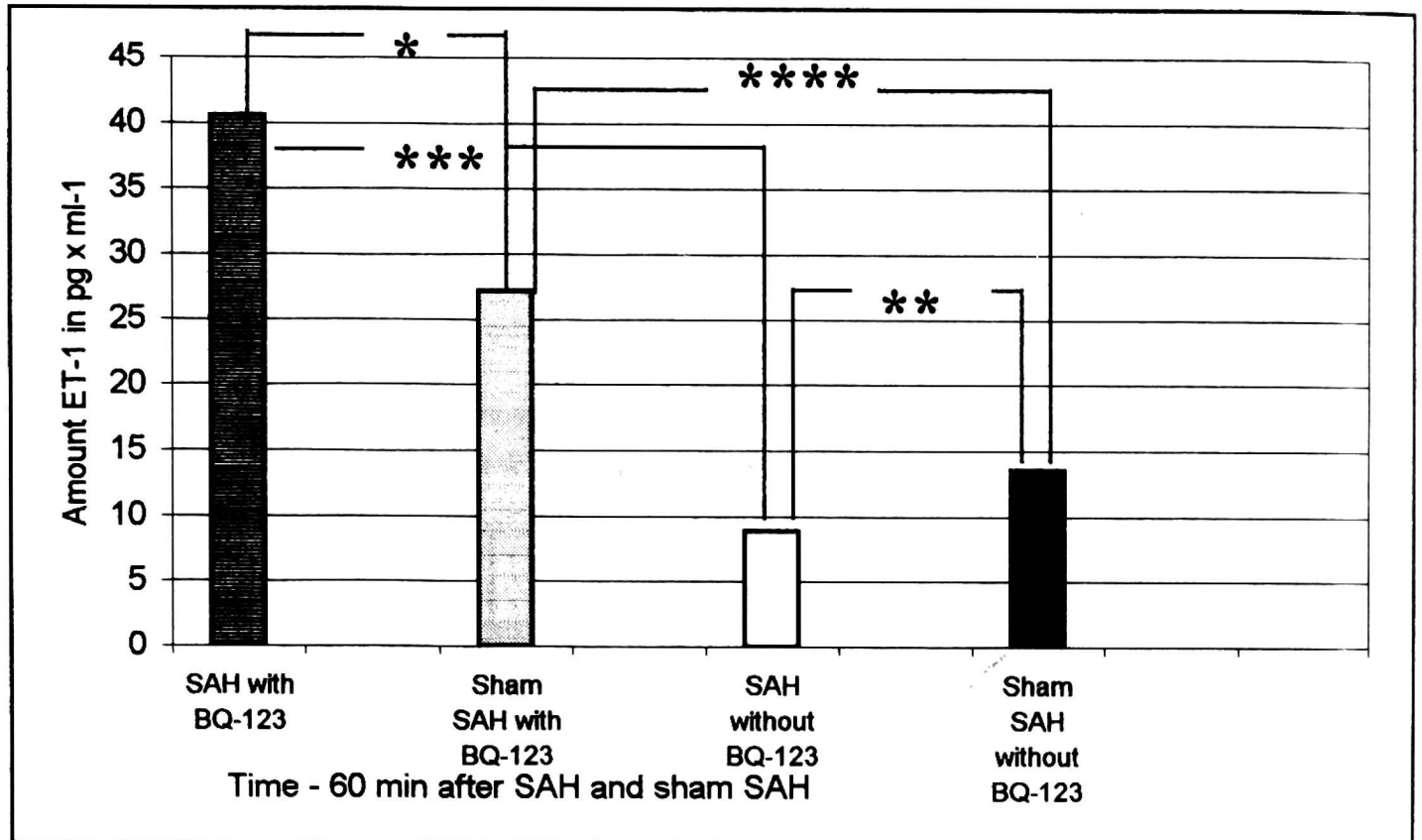
Fig. 3. Bar graphs showing differences in the ET-1 concentrations in the plasma of rats pretreated with ET_A antagonist (40nmol BQ-123 in 50μl CSF), and in the plasma of rats without the antagonist, 60 min after SAH and sham SAH by aCSF. In the group with ET_A antagonist, a significantly higher ET-1 level was present in the animals with SAH (*-p<0.05), whereas in the animals without BQ-123, ET-1 level was significantly lower (**-p<0.05) than in the animals with sham SAH by aCSF. The mean ET-1 concentrations in the plasma of rats pretreated with ET_A antagonist in both groups 60 min after SAH and sham SAH was a statistically significant higher in comparison with level ET-1 noted in these groups animals, which not obtained BQ-123 (***-p<0.005 and ****-p<0.005 — respectively).

DISCUSSION

Both SAH and increased intracranial pressure, which resulted from administering aCSF to CM, doubled, and even tripled, the ET-1 concentration in the rat plasma. The increase was present 30 min after SAH, and, after further 30 min the ET-1 concentration returned to the value similar to the one noted before the hemorrhage, that is in the period of acute vasospasm. Also, in the sham-SAH group, the ET-1 concentration, with the highest level noted after 30 min, decreased considerably after 60 min, but it was still higher than in the group with SAH. Enhanced ET-1 production results from the vascular epithelium injury, and the ET-1 present in blood is only a small part of the amount secreted. Considerably bigger ET-1 amounts permeate the nearest vascular wall environment where ET-1 binds to the receptors situated on the cellular membranes of the smooth muscles (21). The binding leads to acute vasospasm, and the plasma ET-1 concentration decrease observed in the experiment in that period may be due to the fact that ET-1 released binds to its

ET_A receptor. However, there are also additional factors contributing to the elevated ET-1 concentration in plasma: these are agents secreted from the clots in the blood extravasated to subarachnoid cisterns — and oxyHb is the most important of such factors. OxyHb is a potent spasmogenic agent, which stimulates ET-1 synthesis, and it is considered to be the main factor causing acute vasospasm following SAH (22). In the sham SAH there are no main factors damaging the epithelium cells, which may be an explanation for the fact observed in the present study i.e. for a decreased binding of ET-1 released to its receptors and, consequently, higher ET-1 level in the rat plasma 60 min after the SAH.

Such an explanation seems to be confirmed by the results obtained in the rats which were given ET_A receptors antagonist — BQ-123 — before SAH and sham SAH. The levels of the ET-1 released rose abruptly after 30 min, and later, after 60 min, the levels kept rising significantly; it must be added, though, that in the rats with SAH the ET-1 level was statistically significantly higher. The results obtained demonstrate that SAH, which causes endothelial damage by the action of many different factors released from the clots, stimulates ET-1 production more intensively than a rise in the intracranial pressure itself caused by administering aCSF. Also, ET-1 concentration in plasma keeps rising because ET-1 cannot bind to its specific ET_A receptor as the receptor is blocked by its antagonist — BQ-123.

ET-1 produced in excess as a result of a SAH vascular epithelium damage, is a peptide belonging to factors which regulate the vascular tone and diameter, and are characterized by the most effective contractile action. Increased ET-1 concentration after SAH may stimulate further ET-1 production due to a feedback loop present, and it may be a reason for a growing vasospasm leading to cerebral ischemia (2).

A prolonged endothelin action is also caused by a slow, a sting up to 48 hours, dissociation of these peptides from ET complex — ET_A and ET_B receptors (23). The use of selective inhibitors of the enzyme converting big-endothelin to ET-1, and application of ET-1 receptors antagonists may prevent a vasospasm after SAH, and protect neurons from being ischemically damaged (24, 25).

As it has been shown in the experiment, an increased intracranial pressure itself, which tends to subside quickly, may be a reason for a considerable ET-1 production. Already in the period of a growing acute vasospasm, however, the ET-1 level was lower in comparison with the ET-1 concentration in rats after SAH. Blocking the ET_A receptors may have an effective action preventing the ET-1 released from being bound, and it may also prevent the occurrence of acute vasospasm. It must be added, though, that the action of ET_A receptors antagonist is effective only if it is administered to CSF intracisternally, and not intravenously (25). While considering the usefulness of the method, it should be

also noted that the specific ET$_A$ receptors antagonist ought to be administered almost immediately after SAH in order that the acute vasospam dependant on ET-1 does not develop.

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