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INFLUENCE OF THE CORTICOTROPIN RELEASING HORMONE (CRH) ON THE BRAIN-BLOOD BARRIER PERMEABILITY IN CEREBRAL ISCHEMIA IN RATS

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The increase in the blood-brain barrier (BBB) permeability and a developing cerebral oedema due to the ischemic infarction appear a few hours, and intensify during a few days, after closing the carotid arteries. It fails to be clear, however, what causes the increase in the microvessels damage, and whether the damage is a secondary result of the vasoactive substances released by the neurones and glia cells damaged by the ischemia. CRH, which plays an essential role in integrative the nervous, endocrine, and immunological systems, has a positive effect on the decrease in the permeability of the BBB damaged by various physical and chemical factors. Therefore, the examination of the CRH role in the cerebral ischemia may prove useful for explaining the processes taking place in the foci of the cerebral infarction and their environment. The experiment was carried out on rats which, 20 minutes before closing of both internal carotid arteries, was administered 10μg CRH to cerebrospinal fluid via cisterna magna of the brain. The BBB permeability was measured 30 minutes, 3 hours, 3 days, and 7 days after closing the arteries. The experiment has shown the CRH protective effect on the BBB and its consequent effect on the decrease in the BBB permeability which appears in the 3 hours after closing the arteries ($p<0.05$), and is high significant during the chronic phase of the cerebral ischemia ($p<0.03$). It can be thus concluded that CRH, by affecting directly the endothelium of the cerebral vessels, decreases the endothelial damage in the acute phase of the ischemia. The decrease is noted to be more significant in the chronic phase of the ischemia; such an effect can be attributed to CRH stimulating the hypothalamic-adrenal axis, and to the secondary activation of the mechanisms decreasing the BBB permeability.

Key words: *cerebral ischemia in rats, BBB permeability, and influence of the CRH*

INTRODUCTION

CRH, produced in the paraventricular nucleus of the hypothalamus, plays an essential role in the mechanisms of cooperative between the nervous, the endocrine, and the immunologic systems (1-4). CRH has also been shown to have a positive effect on decrease in the permeability of the blood-brain barrier (BBB) damaged by various factors (5-8). Also, CRH has been proven to directly effect a decrease in endothelial permeability in a network of fine peripheral vessels, and in inflammatory oedema resulting from the activity of different physical and chemical factors (6, 8-10). The same positive CRH activity has been noticed in rat cerebral cortex vessels damaged by low temperature (5).

Increase in the BBB permeability and a developing cerebral oedema caused by ischemic infarction is apparent in a few hours, and increase in a few days (11). It fails to be clear, however, what causes the increase in the damage, and whether the damage is a secondary result of vasoactive substances released from neurones and glia damaged by ischemia (12). Increase in the BBB permeability may also result from the production and activity of vasoactive neurotransmitters or eicosanoids (13,14). Increase in the BBB permeability for proteins have been shown to depend on hypercapnia, during which protein extravasation is directly correlated with the increase in $PaCO_2$ (15). It has been suggested that chronic BBB damage and vasogenic oedema may both be a natural result of inflammatory reaction to ischemic cerebral damage. Increase in the BBB permeability may be attributed to leukoterienes and other substances produced as the result of lipid peroxidation, arachidonic acid release and its oxidative metabolism, and thrombocytes and leukocytes accumulation in damaged tissues (3, 16,17).

Subarachnoid hemorrhage (SAH) in men and in experimental animals leads to changes in BBB permeability in large vessels (7, 13, 18, 19) and in the network of cerebral microvessels (20,21). A correlation between the increase in the BBB permeability and critical neurological condition is present in SAH patients with poor prognosis. Lipid peroxidation is considered a basic cause of the damage of the endothelium and the increase in the BBB permeability (21,22). Our previous research has demonstrated that CRH given to cerebrospinal fluid (CSF) in rats 20 minutes before SAH, significantly decreases the BBB permeability during chronic vasospasm characterised by increase in the ischemia focuses in the brain (7). It can be assumed that CRH administered to CSF will have a positive effect on changes in BBB permeability in different phases of cerebral ischemia caused by carotid artery closure. Vasospasm resulting from SAH, similarly to changes caused by carotid artery closure, leads to cerebral ischemia and increase in the BBB permeability for proteins (11,23). The present research is aimed at better understanding of the role of CRH in these processes and their results.

MATERIALS AND METHODS

Animals

The experiments were carried out after the researchers had been granted the permission from Bioethical Board of Silesian Medical University (NN-043-49/95). Subjects were male Wistar rats weighing 220-250 g. The animals were divided into groups of 2 per cage and maintained on a 12-hour light/dark cycle in a temperature and humidity controlled environment (18°C, 70%) and had free access to standard food and tap water. All the experiments were carried out between 2 p.m. and 4 p.m. on animal's anaesthetised intraperitoneally with Ketamine (100 mg × kg^{-1} of body weight).

Technique of cisterna magna (CM) cannulation

Cannulation of the brain cisterna magna (CM) was performed according to the typical technique with a slight modification added by the authors as described previously (23).

The cannula was used for administering CRH to CSF 20 minutes before carotid arteries closure. The control group was administered 0.9% NaCl solution in the same way.

Technique of evoking cerebral ischemia

In anaesthesia evoked by peritoneally administered Ketamine in an earlier established dose, carotid arteries were bilaterally revealed, and, after dissection of both internal carotid arteries, they were given microarterial clamps in order to close them and thus evoke ischemia of both cerebral hemispheres. Finally, the sheaths were sewn together.

Study protocols

The cisterna magna cannulation was conducted 7 days before the actual experiment as, after that time, the changes caused by the cannulation disappeared and thus they were unable to affect the experiment. 20 minutes before closing the carotid arteries, the rats were administered 10μg CRH (CRH produced by Peninsula Lab. Inc.) dissolved in 100μl 0.9% NaCl; the solution was administered through the cannula inserted into the CM. An equivalent amount of 0,9% NaCl was given to the control groups of rats from which 100μl CSF had been earlier collected.

The animals were divided into 4 experimental groups (CRH) and 4 control groups (0.9% NaCl). According to the time after evoking the cerebral ischemia and the time of the BBB permeability measurement. The four groups were the following: 1). Group IA: acute phase of the cerebral ischemia, the BBB permeability measured 30 minutes after closing the carotid arteries; 2). Group IB: acute phase of the cerebral ischemia, the BBB permeability measured 3 hours after closing the carotid arteries; 3). Group II: subacute phase of the cerebral ischemia, the BBB permeability measured 3 days after closing the carotid arteries; 4). Group III: chronic phase of the cerebral ischemia, the BBB permeability measured 7 days after closing the carotid arteries.

Changes in the BBB permeability in different phases of the cerebral ischemia in rats given CRH and in rats given 0.9% NaCl were compared to the BBB permeability measured in an additional control group in which neither cerebral ischemia was evoked nor CRH or 0.9% NaCl were administered to CSF. In each group there were 8-10 rats.

Examination of BBB permeability and statistical analysis

Evans blue - 50 mg/kg of body mass, 2% solution - was administered to rats through a dissected femoral vein. 2 hours later in animals anaesthetised with Ketamine, the thorax was opened and a needle was inserted to the left heart ventricle. The needle was connected through a catheter with a system used for washing out the blood and Evans blue contained in the vascular system and organs. This way was used for administering 0.9% NaCl, 100ml/100mg-body mass, pressure 100 cm water, to the rat vascular system. The excess of the salt solution together with Evans blue washed out from the tissues, poured out through a prepared right heart atrium during 30 minutes. Then, after opening the cranial vault, the brain was taken out. Cerebral hemispheres, separated from the cerebellar hemispheres, were weighed and put into formamid (Merck-Schuchardt) - 1ml formamid per 100 mg tissue. After 72 hours, the cerebral hemispheres were removed from the formamid which was analysed spectrophotometrically (Shimazu), 625mm, for Evans blue.

The amount of Evans blue in 1mg of the tissue was measured on the basis of the standard curve determined, and the values obtained were statistically analysed using the t-Student test for non-paired groups with the $p<0.05$ level of significance.

RESULTS

The amounts of Evans blue extracted from rat's brains are show in *Table 1* and compared on the *Figs 1* and *2*. The BBB permeability measured 30 minutes after evoking the cerebral ischemia increased rapidly in both groups in relation to the level of the permeability before closing the carotid arteries. The BBB permeability in the group with CRH administered to CSF and in the control group given 0.9% NaCl did not differ significantly. In both groups the increase in the BBB permeability was statistically highly significant (CRH group - $p<0.001$; 0.9% NaCl group - $p<0.0001$).

Further substantial increase in the BBB permeability for Evans blue occurred 3 hours after clamping the vessels; such an increase proved a growing BBB damage caused by the ischemia, and the increase was statistically significant (CRH group - $p<0.002$; 0.9% NaCl group - $p<0.00002$). The differences between the control group,

Table 1. The BBB permeability for Evans blue after cerebral ischemia.

		The level of the BBB permeability for Evans blue (amount μg/mg tissue)				
Groups of rats'		Before ischemia	30' after ischemia	3 h after ischemia	3 days after ischemia	7 days after ischemia
Experimental groups (with 10 μg CRH in 100 μl 0.9% NaCl into CSF via CM)	Average	0,28 n = 8	1,96 n=7	4,23 n=9	4,08 n=7	1,64 n=9
	SD	0,14	0,78	1,87	1,62	1,35
Control groups (with100 μl 0,9% NaCl into CSF via CM)	Average	0,28 m=8	1,75 n=9	5,48 n=10	4,36 n=16	4,16 n=9
	SD	0,14	0,72	1,25	1,99	3,24

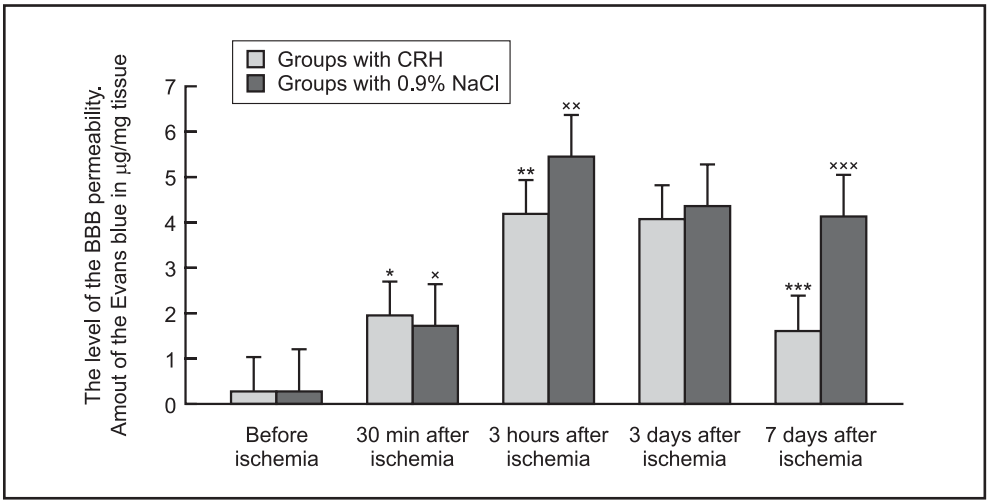

Fig. 1. Bar graphs showing changes in the BBB permeability for Evans blue in acute, subacute, and chronic phases of the cerebral ischemia in rats. In the acute phase, i.e. 30 minutes after closing the arteries, there was a statistically significant increase in the BBB permeability for Evans blue in relation to the Evans blue level in groups before the cerebral ischemia (*****-p<0.0005 and **x**-p<0.0001). 3 hours after closing the arteries there was a further statistically significant increase in the BBB permeability in relation to the permeability noted 30 minutes after closing the arteries (******-p<0.002 and **xx** -p<0.00002). In the chronic phase of the ischemia, i.e. 7 days after closing the arteries, in CRH group was a statistically significant decrease in the BBB permeability in relation to the permeability noted 3 days after closing the arteries (*******-p<0.0005). The level of the permeability was even lower than the level noted 30 minutes after closing the carotid arteries but the differences failed to be statistically significant. In control group a similarly high level of the BBB permeability persisted in the subacute and chronic phases of the cerebral ischemia, i.e. 3 days and 7 days respectively, after closing the arteries. They're still significantly higher in comparison with the values obtained 30 minutes after closing the carotid arteries (**xxx-**p<0.05)

given 0.9% NaCl, and the experimental group, administered CRH, noted in the acute phase of the ischemia, i.e. 3 hours after evoking the condition, were statistically significant (p<0.05; *Fig.2*). In the both group the BBB damage in the subacute phase of the cerebral ischemia, i.e. 3 days after closing the arteries, was serious similarly to the damage in the acute phase, i.e. 3 hours after closing the arteries, thus the difference was not statistically significant *(Fig.1, Table 1)*.

The differences between the two groups grew considerably in the chronic phase of the cerebral ischemia, i.e. 7 days after closing the arteries *(Fig. 1, 2)*. In the control group the Evans blue value measured spectrophotometrically was similar to the values measured 3 hours and 3 days after closing the carotid arteries and still significantly higher in comparison with the values obtained 30 minutes after closing the carotid arteries (p<0.05; *Fig.1*). On the other hand, in the animals given CRH to the CSF via CM, there was a further considerable decrease in the BBB permeability, and the

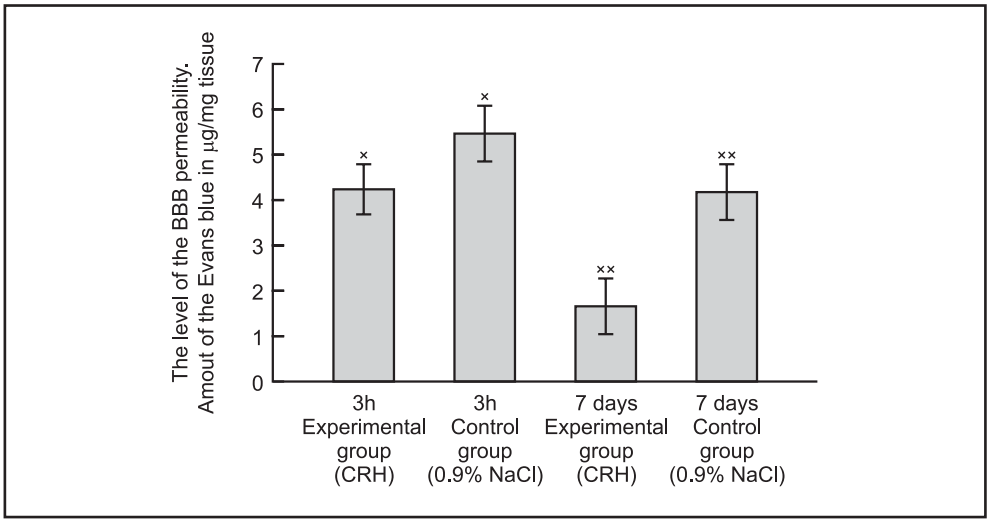

Fig. 2. The bar graphs showing level of the BBB permeability for Evans blue in the acute and chronic phase of the cerebral ischemia, i.e. 3 hours and 7 days after closing the carotid arteries. The differences between the control group, given 0.9% NaCl, and the experimental group, administered CRH, noted in the acute phase of the ischemia, i.e. 3 hours after evoking the condition, were statistically significant (**x-**p<0.05). In the chronic phase in the experimental group there was a considerable decrease in the BBB permeability; such a decrease was not noted in the control group, and the differences observed were statistically significant (**xx-**p<0.03).

differences observed were highly statistically significant in relation to the values obtained 3 days after closing the carotid arteries (p<0.0005; *Fig.1*). In this group, the level of the BBB permeability for Evans blue returned to a level similar to the one noted 30 minutes after evoking the ischemia. The differences between the control group, given 0.9% NaCl, and the experimental group, administered CRH, noted in the chronic phase of the ischemia, i.e. 7 days after evoking the condition, were statistically significant (p<0.03; *Fig.2*).

DISCUSSION

The results obtained show that cerebral ischemia in rat's results in the BBB damage as early as 30 minutes after closing the carotid arteries; the damage intensifies considerably a few hours after closing the arteries, and persists for days. CRH given to CSF has a positive effect on the decrease in the BBB damage in the acute phase of the ischemia, and it also causes a highly considerable decrease in the BBB permeability in the chronic phase, i.e. 7 days after closing the carotid arteries.

Thus, the results of the experiment have supported the assumption, that in ischemic BBB damage, similarly as in SAH-caused BBB damage (7,13,21,19,21), CRH causes a decrease in the BBB permeability for Evans blue. The positive CRH effect was

noted as early as 3 hours after closing the carotid arteries and increased considerably in the chronic phase of the cerebral ischemia in rats. At the same time, in the control group given 0.9% NaCl instead of CRH, a considerable BBB damage persisted causing an increase in the BBB permeability for the serum proteins.

The protective influence of CRH on the decrease of the BBB permeability, noted in the 3 hours after closing the carotid arteries, may suggest that CRH is capable of decreasing the level of the BBB damage by a direct effect on the cerebral endothelium. The protection is even stronger in the chronic phase of the cerebral ischemia it may be explained by assumption that CRH administered to CSF, triggers secondary mechanisms dependent on the pituitary-adrenal axis stimulation. In the acute phase CRH, or its fragments developing in vivo, may affect the endothelial cells directly by changing its susceptibility to damaging factors, or indirectly by non-selective inhibition of chemical mediators, which increase the vascular permeability (9). The mechanisms of BBB damage during ischemia are complex. Ca^{2+} ions play a crucial role in the BBB permeability (24). Flunarizine, a selective Ca^{2+} channels blocker in smooth muscles (25) and in the endothelium (26), inhibits the increase in the vascular permeability induced by histamine, serotonine, bradykinine, and arachidonic acid derivatives in rat skin (27). A decrease in cerebral infarctions noted during flunarizine treatment (28) may result from the decrease in the cerebral-vascular permeability and cerebral oedema.

The participation of cytokines in the mechanism of ischemic BBB damage is also to be discussed. In the first 4 hours of acute cerebral ischemia syndrome in man, the level of interleukin-6 in blood serum increases significantly, and the extent of the increase in the cytokines level is correlated with the extent of the neurologic damage (29). Also, in the cerebral ischemia due to a vasospasm after SAH in man, the level of interleukins IL-1α and IL-1β in CSF increases significantly 3 days and especially 5 days after the hemorrhage (30,31). CRH triggers the production and secretion of interleukin-1 in macrophages; interleukin-1, in turn, induces the release of CRH in the hypothalamus (2,3,8,32). We have shown in our previous study, that the increase of CRH in CSF can be seen in-patients with cerebral ischemia in the course of vasospasm after SAH (33). CRH release may have a positive effect on decreasing the BBB permeability but, at the same time, it triggers cytokines secretion and thus intensifies inflammatory reactions. The result of which may be a vascular obliteration and intensification of vasospasm leading to an increase in the extent and area of the cerebral ischemia (30).

In the chronic phase of cerebral ischemia, effect of the CRH may result from its triggering the ACTH release from the hypophysis. ACTH, in turn, activates the adrenal cortex to produce corticosteroids, which have a sealing effect on the endothelium of the cerebral vessels (34). Administration of glucocorticoids, dexametazone for example, resulted in a diminished cerebrovascular permeability for α-aminoisobutiric acid (AIB) and sucrose, which suggests that adrenal corticoid hormones may play an

important role in regulating the BBB permeability (34). Long and Holaday (35) report that bilateral adrenalectomy, but not medullactomy, increases the penetration of I^{125} - labelled cattle albumin (BSA) - to the brain in animals; moreover, they say that this condition is reversible on corticosteroids administration. Another crucial discovery was made by Rudman and Kunter (36) who have found out that ACTH suppression, or its administering to the cerebral ventricles, (both are connected with a lower corticosteroids secretion) increases the penetration of albumin, sucrose, or mannitol from the blood to CSF and the brain.

From all the findings mentioned above it can be concluded that CRH may play an important role in regulating the BBB permeability. In the acute phase of ischemia, CRH may directly affect the endothelial cells changing their susceptibility to damaging factors, or it may inhibit the chemical mediators of the BBB damage in a non-selective way. In the chronic phase of ischemia, however, CRH, administered a few days earlier, cannot be present in CSF in a concentration allowing such a direct positive effect on the BBB, all the more so as a statistically significant decrease in the BBB permeability was noted during this phase. The protective effect of the CRH in the chronic phase of the cerebral ischemia in rats may be attributed to the mechanism of hypothalamic-adrenal axis stimulation, which leads to an increased production of glucocorticosteroids and has a positive effect on the decrease in the BBB permeability for serum proteins.

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