Role of corticotrophin releasing hormone in cerebral infarction-related gastrointestinal barrier dysfunction

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INTRODUCTION
Gastrointestinal barrier dysfunction always occurs in the presence of cerebral infarction, and "stress ulcer" is one of the important manifestations. To the present, the pathogenesis of such stress ulcer is still unknown, and the treatment has to make reference to treatment for other-factor induced ulcers, such as inhibiting gastric acid. O'Donnell et al[1] reported that antacids can't reduce the mortality of stress ulcer in critically ill patients, but would cause such complications as hospital-acquired pneumonia,[2] intestinal flora, and translocation.[3]

Corticotropin-releasing hormone (CRH) is a central regulator of hormonal stress response, causing stimulation of corticotropin and glucocorticoid secretion. CRH is also believed to mediate stress-induced behaviors, implying a broader, integrative role for the hormone in the psychological stress response. CRH regulates stress response as a hypothalamic hormone in regulating the activity of the hypothalamus-pituitary-adrenal axis,[4,5] as a neurotransmitter in activating the sympathetic adrenal medulla system,[6] and as a hormone directly activating its receptors in the periphery to

BACKGROUND: Corticotrophin releasing hormone (CRH) is believed to mediate stress-induced behaviors, implying a broader, integrative role for the hormone in the psychological stress response, and studies on CRH in physical stress are few. This study was undertaken to investigate whether CRH plays an important role in cerebral infarction-related gastrointestinal barrier dysfunction.

METHODS: Thirty male Wistar rats were randomly divided into a pseudo-operation group (group C, n=10), a cerebral infarction group (group I, n=10), and a cerebral infarction + ic α-helical-CRH (9-41) group (group Aic, n=10). Urine samples were collected to determine the levels of epinephrine, norepinephrine, cortisol, and sucrose. At 24 hours after establishment of the models, blood samples were taken to determine the activity of diamine oxidase (DAO) and the concentration of D-lactic acid (D-lac). The stomach was taken to determine gastric Guth score, and the hypothalamus was also taken to determine tissue CRH protein expression using Western blotting.

RESULTS: The hypothalamus CRH protein, the indicators of stress, the plasma DAO activity and plasma D-lac, urine sucrose exertion and gastric Guth score in group I were higher than those in groups Aic and C.

CONCLUSIONS: After cerebral infarction, CRH in the hypothalamus was increased, the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system were activated, gastrointestinal permeability was increased, and gastrointestinal barrier function was destroyed. CRH receptor antagonist alleviated the gastrointestinal barrier function.

KEY WORDS: Corticotrophin releasing hormone; Cerebral infarction; Gastrointestinal barrier; Stress
mediate stress response. Animal experiments showed that peripheral injection of CRH causes changes of colon function, including changes of permeability, release of mucus, and ion secretion, but these changes can be blocked by CRH antagonists.[7,8] Most studies concerning CRH mainly focus on psychological stress, but studies on CRH in physical stress are few. In this study we used acute cerebral infarction (ACI) as a stress model because ACI has a relatively stable homodynamic status. The present study was to explore whether cerebral infarction related gastrointestinal barrier dysfunction can be alleviated by CRH receptor antagonists.

**METHODS**

**Animals**

Thirty male Wistar rats, SPF-grade, weight 220-250 g, were obtained from the Animal Experiment Center of Beijing Union Medical College Hospital, Beijing, China. They were kept in separate cages (three rats/cage) at room temperature of 20-21 °C. Regular light (light 8:00-20:00), food and water were given. The feeding environment was quiet and well-ventilated. The rats were monitored daily by the same investigator for one week before the study. The experimental procedures were performed at the same time per day in order to minimize the effect of circadian rhythm. The procedures were approved by the Animal Care Committee of Peking Union Medical College.

**Experimental groups**

Thirty rats were divided into a pseudo-operation group (group C, n=10), a cerebral infarction group (group I, n=10), and a cerebral infarction + ic α-helical-CRH (9-41) group (group Aic, n=10).

**Agents and doses**

α-helical CRH-(9-41) (Sigma, C2917) was dissolved according to the manufacturer’s instructions, aliquoted, and frozen at -80 °C for use. Before the experiment, the peptides were diluted in sterile saline for intracerebroventricular injection. The rats were injected with either 10 μg saline (group I and group C) or 10 μg α-helical CRH-(9-41) (group Aic) at 30 minutes before cerebral infarction was induced. The same doses were used to induce significant suppression of the CRH activity both in the central nervous system and the periphery.[9]

**Procedures**

**Intracerebroventricular injection**

Each animal was fasted overnight for surgery. The rats were anesthetized with intraperitoneal injection of 3% pentobarbital sodium (40 mg/kg).

After the routine preparation, the rats were anesthetized, and a tiny hole (0-8 mm o.d.) was drilled (0.8 mm after anterior fontanelle, 1.5 mm left, 3.5-4.0 mm sub-cranium) according to the stereotaxic atlas of Paxinos-Watson.[10] The infused volume was 2 μL.

**Establishment of infarction models**

The occlusion of the middle cerebral artery (MCA) was produced by the Zea Longa technique.[11] A thread-occluder, prepared according to the reported method,[12] was inserted through the general carotid artery and the internal carotid artery to occlude the MCA. The pseudo-operation group was treated similarly as the infarction group except the use of the occluder.

After the middle cerebral artery occlusion (MCAO), 1.0 g/mL of sucrose (1.0 mL)[13] was orally administered to each rat, and then the rats were placed into metabolic cages. After the rats recovered from anesthesia, neurological symptom scores were calculated with the method of Zea Longa[11] to confirm the infarction.

**Specimen collection**

At 24 hours after establishment of infarction models, urine was collected to test epinephrine, norepinephrine and cortisol with the ELISA kit (Adlitteram Diagnost, USA); urinary sucrose was measured using the enzymatic method (Megazyme, Ireland). Subsequently the rats were anesthetized again by intraperitoneal injection of 3% sodium pentobarbital (40 mg/kg). A sagittal laparotomy was performed, and abdominal aorta blood was collected in ice-chilled sterile tubes and put in a cooling centrifuge at 1000×g to obtain plasma for the assay of D-lac, diamine oxidase by the reported method.[14, 15]

The whole stomach was separated and washed in ice cold saline. The stomach was separated and washed in ice cold saline. The stomach was separated and washed in ice cold saline. The stomach was separated and washed in ice cold saline. The stomach was separated and washed in ice cold saline. Then gastric Guth score was calculated with a magnifying lens and a micrometer. All samples were coded and blindly scored. At last, the rats were killed by bloodletting; the left side of the hypothalamus was separated rapidly under a microscope, and put in a liquid nitrogen tank for testing CRH protein with the Western blotting method.
Determination of CRH protein with Western blotting

Hypothalamus tissue was lysed, and proteins were quantified spectrophotometrically. Equivalent amounts (50 μg) of total cellular protein were fractionated by SDS-PAGE and blotted onto nitrocellulose membranes. Membranes were sequentially incubated with primary antibody to CRH (goat anti-rat CRH, diluted in 1:200). HRP-conjugated rabbit anti-goat was used as a secondary antibody (1:3000). Chemiluminescence was performed and the membrane was put into a dark cassette where a medical X-ray film was placed for exposure. Photos were taken and gray scale was analyzed using the Image pro plus software. Actin was used as a negative size-matched control.

Statistical analysis

All data were expressed as means ± SE; Kolmogorov-Smirnov normality test was firstly used to ensure all the data in normal distribution. Levene statistics was then used for homogeneity of variance test in those measurement data of normal distribution; indicators of the measurement were compared by one-way ANOVA, while indicators of the ranked data were compared by the Mann-Whitney U test. All of the tests were two-tailed tests. A P value less than 0.05 was considered statistically significant.

RESULTS

NSS

All the rats in groups I and Aic had infarction with NSS 2.6±0.5 and 2.4±0.5, respectively, whereas the rats in group C did not show infarction.

Relative CRH protein content in the hypothalamus

Relative CRH protein content in the hypothalamus of the rats was significantly increased after cerebral infarction (P<0.01). It was significantly lower in group Aic than in group I (P<0.05), but there was no significant difference between groups Aic and C (Figure 1).

Changes in urinary cortisol and catecholamine

The levels of epinephrine, norepinephrine and cortisol in 24-hour urine in group I were significantly higher than those in group C. They were significantly lower in group Aic than in group I (P<0.05), but no significant difference was observed between group Aic and group C (P>0.05) (Figure 2).

Changes in urine sucrose exertion and gastric Guth score

Urine sucrose exertion and gastric Guth score in group I were significantly higher than those in group C and group Aic (P<0.01). Compared to group C, gastric Guth score was significantly higher in group Aic (P <0.01),

Figure 1. Relative CRH protein content in rats’ hypothalamus.

Figure 2. Comparison of cortisol, epinephrine and norepinephrine in 24-hour urine between the three groups.
but there was no significant difference in urine sucrose between the two groups (Figure 3). The change of urine sucrose was consistent with gastric Guth score: group I > group Aic ≥ group C.

**Changes in plasma diamine oxidase activity and D-lac**

The levels of plasma diamine oxidase activity and D-lac in group I were significantly higher than those in group C ($P<0.05$). They were significantly lower in group Aic than in group I ($P<0.05$), but there was no significant difference between groups Aic and C (Figure 4). The change of plasma diamine oxidase activity was consistent with that of D-lac: group I > group Aic ≈ group C.

**DISCUSSION**

There are various models of acute stress, but man-made psychological stress models such as restraint stress, water-deprivation stress and sleep-deprivation stress are often used. In fact, gastrointestinal changes are different in different stress models.[16] Differences exist in psychological stress models between laboratory and clinical studies, and the strength of physical stress is usually longer than that of psychological stress. In this study, we used stress models of acute cerebral infarction, which is common in clinical study. Massive cerebral infarction often occurs in the middle cerebral artery occlusion (MACO), hence in this study we established the MACO models using a monofilament method from the internal carotid artery[11] to observe changes of gastrointestinal barrier function. Confirmed by NSS, all the 20 rats in group I and group Aic developed symptoms of neurological deficits, suggesting that the models were successfully established.

In this study, we selected 24 hours after the establishment of models as the observation point. At this point, the urine sucrose exertion was significantly higher in group I than in group C, and this indicated that gastroduodenal mucosal barrier was damaged during cerebral infarction. The gastric Guth score was also significantly higher in group I than in group C, and 3 rats developed an ulcer in a large area, which confirmed the barrier impairment indicated by urine sucrose exertion. The 24-hour plasma diamine oxidase activity was significantly higher in group I than in group C, and plasma D-lac concentration was significantly higher in group I than in group C, suggesting the impairment of intestinal mechanical barrier function and the increase...
of permeability. The above changes were consistent with those reported i.e., cerebral infarction can cause damages to the gastrointestinal tract.

In our study, circulatory disorder, anoxia, infection and malnutrition of the digestive tract were excluded because of gastro-intestinal barrier dysfunction, and neuroendocrine was found to be associated with the responses to the dysfunction. To determine the role of neuroendocrine, it is necessary to detect whether cerebral infarction activates the stress system or not. The present study showed that the levels of hypothalamic CRH protein, urinary epinephrine, norepinephrine and cortisol in the brain were significantly higher in group I than in group C. We concluded that the hypothalamic-pituitary-adrenal axis and the sympathetic nervous system are activated. The factors affecting the activation include cerebral angiemphraxis, loss of nerve function and secondary cerebral edema. As a result, cerebral infarction causes both gastrointestinal barrier dysfunction and stress system activation. Thus stress-induced mucosal disease is associated with gastrointestinal barrier dysfunction.

Role of α-helical-CRH (9-41) before infarction

In group Aic, central and peripheral CRH receptors were antagonized before cerebral infarction. Urine sucrose exertion was significantly decreased in group Aic compared with group I. But there was no significant difference between group Aic and group C. These suggested that the change of gastroduodenal mucosal barrier function was completely reversed after the administration of α-helical-CRH (9-41) in the brain. The Guth score of gastric body was also significantly lower in group Aic than in group I, but it was higher than that in group C. The reason may be that CRH has a better protective effect on gastric barrier function than on stomach structure or CRH has a better protective effect on the duodenum than on the stomach. At the same time, at 24 hours after the administration of α-helical-CRH (9-41) in the brain, the plasma diamine oxidase activity and plasma D-lac were decreased more significantly in group Aic than in group I. However there was no significant difference when group Aic compared with group C. This indicated that the intestinal barrier function and permeability were reversed after CRH receptors were antagonized, and the probability of bacterial translocation was decreased.

After the administration of α-helical-CRH (9-41), the levels of hypothalamic CRH protein, 24-hour urine epinephrine, norepinephrine and cortisol in group Aic decreased significantly compared with group I, but there was no significant difference when compared with group C. This finding indicated that the hypothalamus-pituitary-adrenal axis and the sympathetic nervous system were protected after intraventricular administration of α-helical-CRH (9-41) which was able to interfere with the stress response induced by cerebral infarction. The mechanisms of protective effect of α-helical-CRH (9-41) on infarction-related gastrointestinal barrier dysfunction are described.

The central administration of α-helical-CRH (9-41) interfered the stress response induced by the cerebral infarction, and thus alleviated the gastrointestinal barrier dysfunction. In our experiment, the gastrointestinal barrier dysfunction caused by cerebral infarction changed in accordance with cortisol and catecholamine. Presumably after central CRH was antagonized, the release of glucocorticoid was inhibited by suppressing the activation of the hypothalamus-pituitary-adrenal axis caused by central inhibition of CRH, and this alleviated the negative effect of glucocorticoids on epithelial healing. However, we believed that CRH did not play a role after the administration of corticosteroids and catecholamine because studies showed that adequate doses of corticoid hormone inhibitors could not improve the severity of stress ulcer but sympathectomy increased the occurrence of stress ulcer[23]. Besides, adrenalectomy and hypophysectomy could not inhibit stress-induced intestinal response, suggesting that neither adrenal factors nor pituitary factors mediate the stress-induced intestinal tract.[24]

After central administration of α-helical-CRH(9-41), hypothalamic CRH protein level was decreased more significantly in group Aic than in group I. This may be connected with the inhibited positive feedback of central CRH caused by α-helical-CRH (9-41), which thereby inhibited the increase of CRH content after increased infarction.[25]

The peripheral effects of CRH include the activation of local mast cells and nerves on gastrointestinal barrier;[26,27] the retention of intestinal contents and bacteria translocation by inhibiting the motility of the stomach and intestine;[28,29] intestinal barrier dysfunction induced by hyperkinesias of colonic movement;[30] the hypersensitivity of the intestinal tract and increased stress stimulation to the stress center, which may aggravate the gastrointestinal barrier dysfunction. At present, the effects of peripheral CRH on stress-induced gastrointestinal barrier dysfunction have attracted more and more attention.

In this study the central administration of α-helical-
CRH (9-41) lowered the severity of cerebral infarction, and alleviated cerebral infarction-related gastrointestinal barrier dysfunction. Studies suggest that CRH is involved in stroke injury: CRH-mRNA increases rapidly after stroke\(^3[33,34]\) and the central role of CRH receptor antagonist, α-helical-CRH (9-41) after stroke can reduce ischemic injury.\(^3[35-37]\) Its mechanism however remains unclear.

The protective role of CRH receptor antagonist in stress related gastrointestinal barrier dysfunction was found similarly in a psychological stress model,\(^1\) ischemic injury. Antagonist, α-helical-CRH (9-41) after stroke can reduce receptor antagonist significantly reduced the cerebral infarction models, administration of CRH it was limited to the prevention and treatment of mental illness and some psychosomatic diseases. In our stress–cerebral infarction models, administration of CRH receptor antagonist significantly reduced the cerebral infarction-related gastrointestinal barrier dysfunction, suggesting that CRH plays an important role. This may be an explanation for the mechanism of stress-related mucosal disease.

Funding: The study was supported by a grant from Doctoral Fund of the Education Ministry, China.

Ethical approval: All procedures used in this study were approved by the Animal Care Committee of Peking Union Medical College.

Conflicts of interest: No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

Contributors: Liu YC proposed the study and wrote the first draft of the paper. All authors contributed to the design of the study, the interpretation of the data, and further drafting.

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