Possible inhibitory role of prolactin-releasing peptide for ACTH release associated with running stress

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Ohiwa N, Chang H, Saito T, Onaka T, Fujikawa T, Soya H. Possible inhibitory role of prolactin-releasing peptide for ACTH release associated with running stress. Am J Physiol Regul Integr Comp Physiol 292: R497–R504, 2007. First published August 17, 2006; doi:10.1152/ajpregu.00345.2006.—Exercise around the lactate threshold (LT) produces a variety of beneficial physiological and psychological effects on our body (22, 40). The LT is a work rate at which the steady state of blood lactate accumulation breaks down (44) and the plasma ACTH level begins to increase during graded running (7, 17, 29). Because it is well known that the ACTH release is a stress response, defined as “running stress,” we previously demonstrated that running stress is associated with activation of certain regions of the brain, e.g., the paraventricular hypothalamic nucleus (PVN) and supraoptic nucleus, that are hypothesized to play an integral role in regulating stress-related responses, including ACTH release during running stress. Thus we investigated the role of prolactin-releasing peptide (PrRP), found in the ventrolateral medulla and the nucleus of the solitary tract, which is known to project to the PVN during running-induced ACTH release. Accumulation of c-Fos in PrRP neurons correlated with running speeds, reaching maximal levels under running stress. Intracerebroventricular injection of neutralizing anti-PrRP antibodies led to increased plasma ACTH level and blood lactate accumulation during running stress, but not during restraint stress. Exogenous intracerebroventricular administration of low doses of PrRP had the opposite effects. Therefore, our results suggest that, during running stress, PrRP-containing neurons are activated in an exercise intensity-dependent manner, and likewise the produced endogenous PrRP attenuates ACTH release and blood lactate accumulation during running stress. Here we provide a novel perspective on understanding of PrRP in the endocrine-metabolic response associated with running stress.

EXERCISE AT AROUND LACTATE threshold (LT) produces a variety of beneficial physiological and psychological effects on our body (22, 40). The LT is a work rate at which the steady state of blood lactate accumulation breaks down (44) and the plasma ACTH level begins to increase during graded running (7, 17, 29). Because it is well known that the ACTH release is a stress maker (27), moderate running can generate one kind of stress, termed running stress, if it persists above the LT. To date, little is known about its regulatory mechanism; however, it is important in understanding the mechanisms behind the variety of physiological effects caused by exercise around the LT.

To examine the regulating mechanism underlying running stress, we established the rat treadmill running model and used it to identify anatomic activation of the paravascular part of the hypothalamic paraventricular nucleus (pPVN) and the supraoptic nucleus (SON) during running stress (32, 39). The next step in our studies will be to identify the nuclei or neuropeptides that regulate ACTH release during running stress via pPVN and SON activation. The ventrolateral medulla (VLM) and the nucleus of the solitary tract (NTS) that project to the paraventricular nucleus (PVN) are known to play a crucial role in multiple stresses (4, 37, 43). Furthermore, recently we identified that the A2 noradrenergic cell group in the NTS and the A1 noradrenergic cell group in the VLM were activated in running stress (24, 25).

Prolactin releasing peptide (PrRP) was recently isolated as the endogenous ligand for the orphan G protein receptor GPR10/hGPR3 (11) and proved to be localized, in a highly restricted distribution, in a certain A2 noradrenergic cell group in the NTS, the A1 noradrenergic cell group in the VLM, and the dorsomedial hypothalamic nucleus (DMH; see Ref. 18). These PrRP neurons project to the PVN and SON (23) in which the PrRP receptors are known to be present (31). Intracerebroventricular administration of PrRP stimulated ACTH release (20), and accumulation of the Fos protein, an immediate early gene product, in PrRP neurons markedly increased under several stress conditions (19, 46), providing a clear possibility that PrRP neurons are involved in running stress-induced ACTH secretion. Thus we first investigated whether running stress activates PrRP neurons by elucidating the increased expression of Fos protein. We further demonstrated the modulation of ACTH release associated with running stress by immunoneutralization of endogenous PrRP or by exogenous addition of low-dose PrRP. The results provide the important perspective that brain PrRP has an inhibitory effect on ACTH release and blood lactate accumulation during running stress.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (Saitama Laboratories) were housed in group cages illuminated from 0800 to 2000 (12:12-h cycle). Room temperature varied from 21 to 23°C. Tap water and laboratory feed were available ad libitum. All rats were housed at laboratory conditions for at least 3 days and were weighted and handled daily throughout the course of the study. All procedures were performed in accordance with the Institutional Guidelines for Animal Care at the University of Tsukuba, which confirm to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

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**Treadmill Running Habituation**

The protocols of the habituation and the exercise test were the same as in our previous study (24, 31). Briefly, rats were initially trained to run 5 days/wk for 2 wk with a graded increase in speed and duration on a treadmill (KN-73; Natsume, Tokyo, Japan), before being subjected to exercise testing. The running duration per session was 30 min, and the running speed was gradually increased from 10 to 25 m/min. Increases in velocity and/or duration were imposed only when the rats were able to maintain their current exercise intensity.

**Surgery**

Before treadmill running habituation, rats in experiments 2 (running stress group) and 3 were anesthetized with pentobarbital sodium and placed in a stereotaxic frame. A stainless steel guide cannula (22 gauge) was inserted in the brain with the cap in the left lateral cerebral ventricle and secured to the skull with screws and dental cement. The cannula was located at 0.8 mm caudal to the bregma, 1.4 mm lateral to the middle line, and 3.6 mm below the skull. After cannula implantation (2 days), the rats received running habituation as described above. After running habituation, rats in experiments 2 and 3 were anesthetized with 50 mg/kg of pentobarbital sodium. The jugular vein was exposed by dissection, and a catheter, consisting of silicone tubing, was inserted to a distance of 3.5 mm in the vessel. The catheter was fixed by a silk thread in the vessel, and the distal end of the catheter was exteriorized at the nape of the neck. After surgery, rats were kept individually in transparent polycarbonate cages until the start of the running tests.

**Running Stress Studies**

Because our previous studies revealed that the LT is reached if the running speed is ~20 m/min (39), in the present study we used the following running speeds: supra-LT running was 22.5–25 m/min and below-LT running was 13.5–15 m/min. All running studies were performed with at least 2 or 3 days of recovery after completing running habituation (experiment 1) and/or jugular cannulation (experiments 2 and 3) in the morning between 0900 and 1100.

**Experiment 1**

Rats were subjected to test run on the treadmill to examine the activation of PrRP neurons in running stress. In this experiment, rats were left on the treadmill for 30 min without running (control) or had to run at either above or below the LT. After completion of the test session, the animals were returned to their home cage. After the initiation of the test session (120 min), the animals (n = 11) were anesthetized with pentobarbital sodium (50 mg/kg ip) and perfused first with saline and then with 5% acrolein in a 0.1 M phosphate buffer (PB, pH 7.4) for immunohistochemistry. To confirm the physiological impact of running, other animals (n = 9) were decapitated immediately after the test run. Trunk blood was collected for blood lactate and glucose measurements. The remaining blood was centrifuged, and the plasma was used for measurement of ACTH as described below.

**Experiment 2**

The physiological role of PrRP was examined by intracerebroventricular injection of a monoclonal anti-PrRP antibody (aPrRP) in running stress. On the day of the experiment, the animals were randomly injected intracerebroventricularly with anti-PrRP IgG [P2L-1C, 4.9 mg/ml, 10 μl; Takeda Chemical (18)/control (heat-denatured anti-PrRP IgG)] and placed on the treadmill. The P2L-1C recognizes the COOH-terminal phenylalanine amide structure of PrRP31, which is necessary for PrRPs to interact with PrRP receptor (hGR3), and neutralizes PrRP-induced arachidonic acid metabolite release from hGR3 (21). The antibody and its volume that we used in this experiment were equivalent to those in our previous study (46). After injection (20 min), the rats underwent the exercise test, in which they were compelled to run at 22.5–25.0 m/min for 30 min. Blood samples were collected from the jugular vein catheter at 0 (pre) and 30 (post) min before and during running. A series of 0.5-ml blood samples were withdrawn. After the first sample was taken, the rats received a transfusion of 0.5 ml saline-replaced donor blood obtained from undisturbed cannulated rats. The tip of the catheter was filled with 10% heparinized saline as an anticoagulant. After withdrawal, blood glucose and lactate concentrations were determined in 20-μl blood samples using an automated enzymatic method. The remaining blood was centrifuged, and the plasma was used for hormonal measurement.

To compare the effect of the neutralizing aPrRP on running stress, other rats were subjected to a different type of stress (restraint stress). Male rats received intracerebroventricular cannulation as described above. At least 2 days after intracerebroventricular cannulation, jugular catheters (silicon tubing) were installed 4 days before the restraint stress. On the day of the experiment, the animals were randomly injected intracerebroventricularly with anti-PrRP IgG or control. After injection (20 min), rats were held prostrate in the plastic restrainer (diameter of 55 mm) for 30 min (the same time course for running stress procedure). Blood samples were collected from the jugular vein catheter at 0 and 30 min before and during restraint stress.

**Experiment 3**

To confirm the effect of the immunoneutralization on running stress, we further examined augmentation of brain PrRP in the same experimental model as in experiment 2. Rats were randomly injected with intracerebroventricular PrRP (1 nmol/10 μl in sterile 0.1 M PBS, pH 7.4)/vehicle (sterile 0.1 M PBS, pH 7.4 alone) before being subjected to running. The duration of the running stress and blood sampling time points was the same as in experiment 2. Because it was still unclear whether 1 nmol PrRP stimulates ACTH release (33) or not (20), we confirmed that 1 nmol PrRP itself failed to induce plasma ACTH increase and c-Fos expression in the corticotropin-releasing hormone (CRH)-containing neurons in the pPVN in our preliminary experiment (data not shown).

**Tissue Preparation and Immunohistochemistry**

The brains were quickly removed and immersed for >24 h in PB containing 30% sucrose. Forty-micrometer coronal sections, including the NTS, VLM, DMH, PVN, SON, bed nucleus of the stria terminalis (BNST), and basolateral amygdaloid nucleus (BLA), were cut on a cryomicrotome (HM 505 E; Microm, Walldorf, Germany). A one-in-three series of brain stem sections and one-in-two series of forebrain sections were processed for c-Fos and PrRP. The immunohistochemical method used was described previously by Maruyama et al. (18). Briefly, the cryosections were washed with 0.1 M PBS (pH 7.4) and immersed sequentially in the following solutions: 1) 0.5% sodium metaperiodate in PBS for 20 min; 2) 1% sodium borohydride in PBS for 20 min; 3) 1% normal goat serum and 0.2% Triton X-100 in PBS (TNBS) for 1 h; 4) rabbit polyclonal antibody against Fos protein (diluted 1:48,000; Oncogene, Boston, MA) in TNBS for 24 h at 4°C; 5) biotin-conjugated donkey antirabbit IgG (diluted 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) in TNBS for 2 h; and 6) avidin-biotinylated horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) for 30 min. After serial rinsing in PBS and 0.1 M acetate buffer, the black reaction product was made visible by incubating the section with 4% nickel sulfate hexahydrate, 0.025% 3,3-diaminobenzidine tetrahydrochloride, 0.08% ammonium chloride, 0.4% glucose, and 0.03% glucose oxidase (10,000 IU) in PBS for 20–30 min at room temperature. The reaction was stopped by transferring the sections to 0.1 M acetate buffer and rinsing two times (5 min each) in PBS. To visualize cytoplasmic PrRP, the sections were then incubated with primary antibody to PrRP (80 µg/ml P2L-1T; Takeda Chemical; see Ref. 19) in PBS for 24 h at 4°C. The sections were then treated with biotin-conjugated donkey antismouse IgG.
Analysis of Fos Immunolabeling

Ethanol series (60–100%), delipidated in xylene, and coverslipped sections were mounted on slides, air-dried, dehydrated in a graded ethanol series (60–100%), and coverslipped with Mount-Quick (Daido Sangyo). The following staining procedure was performed using the above procedure except for visualization. Neurons were visualized using the brown reaction product in the cytoplasm of neurons after incubating the sections in the above reaction solution with nickel omitted. The brown reaction product in the cytoplasm of neurons after incubating the sections in the above reaction solution with nickel omitted. The two to three reactive (ir) cells was examined by light microscopy. For a cell to be counted by eye and without previous knowledge of treatment for each animal. In the NTS, VLM, and DMH, the percentage of PrRP-ir cells was determined by RIA with specific anti-ACTH antisera (IgG, Nashville, TN), as previously described (46). The coefficients of inter- and intra-assay variation were within 5%.

Biochemical Determinations

Blood lactate and glucose were measured using an automated glucose-lactate analyzer (2300 Stat Plus; Yellow Springs Instruments). Plasma samples were obtained by centrifugation and stored at −80°C until treatment. The plasma concentration of ACTH was determined by RIA with specific anti-ACTH antisera (IgG, Nashville, TN), as previously described (46). The coefficients of inter- and intra-assay variation were within 5%.

Statistical Analysis

Data are expressed as means ± SE. Comparison between different groups was performed using two-way repeated ANOVA followed by Tukey’s multiple-comparison test. The statistical significance between two groups was evaluated using Student’s t-test. P < 0.05 was regarded as statistically significant.

RESULTS

Experiment 1

Blood lactate and plasma ACTH concentration after treadmill running. We had previously shown that supra-LT running induces both ACTH release and elevation of blood lactate accumulation (32, 39). These two parameters had been established as indexes of running stress. Supra-LT was consistently associated with significantly higher levels of blood lactate and plasma ACTH concentrations (P < 0.01) than either the control or below-LT running groups (Fig. 1).

Effect of running stress on c-Fos expression in PrRP-ir cells. Induction of c-Fos expression in PrRP-ir cells in rats subjected to below-/supra-LT running is shown in Fig. 2, A and B. Supra-LT running dramatically increased the number of c-Fos/PrRP-ir cells, although the patterns were different between medullary and hypothalamic sections (Fig. 2A). For the medullary PrRP-ir cells in both VLM (a–c) and NTS (d–f), c-Fos expression gradually increased proportional to running speed, with significantly higher percentage of c-Fos-ir cells in the supra-LT compared with below-LT group (P < 0.01; Fig. 2B). In contrast, c-Fos expression in PrRP-ir cells in DMH (g–i) was significantly elevated for both below- and supra-LT groups (P < 0.01). In this case, there was no clear difference between below- and supra-LT groups.

Effect of running stress on c-Fos expression in PrRP-ir loci. Response to running stress in other areas of the brain where PrRP-ir nerves are also distributed is shown in Fig. 3. Supra-LT running resulted in a significant increase in c-Fos-ir...
cells, although this trend differed between the different brain sections. For PVN and the SON, supra-LT running showed a significant increase in c-Fos-ir cells, but this induction was not observed in the control or below-LT groups. Contrarily, the number of c-Fos-ir cells in the BNST was markedly elevated compared with the controls, regardless of running speed. We failed to detect any change in c-Fos-ir cells in the BLA despite the PrRP-ir nerves being stained.

Fig. 2. Running stress induced c-Fos induction in prolactin-releasing peptide (PrRP)-immunoreactive (ir) cells. A: photomicrographs of double immunostaining with PrRP and c-Fos antibodies in the ventrolateral medulla (VLM; a-c), nucleus of the solitary tract (NTS; d-f), and the dorsomedial hypothalamic nucleus (DMH; g-i). PrRP-ir cells and c-Fos-ir cells are stained brown and black, respectively. Scale bars = 50 μm. B: quantitative assessment of c-Fos expression in the PrRP-ir cells. Values are means ± SE. P < 0.01 vs. controls and below-LT (#) and vs. control (**) n = 3–4.
Experiment 2

Response to running and restraint stress after intracerebroventricular injection of aPrRP. To investigate whether brain PrRP plays a role in response to running stress, we immunoneutralized endogenously released PrRP by intracerebroventricular injection of aPrRP. After being subjected to supra-LT running, blood lactate and plasma ACTH concentrations were significantly higher in rats intracerebroventricularly injected with aPrRP than in the controls (Fig. 4A). In contrast, during restraint stress, immunoneutralization of PrRP did not show any significant effect in any of the blood/serum parameters tested (Fig. 4B). Blood glucose concentrations did not show any significant changes during running stress or restraint stress.

Experiment 3

Exogenous intracerebroventricularly delivered low dose of PrRP attenuates running stress response. To validate the results observed from intracerebroventricular administration of aPrRP, we further examined whether exogenous PrRP, which is believed to have a synergistic effect with endogenously released PrRP, attenuates plasma ACTH concentrations after running stress. After the rats were injected intracerebroventricularly with either 1 nmol of PrRP or...
vehicle, then subjected to running stress, we demonstrated that PrRP caused a significant decrease in blood lactate and plasma ACTH concentrations, which have been used as indexes of running stress (32, 39). The running paradigm was composed of both below- and supra-LT, which are typical exercise intensities that induce different hormonal and metabolic responses to running (44, 45), as shown in Fig. 1.

The percentage of Fos-ir cells in the PrRP neurons after supra-LT running was equivalent to water immersion-restraint stress (19). These results, together with our previous finding that both A2 and A1 cell groups were activated according to the running speed increase (25), suggest that PrRP and norepinephrine may be involved in not only ACTH release induced above the LT but also other responses, such as the cardiovascular response (23, 34), associated with below-LT running. It is of interest that PrRP neurons in the DMH are highly activated by below-LT running, also suggesting that the PrRP neurons respond to stimuli other than running stress induced above the LT.

Increases in Fos expression in the PVN and SON, where PrRP-ir nerves are distributed, suggest a possible role for activated PrRP neurons in regulating the running stress response. The BLA is known to be activated by stressors such as aversive conditioned stimuli (12) or anxiogenic drug treatment (36). However, the BLA did not show any significant changes in Fos expression to running stress, suggesting that running stress appears to be different from the other stressors that induce BLA activation. It is unclear whether Fos-ir cells receive dense terminals projecting from PrRP neurons. Further experiments, using triple immunofluorescence staining with PrRP, an axonal marker and c-Fos, will be required to examine this issue.

Because PrRP-ir nerves contact with CRH neurons in the pPVN and intracerebroventricular (20) or intra PVN (35) administration of PrRP stimulates ACTH release, PrRP appears to facilitate ACTH release via CRH. However, immunoneutralization of brain PrRP failed to induce significant attenuation of ACTH release in conditioned-fear stimuli (46), which is consistent with our findings (see Fig. 4). The reason for this discrepancy is unclear at present; however, it is possible that the role of brain PrRP in ACTH release depends upon the type of stress stimuli used. We previously reported that running stress activates AVP neurons in the pPVN and the SON (32). Other previous studies have also shown that AVP, rather than CRH, might play a major role in the facilitation of ACTH release during running (1, 38), suggesting that the running stress may differ from other types of stress in which ACTH secretion is mainly regulated by CRH. It has also been reported that PrRP may not directly influence CRH neurons in the PVN, since only a small percentage of PrRP-R-expressing cells present in the PVN contain CRH (16). Running stress is thus not likely to activate the facilitative pathway of PrRP-induced ACTH release via CRH in the PVN to a greater degree than other types of stress.

The precise mechanism underlying inhibitory modulation by PrRP on running stress-induced ACTH release remains to be clarified; however, since the BNST receives dense projections from PrRP neurons (13, 18), which is highly responsive to
running, brain PrRP might attenuate running stress-induced ACTH response via an inhibitory GABAergic pathway in the BNST (6, 9, 10).

The fact that brain PrRP affects not only ACTH release but also blood lactate accumulation is of interest. Running affects hormonal response and blood lactate accumulation, and attenuation of these responses is particularly observable after training (3, 30). Activation of the sympathoadrenal axis before initiation of running also causes significant attenuation of blood lactate concentration that leads to improved running performance (8, 41). Furthermore, chemical sympathectomy (5) or postganglionic blockade (42) led to a marked increase of blood lactate concentration during running and impaired its performance. Because medullary PrRP neurons exclusively colocalize in catecholaminergic neurons (2, 19, 31) and synergistically act with norepinephrine (19), it is possible that the PrRP amplifies the effects of norepinephrine and improves both energy efficacy and running performance, resulting in attenuation of ACTH release and blood lactate accumulation during running. The specific interaction between PrRP and norepinephrine during running stress needs to be examined in future studies.

A variety of factors (including sympathetic activity as described above), many of which are modified during running, involve ACTH release and blood lactate accumulation. These also include muscle contraction, which activates neurons in the NTS and the A1 (15). On the other hand, increased lactate alone does not affect ACTH release (14, 26), despite the fact that energy efficacy and running performance, resulting in attenuation of ACTH release and blood lactate accumulation during running, involve ACTH release and blood lactate accumulation. These present results are the first evidence that brain PrRP significantly participates in the endocrine and metabolic responses associated with running stress.

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