Delineation of responsive AVP-containing neurons to running stress in the hypothalamus

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Saito, Tsuyoshi, and Hideaki Soya. Delineation of responsive AVP-containing neurons to running stress in the hypothalamus. Am J Physiol Regul Integr Comp Physiol 286: R484–R490, 2004.——Running becomes a stress, termed running stress, if it persists above the lactate threshold (LT) and results in enhanced plasma ACTH level in humans. Although the exact underlying regulation mechanism is still uncertain, hypothalamic AVP has been shown to play a dominant role in running-induced ACTH release. It is still not known, however, whether running stress activates the hypothalamic AVP-containing neurons that are involved in the activation of the ACTH response. For this reason, we applied our rat running stress model, in which both plasma ACTH and osmolality levels increase just above LT running (supra-LT running), to delineate which hypothalamic AVP neurons were responsive to running stress. Rats were previously habituated to running and then subjected to a 30-min run either just below or above the LT. Plasma samples were collected from these animals to determine ACTH and osmolality levels.Brains were prepared for immunocytochemistry and enzyme immunoassay for the stalk median eminence (SME) AVP content. Only supra-LT running resulted in an increase in the number of Fos/AVP-immunoreactive neurons in both the paraventricular paraventricular nucleus (pPVN) and the magnocellular supraoptic nucleus (SON) accompanied by increased ACTH and plasma osmolality levels. Similarly, running reduced the SME content of the AVP. Thus it was found that AVP-containing neurons located in both the pPVN and SON are responsive to running stress just above the LT.

EXERCISE at around lactate threshold (LT) produces a variety of physiological effects on our body (16, 42). The LT is a work rate at which the steady state of blood lactate accumulation breaks down and the plasma corticotropin (ACTH) level begins to increase during graded running (6, 15, 33). It is well known that the ACTH secretion increase is a marker of stress (29); plasma ACTH and osmolality levels increase just above LT running (supra-LT running), to delineate which hypothalamic AVP neurons were responsive to running stress. Rats were previously habituated to running and then subjected to a 30-min run either just below or above the LT. Plasma samples were collected from these animals to determine ACTH and osmolality levels. Brains were prepared for immunocytochemistry and enzyme immunoassay for the stalk median eminence (SME) AVP content. Only supra-LT running resulted in an increase in the number of Fos/AVP-immunoreactive neurons in both the paraventricular paraventricular nucleus (pPVN) and the magnocellular supraoptic nucleus (SON) accompanied by increased ACTH and plasma osmolality levels. Similarly, running reduced the SME content of the AVP. Thus it was found that AVP-containing neurons located in both the pPVN and SON are responsive to running stress just above the LT.

In general, hypothalamic regulation underlying the stress-induced ACTH response is mainly orchestrated by cooperation between corticotropin-releasing hormone (CRH) and AVP secreted from neurons in the paraventricular part of the hypothalamic paraventricular nucleus (pPVN) (2, 3, 21, 43). In addition, the contribution of magnocellular (m) origin AVP cannot be ignored because several studies still support the hypothesis that AVP from the supraoptic nucleus (SON) and mPVN can reach the portal blood through the median eminence, which would in turn act on the pituitary corticotropes to stimulate ACTH release (5, 7, 10, 20, 48, 49).

Little is known about the regulating mechanism underlying running stress, although a few studies propose the hypothesis that AVP rather than CRH plays the dominant role in regulation (1, 37). This is supported by the facts that AVP, not CRH, increases concurrently with increased ACTH in the pituitary venous flow in galloping horses, and that in human study, CRH receptor occupations with excess ovine intravenous CRH challenge resulted in no alterations in running-induced ACTH response. Because supra-LT running increases plasma osmolality, which is a potentiating factor in the activation of magnocellular vasopressinergic function, AVP originating from mPVN and SON in addition to pPVN may together be involved in the regulation of the running-induced ACTH response. Thus it is hypothesized that AVP-containing neurons in the PVN, pPVN, and SON would be activated with running just above the LT.

In the present study, to delineate AVP-containing hypothalamic neurons responsive to supra-LT running, we forced rats to run either below LT or supra-LT and then removed their brains for immunocytochemistry and c-Fos/AVP testing. Here we report that running just above the LT apparently activates AVP-containing neurons in discrete hypothalamic regions such as the pPVN and SON, suggesting that these brain loci are responsive to running stress just above the LT.

MATERIALS AND METHODS

Animals

Adult male Wistar rats (250–300 g) were housed individually under standard lighting conditions (light on from 0800 to 2000) and temperature (22–24°C). Water and food were available ad libitum except during testing. All rats were housed under laboratory conditions for at least 3 days and were weighed and handled daily throughout the course of the study. All procedures were approved by the University of Tsukuba’s Experimental Animal Use Committee.

Treadmill Running Habituation

All animals were habituated to the treadmill apparatus (Natsume, Tokyo) by seven periods of treadmill running at night. Animals ran on the treadmill for no more that one session per day for a total of seven sessions over 10 days. The running duration per session was 30 min, and treadmill speed was gradually increased from 10 to 25 m/min. During running habituation, of 50 animals, only 14 required a weak electrical stimulation to encourage them to start running. It is impor-
tant to note that no electrical stimulation was used during the runs in the experiments.

Surgery

After running habituation, rats in experiment 1 (except for the time course change study) were anesthetized with 50 mg/kg pentobarbital sodium. The jugular vein was exposed by dissection, and a catheter, consisting of a 1-cm piece of silicone tubing and PE-50 polyethylene tubing, was inserted to a distance of 3.5 mm into 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

Previous studies revealed that LT is reached if the running speed is ~20 m/min (38). Therefore, we used in the present study the following running speeds: supra-LT running was 25 m/min and below-LT running was 15 m/min. All running studies were performed after at least 2 or 3 days of recovery after completing running habituation in the morning between 0900 and 1100.

Experiment 1

Rats were subjected to a test run on the treadmill to examine the effects of the time course change and the effects of speed change.

Time course change study. In the time course study, rats were compelled to run for 30 min at supra-LT, and then rats were perfused with paraformaldehyde at 30, 90, 120, and 180 min after onset of the test run.

Speed change study. In the speed change study, rats were left on the treadmill for 30 min without running or had to run at either above or below the LT, respectively. To prevent blood coagulation, 0.1 ml heparin (100 IU) was injected intravenously 10 min before the test. Serial blood sampling of 0.5 ml was then performed three times: at 5 min before running and at 30 min (at end of running) and 40 min (after 10 min recovery from running), respectively, after onset of the test runs for determination of blood lactate and plasma ACTH concentrations. Thereafter, rats were returned to their home cage and 90 min after the test run were perfused transcardially with 0.1 ml heparin and 100 ml 0.9% saline followed by 300 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) under deep anesthesia with pentobarbital sodium (100 mg/kg).

Speed change study for measurement of plasma osmolality. Rats were subjected to running at zero speed, below LT, and supra LT under the same conditions as above to assess plasma osmolality. Blood samples (0.2 ml) were withdrawn for determination of blood lactate and osmolality at the same time points as listed above.

Experiment 2

Rats were subjected to treadmill running for 30 min at zero speed (control) or above the LT and then decapitated. Immediately after decapitation, trunk blood was collected into a microcentrifuge tube containing 10 μl of 100 mg/ml EDTA for measurement of lactic acid. The brains were then removed from the skull, and the stalk median eminence (SME) was isolated as described below.

Immunocytochemical Staining

Brains were removed and postfixed in the same solution as perfusate (paraformaldehyde) for 1 h (4°C). Thereafter, brains were placed in 20% sucrose (0.1 M phosphate buffer, pH 7.4, 4°C) overnight, frozen on dry ice, and stored at ~80°C until sectioning. Forty-micrometer serial coronal sections of the forebrain including the PVN or SON were cut on a cryomicrotome (HM 505 E, Microm, Walldorf, Germany). To visualize nuclear Fos and cytoplasmic AVP, a one-in-three series of forebrain sections was subjected to dual immunoperoxidase staining. The sections were rinsed three times (15 min each) in 0.1% Triton X-100 in 0.1 M phosphate buffer (PB-T) followed by 0.3% hydrogen peroxide for 15 min to quench endogenous peroxidase activity and rinsed three times (10 min each) with PB-T. The sections were then placed for 30 min in PB-T containing 1% normal goat serum. They were then incubated in a primary antibody directed against c-Fos (1:1,000, rabbit polyclonal; Oncogene Research Products, San Diego, CA) for 24 h at 4°C. At the end of this incubation period, the sections were rinsed three times (10 min each) in PB-T and then incubated in biotinylated goat anti-rabbit IgG (1:200; VECTASTAIN Elite ABC kit, Vector Laboratories, Burlingame, CA) for 1 h. Thereafter, sections were rinsed three times (10 min each) in PB-T and incubated with ABC solution (1:50; VECTASTAIN Elite ABC kit) for 1 h. After serial rinsing in PB-T 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 PB-T for 20–30 min at room temperature. The reaction was stopped by transferring the sections into 0.1 M acetate buffer and rinsing twice (5 min each) in PB-T. To visualize cytoplasmic AVP, the sections were then incubated in a second primary antibody to AVP (a gift from Dr. S. Tanaka, Shizuoka University) for 24 h at 4°C. 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 sections were mounted on slides, air-dried, dehydrated in a graded ethanol series (60–100%), delipidated in xylene, and coverslipped with Mount-Quick (Daido Sangyo, Tokyo).

Analysis of Fos Immunolabeling coveredslipped

The number of Fos-immunoreactive (ir) cells was counted by eye and without previous knowledge of treatment. For a cell to be considered Fos-ir and AVP-ir, the cytoplasm was required to be stained brown and the nucleus black. The two to three sections containing the PVN or SON that most closely matched the Paxinos and Watson rat brain stereotaxic atlas (30) were counted for each animal. The total number of Fos-ir and double-staining cells was counted in the PVN at ~1.9 mm and the SON at ~1.4 mm from the bregma, respectively. Magnocellular and parvocellular divisions were identified according to the previous study (4, 40). The number of Fos-ir and double-stained cells was then divided by the total number of sections counted to provide a mean cell count per slide for each division.

SME Extraction

The SME was dissected using fine scissors, frozen in liquid nitrogen as described earlier (39), and stored at ~80°C until extraction. In the subsequent experiments, SME samples were placed in 120 μl of acid solution (2 N acetic acid, 0.1% Triton X), homogenized with a handheld homogenizer, and boiled for 5 min. After centrifugation at 3,000 rpm for 10 min, a 10-μl aliquot was taken for protein determination using a BCA kit (Pierce Biotechnology, Rockford, IL) for calibration of the peptide concentration with protein.

Measurements

Blood lactate was measured using an automated glucose-lactate analyzer (2300 Stat Plus, YSI). Plasma samples were obtained by centrifugation and stored at ~30°C until measurement. Plasma osmolality was measured by freezing point depression using an osmometer (Fiske One-T, Fiske). Plasma concentrations of ACTH were measured using commercially available kits (ICN Biomedicals, Costa Mesa, CA) with detection limit of 4 pg/ml. The coefficient of intra-assay variation was 7.2%. Aliquots (90 μl each) obtained from the SME extraction were lyophilized and rehydrated. AVP content

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The number of Fos-ir cells in the pPVN at 120 min after onset of running (in the SME) was measured using commercially available kits for enzyme immunoassay (Peninsula Laboratories, San Carlos, CA) with detection limits of <40 pg/ml. The coefficient of intra-assay variation was 9.1% for AVP. All samples were determined within the same assay kit.

Statistics

Results are expressed as means ± SE. Data were analyzed using the Kruskal-Wallis test followed by the Mann-Whitney U-test, with *P < 0.05 considered to be statistically significant.

RESULTS

Experiment 1

Time course change in number of Fos-ir cells in the pPVN.

The number of Fos-ir cells in the pPVN at 120 min after onset of running above the LT maximally was increased by 711% from that at 30 min after onset of running (259.6 ± 49.9 at 120-min time point and 32 ± 15.5 at 30-min time point; Fig. 1). There was less Fos-ir cells in the PVN before running (in our preliminary experiment); thus we did not present data showing basal level in Fig. 1.

Changes in blood lactate and plasma ACTH concentration while running for 30 min. The blood lactate level while running in the supra-LT group was increased by 181, 236, and 210% from the control, the below-LT group, and before running, respectively (3.1 ± 0.4 at supra-LT group, 1.1 ± 0.1 at control, 0.9 ± 0.1 at below-LT group and 1.0 ± 0.0 mM at before running; Fig. 2A, top). The plasma ACTH level while running in the supra-LT group was increased by 290, 122, and 117% from the control, below-LT group, and before running, respectively (379 ± 66 at the supra-LT group, 97 ± 20 at the control, 170 ± 54 at the below-LT group, and 174 ± 17 pg/ml at before running respectively; Fig. 2A, bottom).

Plasma osmolality levels. The plasma osmolality levels of the rats subjected to supra-LT running for 30 min was increased by 1.9 and 3% from the control and before running, respectively (300 ± 1.8 at the supra-LT group, 294.4 ± 0.8 at the control, and 291.2 ± 1.3 mosmol/kgH2O at before running, respectively; Fig. 2B).

Expression of Fos/AVP-ir cells in PVN and SON during a 30-min run at above the LT. A supra-LT running markedly increased the number of both Fos-ir and Fos/AVP-ir cells in the pPVN, whereas control increased them less (Fig. 3, A and B). Representative examples of Fos-ir and Fos/AVP-ir cells in the supra-LT group were presented in Fig. 3C. In addition, in the SON, a supra-LT running also markedly increased the number of both Fos-ir and Fos/AVP-ir cells, whereas control did not (Fig. 3, E and F). However, there were few or no Fos/AVP-ir cells in the PVN magnocellular division (mPVN) (Fig. 3D). The distributions of Fos-ir, AVP-ir, and Fos/AVP-ir cells were represented as computer-assisted camera lucida mapping (Fig. 4). The number of both Fos-ir and Fos/AVP-ir cells in the supra-LT group in the pPVN and the SON was significantly higher than those of the below-LT and the control groups.
the SON we failed to find Fos-ir for the control and the below-LT groups (Fig. 5).

Experiment 2

Changes in blood lactate and AVP content in the SME during running for 30 min in the control and supra-LT groups. Blood lactate was significantly higher in the supra-LT group than in the control. There was a significant decline in AVP content in the SME in the supra-LT group compared with the control (Fig. 6).

DISCUSSION

In these experiments, we examined which hypothalamic AVP neurons would be activated by supra-LT running and whether AVP would be released from the SME by supra-LT running. We found that supra-LT running, which causes ACTH release and plasma hyperosmolality, 1) activated the pPVN and SON AVP neurons and 2) reduced the AVP content in the SME. These results suggest that the pPVN and SON AVP neurons are both activated with running stress, resulting in increased AVP content in the pituitary portal blood.

The experiment examining Fos expression in AVP neurons in the PVN and SON in response to supra-LT running was estimated at 120 min after application of the stress because, as measured by immunocytochemistry, that was the time point at which Fos expression reached its peak in the PVN. This is in good agreement with other stimuli (26, 28, 35) and with the peak time for Fos expression in the SON (data not shown). At that time point we observed a marked increase in Fos/AVP-ir cells in the pPVN and SON. Although under basal conditions only few AVP neurons are visible in the pPVN in rats (23, 31), we identified a population of AVP-ir neurons (see Figs. 3 and 4). It is likely that the repeated exposure to running during habituation enhanced expression of AVP in CRH neurons in the PVN.
the pPVN similar to that which was shown in response to repeated applications of immobilization stress (13). In this context, it is worth noting that a population of AVP neurons originating in the pPVN projects toward the brain stem and spinal cord and regulates the cardiovascular system (9, 40). Thus AVP neurons in the pPVN might also contribute to the cardiovascular response induced by supra-LT running (27).

The present study is the first ever report that describes the activation by supra-LT running of AVP neurons in the SON. Because AVP neurons in the SON are osmosensitive (32), hyperosmolality associated with stress exposure may have contributed to this activation. Interestingly some of the cells showing Fos labeling in the SON were not AVP-ir neurons. This population could include not only oxytocinergic neurons, which are known to be activated during stress (18), but also glial cells, which are similarly osmosensitive (14) and respond to hypotonic stimulation with increased Fos expression (25).

Our data suggest that, in contrast to the SON, AVP neurons in the magnocellular PVN are not activated. There may be different activating thresholds between the neurons in the PVN and SON in their sensitivity to several factors that vary due to running speed. In fact, SON magnocellular neurons show higher sensitivity to several stimuli (hemorrhage, hypotension) than do mPVN neurons (34, 36). Thus it may be that AVP neurons in the mPVN are activated by more intense running. In any case, it is of interest that supra-LT running appears to selectively activate AVP neurons in the SON but not the PVN. Although, to avoid hypovolemia, we decided not to measure plasma AVP levels simultaneously, our preliminary studies have confirmed that supra-LT running increased plasma AVP levels (data not shown) in a similar pattern to that seen in human (11, 41, 46). At first view, Fos-ir of SON-AVP neurons seems to reflect chiefly the release of AVP into plasma, which in turn controls plasma osmolality.

In experiment 2, we found that the SME AVP content declined significantly in the supra-LT group compared with the control. Although the SME contents of ACTH secretagogues are expressed by the ratio of synthesis to release (47), several lines of studies have indicated that reduced SME contents of ACTH secretagogues were used as an indication of secretion (17, 19). Therefore, the reduction in SME AVP content is indicative of probable increased release into the portal blood stimulating ACTH release. However, our methodological approach for measuring AVP in the SME did not make it possible to discriminate between its internal zone (AVP of magnocellular origin) and external zone (AVP of parvocellular origin). Therefore, we cannot state which zone contributed principally to the changes observed. Further studies are needed to investigate the details of the release patterns of AVP at the SME while running using a microdialysis technique.

The factors producing these activating effects on AVP neurons in the hypothalamus and reduction of AVP content in the SME followed by ACTH release when running above the LT are still unclear. A variety of factors (including osmolality as described above), many of which are modified during running, mediates the release into the blood of ACTH and/or AVP. These also include muscle contraction, which activates AVP neurons in both the parvocellular and magnocellular areas of the hypothalamus (22, 24). On the other hand, hypoglycemia, a potent releaser of the AVP content of the SME (8, 12), was not seen in the present study, since blood glucose levels increase to some extent as a result of running stress. This conclusion is supported by several studies (44, 45).

In summary, we found that supra-LT running, which is exercise at sufficient intensity to induce a stress response, actually increases ACTH and plasma osmolality levels and results in differential activation in AVP-containing neurons. These neurons are activated in the parvocellular PVN and magnocellular SON but not in the magnocellular PVN. We thus suggest that these brain loci are specifically responsive to running stress just above the LT.

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