Involvement of Activin and Inhibin in the Regulation of Food and Water Intake in the Rat

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ABSTRACT. The expression of activin and inhibin has been demonstrated in the hypothalamus, but their physiological roles in the brain remain to be elucidated. In the present study, involvement of activin and inhibin in the regulation of food and water intake was examined. Male rats were deprived of food or water for 12 and 60 hr, and mRNA levels of activin/inhibin α , β A and β B subunits in the hypothalamus were estimated by RT-PCR. Gene expression of α subunit transiently decreased at 12 hr of food deprivation, while it did not change during water deprivation. Food and water deprivation for 60 hr increased mRNA levels of β A and β B subunits, respectively. These results indicated that gene expression of each subunit was independently regulated. Injection of activin A (0.5 and 4.0 μ g) into the third ventricle decreased food intake. Water intake was suppressed by 4.0 μ g, but not 0.5 μ g, of activin A. Intracerebroventricular injection of inhibin A (0.5 and 4.0 μ g) decreased water intake in a dose dependent manner without affecting food intake, suggesting that inhibin could act independently of activin. Taken together, it is suggested that activin and inhibin take part in the central regulation of nutrient and fluid balance, though further study is needed to determine precise molecular species involved.

KEY WORDS: activin, food intake, hypothalamus, inhibin, water intake.

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Activin/inhibin is classified as a member of the transforming growth factor (TGF)- β superfamily [5, 30]. Inhibin is a heterodimer composed of α and β subunits, and activin is a homodimer or a heterodimer of β subunits. There are two forms of β subunit, β A and β B, which form three forms of activin, activin A (β A- β A), activin B (β B- β B) and activin AB (β A- β B), and two forms of inhibin, inhibin A (α - β A) and inhibin B (α - β B). The expression of mRNAs for α , β A, and β B subunits have been demonstrated in many tissues, including the gonad, placenta, pituitary, adrenal, bone marrow and brain [15]. Various experiments have shown that activin/inhibin plays important roles in erythroid differentiation [7], embryogenesis [26], follicular development [29], and spermatogonial proliferation [14], as well as the regulation of follicle stimulating hormone (FSH) secretion [25]. Modification of activin actions by follistatin, an activin-binding protein, has been also documented [16].

Activin/inhibin subunits within the brain have been demonstrated by Sawchenko *et al.* [21, 22], who localized β A subunit immunoreactivity in the nucleus tractus solitarius (NTS) in the rat. β A peptide immunoactivity was also found in nerve terminals of projections from the NTS to the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus. It is now recognized that activin receptors are expressed in many brain regions including the hypothalamus [3], and that activin is related to the control of hypothalamic functions such as oxytocin and corticotropin-

releasing hormone secretion [19]. In addition, activin promotes neural cell survival [23], phenotypic differentiation of cultured neurons [8], while inhibits neuroblastoma and P19 cell neuronal differentiation [10]. Activin is also suggested to participate in recognition of nutrient status because the lever pressing operant behavior induced by L-lysine deficiency was suppressed by infusion of inhibin and follistatin into the lateral hypothalamic area (LHA) [11, 12]. Since infusion of activin, but not inhibin, into the dorsal hypothalamus near the PVN resulted in an apparent increase in water intake and urine volume [24], activin might be involved in central regulation of fluid balance. Taken together, it is suggested that activin in the hypothalamus is related to the regulation of food and water intake.

In the present study, to elucidate the physiological significance of activin and inhibin in maintaining nutrient and water homeostasis, we examined if mRNA expression of activin/inhibin subunits in the hypothalamus was changed by food or water deprivation. The effects of intracerebroventricular (ICV) injection of activin and inhibin on food and water intake were also assessed.

MATERIALS AND METHODS

Animals: Eight-week old male Wistar-Imamichi rats, purchased from Imamichi Institute for Animal Reproduction (Tsuchiura, Japan) and housed under a controlled lighting condition of 14 hr light and 10 hr darkness (lights on from 0500 to 1900 hr) and constant temperature ($22 \pm 1^\circ\text{C}$), were used in this study. The animals were separately kept in plastic cages with stainless-steel lids. All rats were given pellet food (MR Breeder, Nihon-Nosan, Japan) and tap water *ad*

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libitum except when they were subjected to food or water deprivation experiment.

Brain RNA preparations: In the food deprivation experiment, food was withdrawn at 1700 hr, and animals were fasted for 0 (Control), 12 and 60 hr and then sacrificed by decapitation between 0500 hr and 0600 hr. During food deprivation period, water was available *ad libitum*. In the water deprivation experiment, rats were water-deprived and sacrificed as in the food deprivation experiment, while food was available *ad libitum*. The brain was immediately removed, and hypothalamus (anterior border: optic chiasma, posterior border: mammillary body, 2 mm from the center, 4 mm from the bottom) block was cut out in iced PBS and immediately frozen in liquid nitrogen. The blocks were stored at -80°C until total RNA extraction. Total RNAs from each block were extracted using the guanidinium thiocyanate/phenol extraction method (TRIZOL reagent; GIBCO BRL, Gaithersburg, MD, U.S.A.) and stored at -80°C until RT-PCR.

RT-PCR procedure: Levels of activin/inhibin subunit mRNA were determined by RT-PCR. Total RNA ($0.5\ \mu\text{g}$) was reverse transcribed at 42°C by using avian myeloblastosis virus reverse transcriptase and oligo (dT)+ primer in a total volume of $20\ \mu\text{l}$. PCR was performed on synthesized cDNAs in a total volume of $100\ \mu\text{l}$ containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl_2 , 0.01% gelatin, 1 mM dNTPs, 0.2 μM of each primer, and 2.5 units of LA *Taq* polymerase (Perkin-Elmer, Norwalk, CT, U.S.A.). Primer sets used for PCR was as follows: α subunit (5'-ACTACTGCCATGGTAGCT-3' and 5'-TACAAGCACAGTGTGTG-3'), βA subunit (5'-GGTCAACATTTGCTGTAAG-3' and 5'-CTTAAGGTTGGCAAAGG-3') and βB subunit (5'-CAACCAGAACCTATTTG-3' and 5'-CGAAAGTCGATGAAGAA-3'). After the samples were overlaid with light mineral oil, PCR amplification was carried out in a Perkin-Elmer/Cetus Thermal cycler (Perkin-Elmer, Norwalk, CT, U.S.A.). Each cycle consisting of 30 sec at 94°C (denaturation); 60 sec at 55°C (annealing) for α and βA subunit, and 30 sec at 94°C (denaturation); 90 sec at 49°C (annealing) for βB subunit. To determine the optimal cycle number within the multiplication stage, PCR reaction was done every 4 cycles between 16 and 44 cycles for each subunit. According to the results of this preliminary experiment, PCR for α , βA and βB subunits were done at 28, 32 and 36 cycles, respectively. In addition, to provide an appropriate internal control, coamplification of a 983-bp fragment of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was carried out in each sample using the primer pair (Clonetech Amplimer Sets, Clonetech, CA). PCR products were analyzed by agarose gel electrophoresis on NuSeiveGTG (BMA, Rockland, ME, USA) 3.0% gels and visualized with UV light following ethidium bromide staining. Band intensities of RT-PCR products were determined with NIH image analyzer, and data were normalized by the intensities of G3PDH RT-PCR products.

ICV injection of activin and inhibin: Indwelling cannula

(0.9 mm o.d., 0.6 mm i.d.) was placed into the 3rd ventricle using stereotaxic apparatus (Narishige, Tokyo, Japan) under sodium pentobarbital anesthesia (50 mg/kg, i.p.). The coordinate was determined by the atlas of Paxinos and Watson [17], and modified according to the distance between the lambda and bregma. The cannula placement in the 3rd ventricle was confirmed by the spouting of cerebrospinal fluid from the cannula. More than one week of recovery period after implantation of the cannula, recombinant human activin A and inhibin A (Genzyme, Cambridge, MA, U.S.A.) were injected into the 3rd ventricle between 1700 hr and 1830 hr. Each protein was dissolved in saline (0.5 or 4 $\mu\text{g}/2\ \mu\text{l}$) and injected with a 5- μl microsyringe connected to the injection cannula by means of polyethylene tubing (0.8 mm o.d., 0.5 mm i.d.), at a speed of 2 $\mu\text{l}/30\ \text{sec}$. 40 sec after the end of injection, the injection cannula was replaced with the stylet.

Measurements of food intake and water intake: Food and water intake were measured daily at 0930–1030 hr. Food was added daily in order to maintain the same amount in the food container every day. Water bottle was emptied and refilled every day with new tap water to maintain the same condition. Stainless ball-tip water bottles (Natsume, Tokyo, Japan) were used to reduce the amount of spilled water. The amounts of food and water intake during control period were calculated as mean of those of four days before the injection. The effects of ICV injection of activin A and inhibin A on food and water intake were evaluated by comparing the values of control period and those of next morning after injection.

Statistical analysis: For RT-PCR experiments, data were expressed as mean $\pm$ SEM, and analyzed by one way functional ANOVA followed by Fishers PLSD as a post-hoc test. All the other data were expressed as mean $\pm$ SEM, and analyzed by paired *t*-test. Differences at $P < 0.05$ were considered statistically significant.

RESULTS

Changes in activin/inhibin subunit mRNA expression in the hypothalamus during food deprivation are shown in Fig. 1. Gene expression of α subunit significantly decreased at 12 hr but recovered at 60 hr. Although gene expression of βB subunit did not change throughout the experimental period, that of βA subunit progressively increased and the increment reached significant at 60 hr. On the other hand, gene expression of α and βA subunits did not change during water deprivation (Fig. 2). Water deprivation significantly increased βB subunit gene expression at 60 hr.

Injection of activin A of both doses (0.5 μg and 4 μg) into the 3rd ventricle significantly reduced food intake as shown in Fig. 3. Water intake was also significantly suppressed by 4 μg , but not 0.5 μg , of activin A. While ICV injection of inhibin A had no effect on food intake, it significantly decreased water intake in a dose dependent manner (Fig. 4). Injection of saline affected neither food nor water intake.

Fig. 1. Changes in activin/inhibin subunit mRNAs in the hypothalamus during food deprivation. The value of time 0 was defined as 1. Each point and vertical bar represent the mean $\pm$ SEM (n=6/group). * $P < 0.05$ vs. time 0.

Fig. 2. Changes in activin/inhibin subunit mRNAs in the hypothalamus during water deprivation. The value of time 0 was defined as 1. Each point and vertical bar represent the mean $\pm$ SEM (n=6/group). * $P < 0.05$ vs. time 0.

Fig. 3. Effect of the injection of activin A into the third ventricle on food (upper panel) and water intake (lower panel). Each column and vertical bar represent the mean $\pm$ SEM (n=6/group). Open and closed columns represent the value before and after injection, respectively. * $P < 0.05$ vs. before injection.

DISCUSSION

In the present study, we demonstrated that food and water deprivation differently changed gene expression of activin/inhibin subunits in the hypothalamus, i.e. food and water deprivation mainly increased mRNA of βA and βB subunits, respectively. Gene expression of α subunit was not so much affected as β subunits by either treatment. These results suggest that gene expression of each subunit is independently regulated. It is not known in the present study, however, whether β subunits, whose gene expression was changed by the treatments, formed activin or inhibin. To

Fig. 4. Effect of the injection of inhibin A into the third ventricle on food (upper panel) and water intake (lower panel). Each column and vertical bar represent the mean $\pm$ SEM ($n=6$ /group). Open and closed columns represent the value before and after injection, respectively. * $P<0.05$ vs. before injection.

clarify this, analysis at protein level will be needed.

The increase in βA subunit gene expression after food deprivation suggests the physiological importance of this subunit in the maintenance of nutrient homeostasis. Considering the optimal cycle number for PCR, gene expression of βA subunit is expected to be much larger than that of βB subunit. Interestingly, Hawkins *et al.* [11, 12] reported the involvement of activin A in the recognition of nutrient status. They showed that infusion of inhibin and follistatin into the LHA suppressed lever pressing operant behavior under lysine deficiency paradigm. Infusion of activin A did not affect lever pressing behavior, probably due to sufficient

release of endogenous activin A. Taken together, it is suggested that nutrient unbalance caused by food deprivation as well as lysine deficiency leads an increase in βA subunit gene expression and in turn activin A synthesis with a resultant facilitation of feeding behavior and lever pressing for lysine-containing food, respectively.

Following water deprivation, gene expression of only βB subunit was increased. Concerning the regulation of water balance, there is a report showing that long term infusion of activin A, but not inhibin A, into the dorsal hypothalamus near the PVN induced an increase in water intake and urine volume without affecting food intake [24]. It has been also shown that infusion of activin A acutely increases circulating oxytocin levels [18], which is known to have natriuretic actions. These observations suggest the involvement of activin A in the regulation of fluid balance, though the expression of βA subunit was not affected by water deprivation at least at mRNA levels in the present study. Taking our results that βB subunit mRNA level was specifically increased by water deprivation into account, activin B may also participate in central regulation of water balance.

Injection of activin A into the third ventricle significantly decreased food intake at both doses used in the present study. The higher dose of activin A also suppressed water intake. Based on the above assumption that activin A may be a central mediator in the regulation of nutrient balance, activin A would be expected to increase food intake. Our results were not consistent with the reported effect of activin A on water intake mentioned above [24], too. Although it is currently difficult to reconcile the discrepancy, one of the possible explanations is that effects of activin A may vary among brain regions. In above-mentioned studies, the predicted site of action of activin A was either the LHA [11, 12] or near the PVN [24], whereas activin A was injected into the third ventricle in the present study. Since activin receptors have been demonstrated in the extra-hypothalamic sites as well [3], the present results might reflect the actions of activin A on these extra-hypothalamic sites. Alternatively, the effects of activin A may change depending on the doses administered, because activin at high doses is known to suppress its own effects [13]. The possibility that an increase in βA subunit mRNA during food deprivation results in an increase in inhibin A production, thereby stimulates appetite by suppressing the action of activin A, also cannot be ruled out.

On the other hand, ICV injection of inhibin A decreased water intake in a dose dependent manner without affecting food intake. Since inhibin mainly exerts the effect by interfering with the association of activin with its receptor [27], the observed effects of inhibin A might be due to the suppression of the action of endogenous activin A in the dorsal hypothalamus near the PVN. Interestingly, however, recent reports suggest the existence of an inhibin-specific receptor and an inhibin-signaling pathway [4, 6, 9]. Inhibin might be signaling by using activin receptor Type II (ActRII) along with β -glycan (TGF- β Type III receptor), which cannot be blocked by excess activin [2]. It is therefore probable that

inhibin A is specifically involved in the maintenance of water homeostasis apart from the suppression of the activin action.

Food and water intake is controlled by particular areas in the hypothalamus including the LHA, ventromedial nucleus (VMH), PVN, SON and anterior hypothalamic area [1, 28]. It is known that these areas are controlled not only by neural networks, but also by humoral factors. For example, it has been shown that acidic fibroblast growth factor (aFGF) is secreted from the ependymal layer in the 3rd ventricle after food intake, reaches the LHA through the VMH, and suppresses food intake [20]. Since both activin and inhibin could be detected at relatively high concentrations in the cerebrospinal fluid (our unpublished observation), activin/inhibin may play roles in regulating food and water intake as humoral factors in the brain rather than as neurotransmitters.

In conclusion, the present study demonstrated that food and water deprivation affected gene expression of activin/inhibin subunits in the hypothalamus and that ICV injection of activin and inhibin modified food and water intake. These results suggest the physiological involvement of activin and inhibin in the regulation of nutrient and fluid balance. Further study, however, is needed to clarify the sites of actions of activin and inhibin and the mechanisms underlying.

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