Leptin and Ciliary Neurotrophic Factor Enhance the Formation of Gap Junctions Between Folliculo-stellate Cells in Castrated Male Rats

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Summary. We investigated the effects of the leptin and ciliary neurotrophic factor (CNTF) on gap junction formation between folliculo-stellate cells in the anterior pituitary glands of male rats. Thirty-day-old Wistar-Imamichi strain male rats were castrated, and 30 days later they received intraperitoneal injections of either human recombinant leptin or recombinant rat CNTF. They were divided into seven groups according to the injected materials: PBS as a control, either 0.04, 0.2 or 1.0 mg/kg leptin or either 0.004, 0.02, or 0.1 mg/kg CNTF. Five rats from each group were killed 1, 2, 3, 4 and 5 days after the injections, and the pituitary gland was removed from each rat. Then the specimens were prepared for observation by transmission electron microscopy. We quantified the number of follicles and gap junctions and calculated the rate of occurrence of gap junctions as the ratio of the number of gap junctions existing between folliculo-stellate cells per intersected follicle profile in electron photomicrographs. The administration of 1.0 mg/kg leptin and 0.1 mg/kg CNTF to castrated male rats increased the number of gap junctions between folliculo-stellate cells. These observations indicate that the formation of gap junctions within the anterior pituitary gland of male rats is under the influence of leptin and CNTF.

The anterior lobe of a pituitary gland is comprised of hormone-producing granular cells and agranular cells. Agranular cells, characterized by their star-like

morphological features and specific ability of forming follicles, have been named folliculo-stellate cells (RINEHART and FARQUHAR, 1953; KAGAYAMA, 1965; VILA-PORCILE, 1972). Folliculo-stellate cells are also known to be coupled by gap junctions (SOJI and HERBERT, 1990; YAMAMOTO et al., 1993; INOUE et al., 1999; SAKUMA et al., 2001). These junctions are composed of transmembrane channels allowing the free transcellular exchange of small cytoplasmic molecules such as ions, amino acids, sugars, and several second messengers (LOEWENSTEIN, 1981; BENNETT et al., 1991; BEYER, 1993; SPRAY, 1994; WOLBURG and ROHLMANN, 1995; KUMAR and GILULA, 1996). We have proposed that such an intercellular communication via gap junctions might have some fundamental importance in maintaining homeostasis throughout the interconnected folliculo-stellate cells (SAKUMA et al., 2001).

SOJI and HERBERT (1990) performed a quantitative electron microscopic study and calculated that the number of gap junctions found in normal adult rats was about 0.5 gap junction per follicle. They also reported that the formation of gap junctions between folliculo-stellate cells was down-regulated by castration and that the slow rate in gap junction formation in the castrated male rats could be compensated for by testosterone injection. From these findings, they suggested that an intact hypothalamo-pituitary gonadal axis was a prerequisite for the normal maturation of gap junctions between folliculo-stellate cells.

Leptin, the product of the obese (ob) gene and ciliary neurotrophic factor (CNTF), a member of the neuropoietic family of cytokines might share the essential functions in the hypothalamo-pituitary

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gonadal axis in guiding normal sexual maturation (BARASH et al., 1996; CHEHAB et al., 1996; MANTZOROS et al., 1997; YU et al., 1997; CLEMENT et al., 1998; KALRA et al., 1998; STROBEL et al., 1998; CUNNINGHAM et al., 1999; STANLEY et al., 2000; WAUTERS et al., 2000). Moreover, recent studies have revealed that both functional leptin receptors (OB-Rb) (JIN et al., 2000) and CNTF receptors (PEREZ CASTRO et al., 2000) are present in the stable mouse pituitary-derived folliculo-stellate cell line TtT/GF. JIN et al. (2001) also reported that purified populations of rat folliculo-stellate cells express mRNAs for OB-Rb.

The aim of this study was to examine the effects of leptin and CNTF over gap junction formation between folliculo-stellate cells within rat anterior pituitary glands in castrated male rats.

MATERIALS AND METHODS

All animal experiments were performed under the NIH Guidelines for the Care and Use of Laboratory Animals. Male rats of the Wistar-Imamichi strain were prepared in this study. They were maintained under conditions of controlled temperature (22–24°C) and illumination (8:00–20:00 daily). Animals were given a normal diet and ample water. All rats were castrated at 30 days after birth under anesthesia with Nembutal® (pentobarbital). On day 60 of age, all animals received intraperitoneal injections of indicated doses of recombinant human leptin or recombinant rat CNTF dissolved in 0.1 ml of PBS (0.01 mol/L phosphate buffer saline; pH 7.4). The animals were separated into seven groups: Group A received only PBS and served as a control, Group B was injected with 0.04 mg/kg leptin, Group C was injected with 0.2 mg/kg leptin, Group D was injected with 1.0 mg/kg leptin, Group E was injected with 0.004 mg/kg CNTF, Group F received 0.02 mg/kg CNTF and Group G received 0.1 mg/kg CNTF. Each group consisted of twenty-five animals. The recombinant human leptin and recombinant rat CNTF were purchased from CHEMICON International, Inc. (Temecula, CA, USA).

Five rats from each group were killed at ages 61, 62, 63, 64 and 65 days or 1, 2, 3, 4 and 5 days after the injections, respectively. On the scheduled date, the animals were deeply anesthetized with Nembutal® and perfused with a fixative for 5 min through the left ventricle of the heart. The fixative used was 2.5% glutaraldehyde and 2% sucrose in a 0.05 M cacodylate buffer (pH 7.4). After the perfusion, the pituitary gland was removed from each rat, and the ante-

rior lobe of the pituitary gland was dissected into four blocks. They were then refixed by immersion for 1 h in the same solution used for perfusion. The sections were then postfixed for 1 h in 1% osmium tetroxide buffered by 2% sucrose and 0.05 M sodium cacodylate (pH 7.4). After postfixation, they were rinsed in ice-cold water for 5 min and dehydrated in a graded series of ethanol. Following two rinses in 100% ethanol for 10 min each, the specimens were immersed twice in absolute propylene oxide for 15 min each and then embedded in epoxy resin. Two ultrathin sections of 60 nm thickness and separated by at least 100 µm from each other were prepared from each block. They were placed on copper grids, stained with uranyl acetate and lead citrate, and observed using a Hitachi H-7000 transmission electron microscope. In this way, a total of 8 ultrathin sections was investigated for each rat amounting to 40 for each group in this study.

The photomicrographs were selected on the basis of each displaying a minimum of one follicle according to the identification procedures described by SOJI and HERBERT (1990). We confirmed the detail of the trilamellar structure of the gap junction at magnification levels of at least 20,000. Distinction between the types of intercellular junctions was based on the morphological criteria described by STAEHELIN (1974). We determined the rate of occurrence of gap junctions as the ratio of the number of gap junctions existing between folliculo-stellate cells per intersected follicle profile in electron photomicrographs. The number of follicles and gap junctions was quantified using a total of 400 electron photomicrographs that contained at least one follicle from each animal group, and we calculated the mean and standard error for each animal group. The data were statistically analyzed by an analysis of variance followed by the t-test for determining differences between means.

RESULTS

We found many folliculo-stellate cells encircling the hypertrophied gonadotrophs with long cytoplasmic processes (Fig. 1).

In all samples of the present study, gap junctions with the trilamellar structure of plasma membranes of adjacent cells were found between neighboring folliculo-stellate cells. The rate of gap junctions in the control, PBS-injected group rats remained constant (0.16 ± 0.04 to 0.18 ± 0.05) from days 1 to 5 after injections (Figs. 2, 3).

Fig. 1. The electron microscopic appearance of folliculo-stellate cells (*F*) in the castrated male rat anterior pituitary at three days after PBS treatment. Slender cytoplasmic processes (*arrows*) of folliculo-stellate cells are in contact with hypertrophied gonadotrophs. A small follicular lumen (*) can be seen. There are no gap junctions present. $\times 5,000$

Leptin treatment groups

In the groups given 0.04 and 0.2 mg/kg leptin, no significant differences were noted in the presence of gap junctions as compared with the control group rats. In the 1.0 mg/kg leptin group, an increase in the rate of gap junctions was found on days 2, 3 and 4 after injection. Gap junctions between folliculo-stellate cells were observed at an especially high

ratio on day 3 after injections when their numbers reached 0.45 ± 0.07 per follicle (Figs. 2, 4). The rate of occurrence in this group returned to a level similar to the control group on day 5 after the leptin injection (Fig. 2).

CNTF treatment groups

Treatment with 0.004 and 0.02 mg/kg CNTF caused no significant changes in the rate of gap junctions;

Fig. 2. Summary of the number of gap junctions present per follicle in the leptin treatment groups at each of the five ages studied.

Fig. 3. Summary of the number of gap junctions present per follicle in the CNTF treatment groups at each of the five ages studied.

Fig. 4. In a castrated male rat anterior pituitary three days after 1.0 mg/kg leptin treatment, one gap junction (*arrow*) is situated between folliculo-stellate cells forming the same follicles (*). In the *inset*, the area in the rectangle is shown at a higher magnification and illustrates the typical trilamellar structure of the gap junction in more detail. $\times 8,500$, inset: $\times 100,000$

however, injections with 0.1 mg/kg CNTF resulted in an increase in the rate of gap junctions on days 2, 3 and 4. The occurrence of gap junctions between folliculo-stellate cells peaked at a maximum of 0.44 ± 0.07 per follicle on day 3 after injection (Figs. 3, 5) and then returned to a level similar to the control group on day 5 after the administration of CNTF (Fig. 3).

DISCUSSION

There is some evidence that castration results in the morphological alteration of folliculo-stellate cells within anterior pituitary glands (SHIRASAWA et al., 1983; INOUE et al., 1999). SHIRASAWA et al. (1983) carried out morphological investigations of folliculo-stellate cells after castration and thyroidectomy by

Fig. 5. A 63-day-old castrated male rat treated with 0.1 mg/kg CNTF. One gap junction (*arrow*) is seen joining the folliculo-stellate cells near the follicular lumen (*). The region pointed out by the *arrow* is also shown at a higher magnification in the **inset**. $\times 11,000$, inset: $\times 100,000$

using immunohistochemistry and electron microscopy. They found morphological changes in folliculo-stellate cells such as expanded and ramified cytoplasmic processes in the case of castration, but these changes were not seen after the removal of thyroid glands. INOUE et al. (1999) also found many folliculo-stellate cells encircling hypertrophied gonadotrophs with their long cytoplasmic processes in the castrated rats in transmission electron microscopic observations. In our study, the morphological changes of folliculo-stellate cells and their association with hypertrophied gonadotrophs agreed well with these previous investigations.

Concerning gap junctions, previous studies have indicated that a dysfunction of the hypothalamo-pituitary gonadal axis can slow the rate of gap junction formation between folliculo-stellate cells (SOJI and HERBERT, 1990; SAKUMA et al., 2001). In this study, the rate of gap junctions in the control group rats was suppressed to a low level (0.16 ± 0.04 to 0.18 ± 0.05) from days 1 to 5 after injections. This insufficient development of gap junctions is thought to be a result of the influence of the castration. On the other hand, the animals injected with 1.0 mg/kg leptin and 0.1 mg/kg CNTF showed a transient increase of the gap junction rate between folliculo-stellate cells on days 2, 3 and 4 after injections. Though the role of gap junctions between folliculo-stellate cells remains largely unknown, some theories have been proposed regarding their function. The morphological characteristic of folliculo-stellate cells surrounding neighboring granular cells suggests that they may be contributing to the regulation of these cells by paracrine effects (INOUE et al., 1999). The folliculo-stellate cells connected through gap junctions have been shown to communicate by calcium waves (GUERINEAU et al., 1998; FAUQUIER et al., 2001). Therefore, the folliculo-stellate cell network seems to provide a functional syncytium that could rapidly control a variety of Ca^{2+} -dependent cellular processes, including the synthesis and release of bioactive substances having the potential for the coactivation of various hormone-producing granular cells including gonadotrophs. It is possible that the insufficient endocrine condition caused by castration can enhance the gap junctional communication between folliculo-stellate cells and lead to changes in the production of bioactive substances.

Both leptin and CNTF seem to counteract the down-regulation of gap junction formation caused by the disrupted hypothalamo-pituitary gonadal axis. The presence of OB-Rb and CNTF receptors indicates that folliculo-stellate cells are capable of responding to both leptin and CNTF (PEREZ CASTRO et

al., 2000; JIN et al., 2000, 2001). A recent study found that CNTF—in combination with its soluble receptor—can increase the gap junctional coupling of C6 glioma cells which have fewer gap junctions compared with normal astrocytes (OZOG et al., 2002). In the same manner, the intracellular pathways activated by CNTF might enhance the gap junctional coupling of folliculo-stellate cells.

Some investigators have suggested that marked functional similarities such as the influence on the hypothalamo-pituitary gonadal axis exist between leptin and CNTF (KALRA et al., 1998; STANLEY et al., 2000).

Leptin, the product of the obese (*ob*) gene, is a 167 amino acid protein and mainly produced by adipose tissue. It is well known to play a fundamental role in weight homeostasis by regulating appetite, glucose utilization, and energy expenditure (ZHANG et al., 1994; PELLEYMOUNTER et al., 1995; HALAAS et al., 1995; CAMPFIELD et al., 1995; KAMOHARA et al., 1997; SATOH et al., 1997; FRIEDMAN and HALAAS, 1998; SHIODA et al., 1998; FUNAHASHI et al., 1999). In addition to such primary effects on the metabolism, a growing amount of data are emerging that leptin is especially involved in normal sexual maturation by maintaining the normal functions of the hypothalamo-pituitary gonadal axis (SWERDLOFF et al., 1976; COLEMAN, 1978; BRAY and YORK, 1979; BARASH et al., 1996; CHEHAB et al., 1996; MANTZOROS et al., 1997; YU et al., 1997; CLEMENT et al., 1998; STROBEL et al., 1998; CUNNINGHAM et al., 1999; WAUTERS et al., 2000).

Conditions which cause leptin dysfunction are known to result in disturbances in reproductive capacity in the field of clinical medicine (CLEMENT et al., 1998; STROBEL et al., 1998). In experimental animals, infertility and atrophic reproductive organs due to hypothalamo-pituitary hormone insufficiency comprise a well-known feature of leptin-deficient *ob/ob* mice (SWERDLOFF et al., 1976; COLEMAN, 1978; BRAY and YORK, 1979; CHEHAB et al., 1996), and leptin treatment can reverse the prepubertal pattern of gonadotropin secretion in the leptin deficient *ob/ob* mouse (BARASH et al., 1996; CHEHAB et al., 1996; MOUNZIH et al., 1997).

STANLEY et al. (2000) suggests that CNTF also can mediate some of the actions of the hypothalamo-pituitary gonadal axis. CNTF, a 22-kDa pluripotent neurocytokine, belongs to the neuropoietic family and is expressed by glial cells in the peripheral nerve and central nervous system (LIN et al., 1990; IP and YANCOPULOS, 1992). Unlike leptin, CNTF can normalize the obesity-related phenotype both in *db/db* mice which lack functional leptin receptors and in mice

with diet-induced obesity, a condition which is known to be partially resistant to the actions of leptin (GLOAGUEN et al., 1997; LAMBERT et al., 2001). From these findings, CNTF is thought to function by activating the same intracellular pathways with leptin through a different receptor.

The functional leptin receptor (OB-Rb) has many features consistent with the class I cytokine receptor family (HELDIN, 1995; TARTAGLIA et al., 1995; BAUMANN et al., 1996; LEE et al., 1996; NAKASHIMA et al., 1997) and shows high similarity to the gp 130 signal-transducing component of the interleukin-6 receptor (TAGA et al., 1989; HIBI et al., 1990), the granulocyte colony-stimulating factor receptor (FUKUNAGA et al., 1990; LARSEN et al., 1990), the leukemia inhibitory factor receptor (GEARING et al., 1991; IP et al., 1992) and the CNTF receptor (DAVIS et al., 1991; IP et al., 1992). Therefore, leptin and CNTF are thought to activate similar intracellular pathways that activate overlapping signaling molecules, notably the STAT3 transcription factor (KISHIMOTO et al., 1994; STAHL et al., 1995; GHILARDI et al., 1996; VAISSE et al., 1996; CARPENTER et al., 1998).

Clarification of the mechanisms by which leptin and CNTF induce changes in the regulation rate of gap junctions will require further research; however, it can presently be concluded that gap junction formation between folliculo-stellate cells is at least in part regulated by leptin and CNTF in male rats.

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