Effects of Leptin on Hypothalamic Arcuate Neurons in Wistar and Zucker Rats: An *In Vitro* Study

KATSUHIKO NAGAMORI,* MASARU ISHIBASHI,* TAKEMASA SHIRAISHI,† YUTAKA OOMURA,‡ AND KAZUO SASAKI[1]

Department of Bio-Information Engineering, Faculty of Engineering, Toyama University, Toyama 930-8555, Japan; †Foundation for Oriental Medicine Research, Tokyo, Japan; and ‡Department of Physiology, Faculty of Medicine, Kyushu University, Fukuoka 882-0066, Japan

Leptin, a product of the *ob* gene, decreases food intake and body weight in both Wistar and Zucker obese rats when administered centrally or peripherally. To examine whether these leptin effects might be mediated through a neuropeptide Y (NPY) signaling pathway in the medial part of the arcuate nucleus of the hypothalamus (vmARC), the effects of leptin on vmARC neurons in Wistar and Zucker obese rats were examined electrophysiologically using brain slice preparations. Bath application of leptin inhibited about 60% of the vmARC neurons recorded in slices from Wistar rats. Similar inhibitory effects of leptin on vmARC neurons were also observed under low-Ca^{2+}, high-Mg^{2+} Ringer's solution. However, inhibitory effects were almost absent under Ringer's solution containing a protein kinase C inhibitor, chelerythrine chloride. In slices from Zucker obese rats, leptin inhibited only about 25% of the vmARC neurons recorded, and the proportion of neurons inhibited was significantly smaller for these rats than for Wistar rats. These results suggest that reductions in food intake and body weight induced by leptin in both Wistar and Zucker obese rats are partly mediated *via* inhibition of an NPY signaling pathway in the vmARC. Exp Biol Med 228:1162–1167, 2003

Key words: leptin; arcuate nucleus; protein kinase C; Wistar rat; Zucker rat

Leptin, a product of the obese (*ob*) gene (1), is involved in the regulation of food intake and body weight (2). Adipocytes are the major source of leptin production and secretion, and leptin constitutes a feedback signal that informs the central nervous system about the status of energy stores in the peripheral tissues (3). For example, the leptin level in the plasma shows a positive correlation with the adipose tissue mass (4), and it is increased in the fed state and decreased during starvation (5). Leptin-deficient *ob/ob* mice, which have a low level of plasma leptin, show increased food intake and obesity (6). Intraperitoneal or intracerebroventricular injections of leptin inhibit food intake and reduce body weight in normal rats as well as in *ob/ob* mice (2, 7, 8).

Plasma leptin crosses the blood-brain barrier *via* a receptor-mediated transport system (9) and acts on the biologically active long forms of the leptin receptor, which are expressed mainly in a number of hypothalamic sites (10, 11). One of these hypothalamic sites is the arcuate nucleus (ARC), and Håkansson *et al.* (12) reported that leptin receptors are co-localized with neuropeptide Y (NPY) in the ARC, especially in its ventromedial portion (vmARC). As is well known, NPY is a potent orexigenic substance, and indeed acute intracerebroventricular injections of NPY induce robust feeding (13, 14), whereas long-term intracerebroventricular or intrahypothalamic administrations cause hyperphagia and obesity (15, 16). Furthermore, both fasting in normal rats and the genetic leptin deficiency seen in *ob/ob* mice increase NPY mRNA expression in the ARC (17, 18), and this increased expression is attenuated by leptin administration (19, 20). In addition, leptin inhibits both the release and biosynthesis of NPY (21). These results raise the possibility that the effects of leptin on food intake and body weight may be partly mediated through an NPY signaling pathway in the vmARC.

Hyperphagia and obesity are also seen in leptin receptor–deficient *db/db* mice and Zucker obese (*fa/fa*) rats. The former have a point nonsense mutation that generates a new

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[1] To whom requests for reprints should be addressed at Division of Bio-Information Engineering, Faculty of Engineering, Toyama University, 3190 Gofuku, Toyama 930-8555, Japan. E-mail: sasaki@eng.toyama-u.ac.jp

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splice donor site, and this dramatically reduces the expressions of the long forms of the leptin receptor (22). Consequently, *db/db* mice do not respond to leptin (3) and generate an obese phenotype. Unlike *db/db* mice, Zucker obese rats have a missense point mutation that results in a glutamine^{269} to proline^{269} amino acid substitution in the extracellular ligand-binding domain of the leptin receptor (23). Plasma leptin levels are 6-fold higher in Zucker obese rats than in control lean rats (24), and endogenous NPY synthesis is elevated in the ARC of Zucker obese rats (25). A few years ago, it was reported that central, but not peripheral, administration of leptin reduces food intake and body weight in both Zucker obese and lean rats (26, 27). Furthermore, intracerebroventricular administration of leptin was found to decrease NPY levels in the ARC and in the paraventricular nucleus of the hypothalamus (PVN) in both lean and obese Zucker rats (26). These results suggest that an NPY signaling pathway involving leptin in the vmARC still functions to some extent even in Zucker obese rats. For that reason, we examined electrophysiologically how, and in what ways, neurons in the vmARC of Wistar and Zucker obese rats respond to leptin.

Materials and Methods

Animals, Slice Preparation, and Ringer's Solution. The Institutional Animal Care and Use Committee of the Faculty of Engineering of Toyama University approved all experiments. Animals were 5-week-old male Wistar rats (Sankyo Laboratory, Shizuoka, Japan) and 8-week-old male Zucker obese rats (Charles River Japan, Kanagawa, Japan). Following decapitation, the brain was quickly removed from the skull, and frontal hypothalamic slices (400 μm) were cut using a microslicer in oxygenated, ice-cooled normal Ringer's solution. Those slices that included the vmARC were selected, and in each slice only the vmARC was separated from the other tissues. The vmARC slices were then placed into an incubation chamber containing normal Ringer's solution, which was maintained at room temperature (25°C) and continuously oxygenated with 95% O_2–5% CO_2. The composition of the normal Ringer's solution was as follows (m*M*): NaCl, 124.0; KCl, 5.0; $CaCl_2$, 2.4; $MgSO_4$, 1.3; KH_2PO_4, 1.24; $NaHCO_3$, 26.0; glucose, 10.0. To make low-Ca^{2+}, high-Mg^{2+} Ringer's solution, $CaCl_2$ was decreased to 0.1 m*M* and $MgCl_2$ was added at 2.3 m*M*. When chelerythrine chloride (Research Biochemicals International, Natick, MA), a protein kinase C (PKC) inhibitor, was applied in normal Ringer's solution, its concentration was 5×10^{-6} *M*.

Neurophysiological Procedures. After preincubation for more than 1.5 hr, each vmARC slice was transferred into a recording chamber. This was maintained at 36°C ± 0.5°C, constantly aerated with 95% O_2–5% CO_2, and perfused continuously with Ringer's solution at a rate of 1 ml/min. Extracellular action potentials, which were recorded from the vmARC with the aid of a glass microelectrode filled with normal Ringer's solution (electrode resistance, 3–8 MΩ), were fed into a main amplifier *via* a preamplifier. The output signal of the amplifier was monitored on an oscilloscope and recorded on magnetic tape. The signal was also passed through a pulse former and from thence into a computer that calculated the number of action potentials every 1 sec and displayed them on a screen as a histogram. After completion of each experiment, the histogram was saved onto a hard disk and printed on paper by means of a laser printer.

Leptin. Recombinant murine leptin was purchased from Pepro Tech, Inc. (London, England). The agent was first dissolved in distilled water, then diluted with normal Ringer's solution to a concentration of 10^{-7} *M* and stored at −40°C in small aliquots. One aliquot was taken for each experiment and further diluted to the desired concentration by adding Ringer's solution just before use.

Statistics. The statistical significance of differences in the proportions of neurons that were responsive or unresponsive to leptin between different Ringer's solutions or different strains of animals was analyzed using the χ^2 test. Statistical analysis of the latency and duration of leptin-induced responses was performed using a *t* test. The criterion of significance was $P < 0.05$.

Results

Effects of Leptin on vmARC Neurons From Wistar Rats Under Normal and Low-Ca^{2+}, High-Mg^{2+} Ringer's Solution. Single-neuron activity was recorded from 47 neurons in vmARC from Wistar rats under normal Ringer's solution. Of these, 22 were tested with both 10^{-11} *M* and 10^{-10} *M* leptin, 19 with 10^{-11} *M* leptin alone, and the remaining 6 with 10^{-10} *M* leptin alone. The results are summarized in Table I. When 10^{-11} *M* leptin was applied to 41 neurons, 15 (36.5%) were inhibited, 2 (4.8%) were excited, and 24 (58.5%) were unaffected. When 10^{-10} *M* leptin was applied to 28 neurons, 16 (57.1%) were inhibited, 1 (3.5%) was excited, and the remaining 11 (39.2%) were unaffected. Among the 22 neurons tested with both 10^{-11} *M* and 10^{-10} *M* leptin, 8 were inhibited and 14 were not affected by 10^{-11} *M* leptin. The eight neurons that were inhibited by 10^{-11} *M* leptin were more strongly inhibited by 10^{-10} *M* leptin (Fig. 1). Of the 14 neurons that were not affected by 10^{-11} *M* leptin, 6 were inhibited, and 8 were unaffected by 10^{-10} *M* leptin. At 10^{-11} *M* leptin, the mean latency and duration of the inhibitory responses (in 15/41 neurons, 36.5%) were 11.0 ± 1.3 min (mean ± SEM) and 30.8 ± 5.5 min, respectively. The corresponding values in those inhibited at 10^{-10} *M* (16/28, 57.1%) were 9.9 ± 1.8 min and 36.4 ± 7.4 min, respectively.

The leptin effects on vmARC neurons were further examined under low-Ca^{2+}, high-Mg^{2+} Ringer's solution, each slice being exposed to this solution in the recording chamber for at least 30 min before leptin application. Of the 10 vmARC neurons that were exposed to 10^{-10} *M* leptin under low-Ca^{2+}, high-Mg^{2+} Ringer's solution, 6 (60.0%) were inhibited (Fig. 2), 1 (10.0%) was excited, and the remaining 3

Table I. Summary of Leptin Effects on vmARC Neurons in Wistar and Zucker Obese Rats

Animals	Ringer's solution	Leptin				
		Dose	Inhibition	Excitation	No effect	Total
Wistar	Normal	10^{-11} *M*	15 (36.5%)	2 (4.8%)	24 (58.5%)	41 (100%)
	Normal	10^{-10} *M*	16 (57.1%)	1 (3.5%)	11 (39.2%)	28 (100%)
	Low-Ca^{2+}, high-Mg^{2+}	10^{-10} *M*	6 (60%)	1 (10.0%)	3 (30.0%)	10 (100%) ns
	PKC inhibitor	10^{-10} *M*	1 (4.5%)	2 (9.1%)	19 (86.4%)	22 (100%)[a]
Zucker	Normal	10^{-10} *M*	7 (24.1%)	1 (3.4%)	21 (72.4%)	29 (100%)[b]

Note. ns, not significant. [a] and [b] $P < 0.01$ and $P < 0.05$, respectively, versus proportions obtained using 10^{-10} *M* leptin under normal Ringer's solution in Wistar rats.

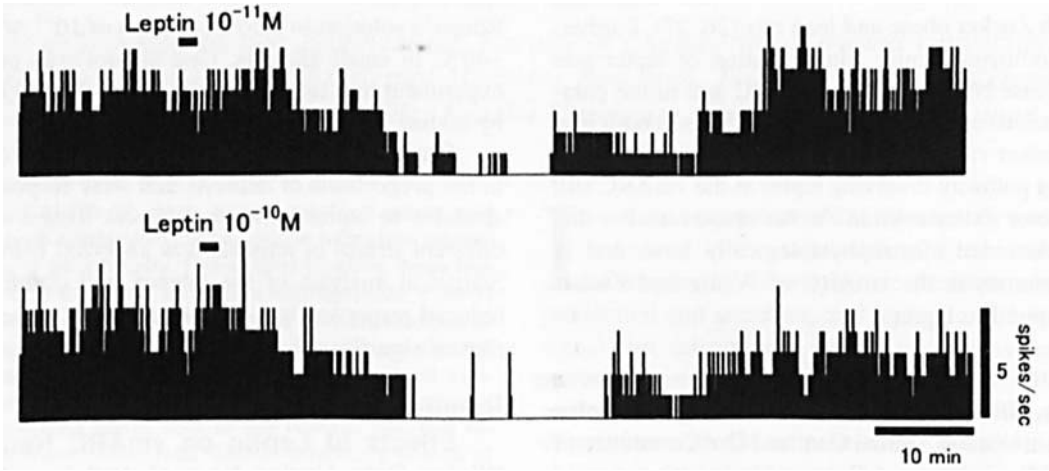

Figure 1. Leptin effect on a vmARC neuron in a slice from Wistar rat; frequency in impulses per second is shown as a function of time. Upper and lower records were continuous, and leptin (applied at 10^{-11} *M* and 10^{-10} *M* for the period indicated) decreased activity in a dose-related fashion.

(30.0%) were unaffected (Table I). The proportions of vmARC neurons excited or inhibited at 10^{-10} *M* leptin were not significantly different between low-Ca^{2+}, high-Mg^{2+} Ringer's solution and normal Ringer's solution (χ^2 test, $P > 0.05$). These results suggest that about 60% of vmARC neurons are dose-relatedly inhibited by leptin *via* direct actions on the membranes of the recorded neurons themselves.

Effects of Leptin on vmARC Neurons From Wistar Rats Under Normal Ringer's solution Containing Chelerythrine Chloride. To examine the possible involvement of PKC in the effects of leptin on vmARC neurons, chelerythrine chloride was applied in normal Ringer's solution. The recording chamber was perfused with this solution for at least 30 min before leptin application. Under the chelerythrine chloride–containing Ringer's solution, 22 neurons in the vmARC were exposed to 10^{-10} *M* leptin. Of these, 1 (4.5%) was inhibited, 2 (9.1%) were excited, and the remaining 19 (86.4%) were unaffected (Table I). The proportion of vmARC neurons showing an inhibitory response to leptin was significantly smaller in Ringer's solution containing chelerythrine chloride than in that without chelerythrine chloride (χ^2 test, $P < 0.001$).

Effects of Leptin on vmARC Neurons From Zucker Obese Rats Under Normal Ringer's Solution. In slices from Zucker obese rats, single-unit activity was recorded from 29 neurons in the vmARC. Bath application of 10^{-10} *M* leptin inhibited 7 (24.1%, Fig. 3) and excited 1 (3.4%), whereas the remaining 21 (72.4%) were unaffected (Table I). The proportion of vmARC neurons inhibited by 10^{-10} *M* leptin was significantly smaller for Zucker obese rats than for Wistar rats (χ^2 test, $P < 0.05$). The mean latency and duration of these inhibitory responses were 6.1 ± 1.0 min and 20.7 ± 5.7 min, respectively, for Zucker obese rats. These values were not statistically different from those obtained using 10^{-10} *M* leptin under normal Ringer's solution in slices from Wistar rats (*t* test, $P > 0.05$).

Discussion

In the previous studies, it has been reported that the electrophysiological characteristics such as membrane potential and input resistance and the responsiveness to leptin in hypothalamic neurons of Zucker lean rats are indistinguishable from those obtained from Wistar or Sprague-Dawley rats (28, 29). In the present study, therefore, we used Wistar rats as lean controls for Zucker obese rats. About 60% of the vmARC neurons tested in slices from Wistar rats were inhibited when 10^{-10} *M* leptin was applied under normal Ringer's solution, and only 4% were excited. Similar results were obtained under low-Ca^{2+}, high-Mg^{2+} Ringer's solution, which was used to block synaptic trans-

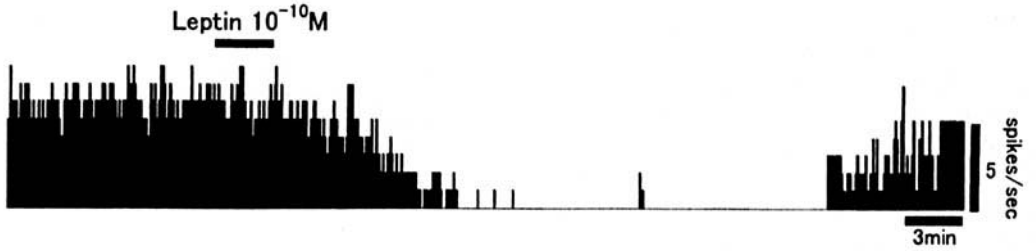

Figure 2. Direct effect of leptin on a vmARC neuron in a slice from a Wistar rat. Single-neuron activity was inhibited by leptin under low-Ca^{2+}, high-Mg^{2+} Ringer's solution, suggesting that leptin exerted a direct inhibitory postsynaptic effect on the recorded neuron.

mission. These results suggest that the main effect of leptin on vmARC neurons is inhibition, and that the inhibition is due to a direct action of this agent on the recorded neurons, not to an indirect action *via* synaptic transmission. Inhibitory responses to leptin in ARC neurons have been reported previously. For instance, Rauch *et al.* (30) found that about 40% of the neurons they recorded in the ARC of Wistar rats were inhibited by bath application of leptin at 10^{-8} to 10^{-6} *M*. The difference in the proportions of inhibited neurons between the present and previous studies suggests that when the recording sites are restricted to the vmARC, in which NPY-containing neurons are concentrated, the proportion of neurons inhibited is greater. This supports the view that the effects of leptin on food intake and body weight may be exerted in part through inhibition of an NPY signaling pathway in the vmARC.

Spanswick *et al.* (29) reported that glucose-receptive neurons in the ventromedial hypothalamus (VMH) of Sprague-Dawley and Zucker lean rats were hyperpolarized by bath application of leptin through activation of ATP-sensitive potassium channels. However, in an *in vivo* study in which leptin was administered iontophoretically in anesthetized rats, about 80% of glucose-receptive neurons in the VMH were excited, not inhibited, by leptin (31). Moreover, inhibition of neurons in the supraoptic nucleus of the hypothalamus (SON) by bath application of leptin was found not to be blocked by co-application of tolbutamide (32), which is known to inhibit ATP-sensitive potassium channel activity (33). Thus, the precise nature of the mechanisms underlying leptin-induced inhibitory responses in hypothalamic neurons remains unclear. In the present study, the proportion of neurons in the vmARC inhibited by leptin was significantly smaller under chelerythrine-containing Ringer's solution than under normal Ringer's solution. This observation suggests that PKC activity may be involved in the cellular mechanisms underlying the inhibitory effects of leptin, at least in the vmARC. As is well known, leptin activates the long forms of the leptin receptor, which belongs to the cytokine superfamily, and further induces activation of signaling pathways such as those involving Janus protein tyrosine kinase and signal transducers and activators of transcription (JAK-STAT), mitogen-activated protein kinase (MAPK), and src-like homology 2 domain-containing protein tyrosine phosphatase (SHP-2) (34). Recent studies indicate that in addition to JAK-STAT, MAPK, and SHP-2, PKC is involved in the signal transduction pathways activated by leptin. For example, Takekoshi *et al.* (35) demonstrated that leptin stimulates catecholamine synthesis in a PKC-dependent manner in cultured chromaffin cells, and that this leptin effect was blocked by addition of a PKC inhibitor.

In the present study, the latency and duration of the inhibitory responses induced by 10^{-10} *M* leptin in slices from both Wistar and Zucker obese rats were relatively long, at 6–10 min and 20–35 min, respectively. Inhibitory responses to leptin with long latencies and durations have been demonstrated in other hypothalamic areas such as the ARC (30), SON (32), and VMH (29). Interestingly, we showed a decade ago that acidic fibroblast growth factor (aFGF), when applied iontophoretically, inhibited neuronal activity in the lateral hypothalamic area with a long latency (7.0 ± 2.3 min) and duration (10.6 ± 4.1 min) and that 1-oleoyl-2-acetyl-glycerol, a direct activator of PKC, had inhibitory effects nearly identical to those of aFGF (also with long latency and duration), suggesting an involvement of PKC in aFGF-induced inhibition (36). These results seem to support the view that leptin-induced inhibition in vmARC neurons is exerted *via* PKC activation.

Cusin *et al.* (26) found that a single intracerebroventricular injection of leptin was sufficient to reduce food intake and body weight in Zucker lean and obese rats, although a higher leptin concentration was required in the

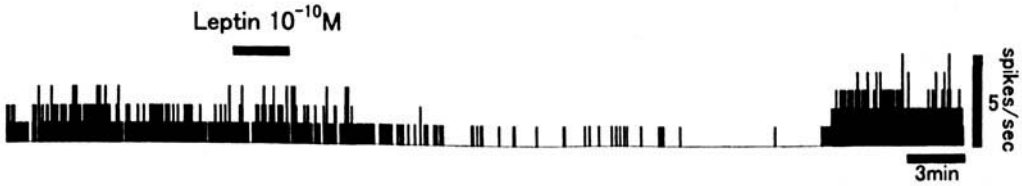

Figure 3. Leptin effect on a vmARC neuron in a slice from a Zucker obese rat. Activity in such vmARC neurons was inhibited by leptin, although the proportion of neurons inhibited was significantly less in these rats than in Wistar rats.

latter than in the former. Similar food intake and body weight reductions in Zucker lean and obese rats have been observed when leptin is administered by single or daily intracerebroventricular injection (27). These results demonstrate that Zucker obese rats are capable of responding to centrally administered leptin. In the present study, about 25% of vmARC neurons in Zucker obese rats were inhibited by 10^{-10} *M* leptin, a proportion significantly smaller than in Wistar rats. Such a reduced electrical responsiveness to leptin in Zucker obese rats has also been reported for SON neurons (32). In accord with this line of evidence, Phillips *et al.* (37) demonstrated that leptin could bind to the receptor in Zucker obese rats, although the affinity between leptin and the receptor was reduced. In addition, the reported finding that intracerebroventricular administration of leptin is able to decrease NPY levels in both the ARC and the PVN (26) suggests that regulation of food intake and body weight by the leptin-NPY signaling pathway operates to some extent even in Zucker obese rats.

In conclusion, we found that in slices from Wistar rats, about 60% of vmARC neurons were inhibited by leptin through a direct postsynaptic action, and that PKC activation may be needed for these inhibitory responses. In addition, we found that even in Zucker obese rats, about 25% of vmARC neurons were inhibited by leptin. These results suggest that the reductions of food intake and body weight known to be induced by leptin in both Wistar and Zucker obese rats are partly exerted through inhibition of an NPY signaling pathway in the vmARC.

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