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SHORT COMMUNICATION

Characterization and Functional Role of Leptin Receptor in Bovine Adrenal Medullary Cells

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ABSTRACT. We report here the characterization and functional roles of the leptin receptor (ObR) in bovine adrenal medullary cells. The plasma membranes isolated from bovine adrenal medulla showed a single class of specific binding sites of ^{125}I-leptin with an apparent K_d of 6.6 nM and B_{max} of 62 fmol/mg protein. ObRa but not ObRb mRNA was detected in bovine adrenal medulla by reverse transcriptase–polymerase chain reaction. Incubation of cultured adrenal medullary cells with leptin (3–30 nM) for 20 min resulted in a significant increase in [^{14}C]catecholamine synthesis from [^{14}C]tyrosine without any change in catecholamine secretion. These findings suggest that leptin stimulates catecholamine synthesis through its receptors in bovine adrenal medullary cells. BIOCHEM PHARMACOL **59**;9:1141–1145, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. bovine adrenal medulla; catecholamine synthesis; ^{125}I-leptin binding; leptin receptor

Leptin, the protein product of the recently cloned *obese* gene, is secreted from adipose tissues and plays an important role in regulating body weight and energy expenditure through its receptor in the hypothalamus, the center of energy homeostasis [1, 2]. In addition to its hypothalamic action, recent studies have shown that leptin acts directly on some extrahypothalamic and peripheral tissues such as pancreatic islets [3] and T-lymphocytes [4].

The ObR‖, first cloned from a mouse choroid plexus cDNA expression library, is a member of the extended class I cytokine receptor family [2]. ObR has at least five isoforms, i.e. ObRa, ObRb, ObRc, ObRd, and ObRe [5, 6]. They share the same extracellular domain and have a single transmembrane domain, except that ObRe has no intracellular domain (it is a putative soluble receptor). ObRa, ObRc, and ObRd possess a short intracellular domain and may serve to transport leptin across the blood–brain barrier or may be implicated in the clearance of leptin from circulation. In contrast to these receptors, ObRb has the long cytoplasmic domain essential for intracellular signal transduction of proteins such as STAT [2, 7].

Catecholamines are neurotransmitters that affect food intake [8]. Furthermore, catecholamines can cause weight loss via a number of mechanisms, including increase of lipolysis, elevation of blood glucose level, inhibition of insulin secretion, and increase of thermogenesis. Several lines of evidence suggest that leptin influences catecholamine secretion from the adrenal medulla by stimulation of the central sympathetic nervous system [9]. On the other hand, the presence of ObR mRNA has been reported in the rat [10], murine [11], human [12], and porcine [13] adrenal medulla.

Regarding the functional studies of ObR in the adrenal medulla, there are two conflicting reports showing effects of leptin on catecholamine secretion. In the first one, performed in human adrenal medullary cells, leptin had no significant effect on catecholamine release [12], while in a more recent study with cultured porcine adrenal medullary cells [13], leptin was found to stimulate catecholamine secretion. To further investigate the characterization and functional role of ObR, we studied the specific binding sites of ^{125}I-leptin and examined the effect of leptin on the secretion and synthesis of catecholamines in cultured bovine adrenal medullary cells.

MATERIALS AND METHODS

Isolation and Primary Culture of Adrenal Medullary Cells

Bovine adrenal medullary cells were isolated and maintained in a monolayer culture (4×10^6/dish, Falcon 35-mm

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‖ *Abbreviations*: K_d, equilibrium dissociation constant; JAK, Janus protein-tyrosine kinase; KRH, Krebs–Ringer–HEPES; MAPK, mitogen-activated protein kinase; ObR, leptin receptor; RT–PCR, reverse transcriptase–polymerase chain reaction; and STAT, signal transducers and activators of transcription.

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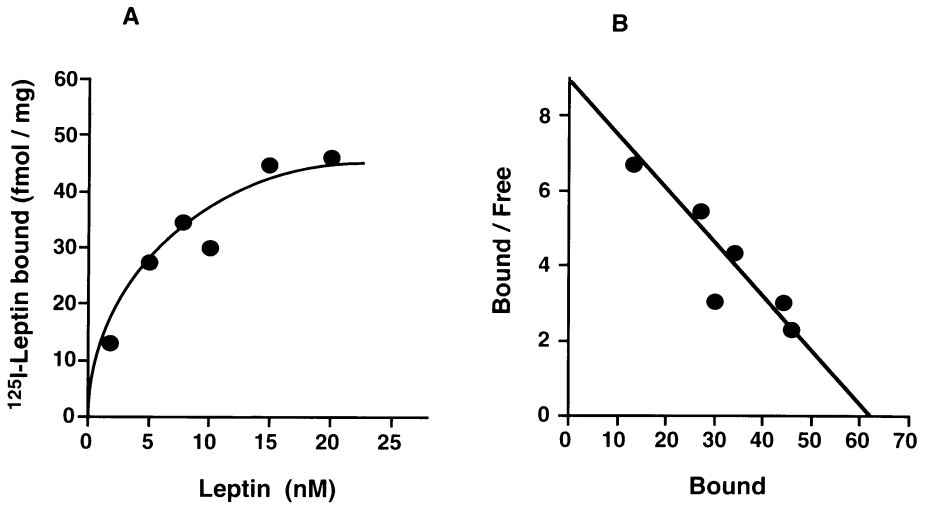

FIG. 1. Specific binding of ^{125}I-leptin to plasma membranes of bovine adrenal medulla. (A) Plasma membranes isolated from bovine adrenal medulla were incubated at 4° for 60 min with increasing concentrations of ^{125}I-leptin (2–20 nM). (B) Scatchard plot analysis of ^{125}I-leptin binding. Data in panels A and B are from one experiment that is representative of six separate experiments. [Bound/Free (fmol/mg protein/nM); Bound (fmol/mg protein)].

dishes) in Eagle's minimum essential medium containing 10% calf serum and antibiotics [14].

RT–PCR of ObR and Its Partial Nucleotide Sequence

mRNA was isolated from bovine adrenal medullary cells by guanidine hydrochloride extraction and oligo(dT) cellulose column separation. The primers 5′-TTGAGAAGTACC-AGTTCAGTC-3′and5′-CAAAGAATGTCCGTTCTC-TTC-3′ for ObRa were designed on the basis of the sequence of human ObRa [15]. RT–PCR was performed with a PC-800 thermocycler (Astec), using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech) and a Takara Ex Taq kit (Takara). The thermocycling conditions were as follows: 94° (45 sec), 59.6° (40 sec), and 72° (1 min) for 32 cycles. The resultant PCR product was subjected to electrophoresis in a 5% SDS-polyacrylamide gel, stained with GelStar Nucleic Acid Gel Stain (Takara), and analyzed using an FLA-2000 fluoroimage analyzer (Fujifilm). The PCR product was cloned further into pGEM-T Easy vector (Promega) and sequenced using an ALF express DNA sequencer (Pharmacia Biotech).

^{125}I-Leptin Binding to Plasma Membranes

Plasma membranes were prepared from bovine adrenal medulla as described previously [16]. ^{125}I-Leptin binding was determined by incubating plasma membranes (180 μg of proteins) in KRH buffer (composition: 125 mM NaCl, 4.8 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM $MgSO_4$, 5.6 mM glucose, 25 mM HEPES–NaOH, pH 7.4, and 0.5 mg/mL of BSA) with 2–20 nM ^{125}I-leptin (human, recombinant) (NEN) for 60 min at 4° in the presence or absence of 100-fold excess concentrations of leptin (human, recombinant) (Calbiochem). After incubation, binding was terminated by the membrane filtration method [16].

Catecholamine Secretion and [^{14}C]Catecholamine Synthesis from [^{14}C]Tyrosine

Cultured adrenal medullary cells (4×10^6/dish) were incubated with or without 1–30 nM leptin (human, recombinant) for 20 min at 37°. Catecholamine secretion was measured as previously reported [14]. Catecholamines (norepinephrine plus epinephrine) secreted into the medium were adsorbed to aluminum hydroxide and estimated by the ethylenediamine condensation method [17]. For measurement of [^{14}C]catecholamine synthesis, cells were incubated with 20 μM L-[U-^{14}C]tyrosine (Amersham International) with or without leptin (1–30 nM). [^{14}C]Catecholamines formed in the cells were separated using a Duolite C-25 column (H^+ type, 0.4×7 cm) [18].

Statistical Analysis

Data are expressed as means ± SD. The statistical evaluation of the data was performed by ANOVA.

RESULTS AND DISCUSSION

Although several recent reports [10–13] have revealed the presence of ObR in the adrenal medulla, there has been little information about the properties of leptin binding sites in this tissue. We studied the specific binding of ^{125}I-leptin in plasma membranes isolated from bovine adrenal medulla. When the membranes were incubated with increasing concentrations (2–20 nM) of ^{125}I-leptin, the specific binding, determined as total binding minus

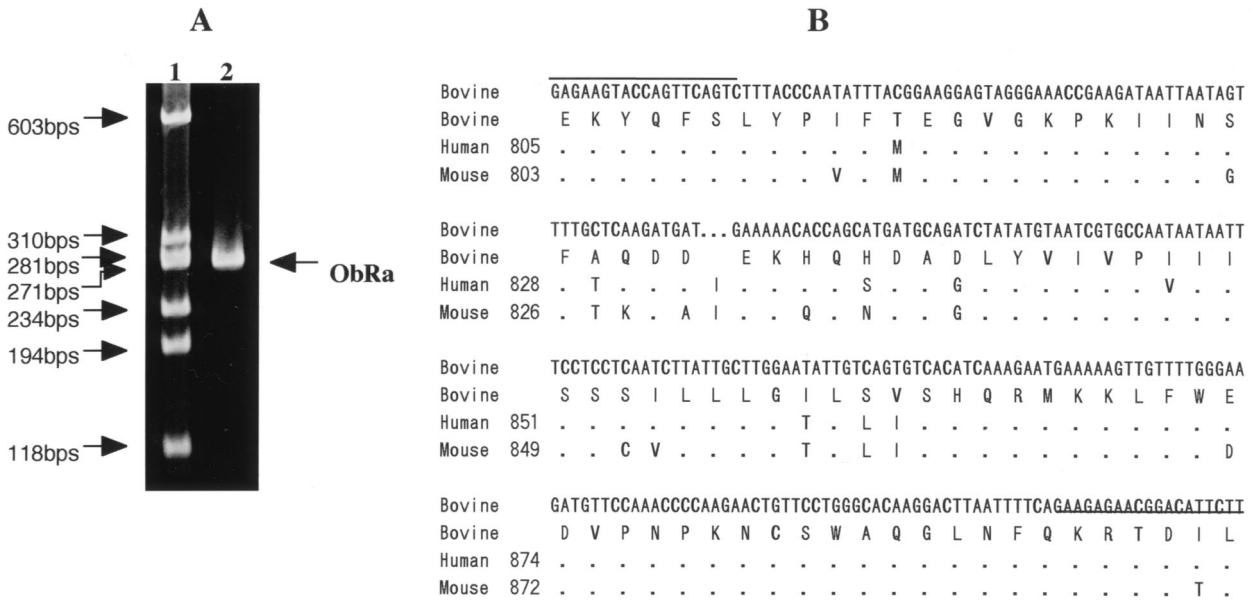

FIG. 2. RT–PCR detection of ObRa mRNA in bovine adrenal medulla (A) and its partial nucleotide and deduced amino acid sequence (B). (A) A PCR product was detected in bovine adrenal medulla using primers designed from the sequence of human ObRa (lane 2). ØX174 DNA–*Hae*III digest size markers are run in lane 1. (B) The bovine ObRa amino acid sequence was deduced from the nucleotide sequence of the RT–PCR product. This sequence of bovine ObRa was compared with those of humans and mice. Identical amino acids relative to those of bovine ObRa are indicated by dots. The synthetic oligonucleotides used for primers are overlined and underlined.

nonspecific binding, was saturable (Fig. 1A). Scatchard analysis showed the presence of a single class of ^{125}I-leptin binding sites with an apparent K_d of 6.6 ± 1.4 nM and B_{max} of 62 ± 16 fmol/mg protein (Fig. 1B).

To clarify the type of ObR in bovine adrenal medulla, ObR mRNA was assayed by RT–PCR. Since none of the bovine ObR types has been cloned, we employed the distinctive regions of the human ObRa segment to design bovine PCR primers. Amplification of bovine adrenal medullary mRNA with these primers resulted in the formation of a single band of PCR product (Fig. 2A). To confirm the PCR product, its nucleic acid sequence was analyzed (Fig. 2B). The deduced amino acid sequence of bovine ObRa showed 90 and 83% identity with the partial sequences of humans and mice, respectively [2, 15]. ObRs occur mainly in two forms, ObRa and ObRb. It remains controversial which type of leptin receptors, ObRa or ObRb, is predominant in the adrenal medulla. Several studies using RT–PCR have shown that ObRb mRNA is expressed highly in the murine, human, and porcine adrenal medulla [11–13], but in the human adrenal tissue [12] there was only weak immunostaining of the ObRb protein. On the other hand, Cao *et al.* [10] reported that the shorter receptor ObRa mRNA was predominant, but a trace of mRNA for the long form ObRb was present in the rat adrenal medulla. In the present study, we tried to amplify ObRb mRNA by RT–PCR using 8 primers designed from human ObRb cDNA, but unfortunately could not detect any of its PCR product (data not shown). The present result, however, does not exclude a possibility of the presence of ObRb mRNA in bovine adrenal medulla.

To obtain evidence for the effect of leptin on adrenal medullary functions, we studied whether leptin may influence the secretion and synthesis of catecholamines in the cells. Leptin, regardless of concentration (1–30 nM), did not affect the secretion of catecholamines from cultured adrenal medullary cells during incubation for at least 20 min (Table 1). The result is consistent with a previous study in human adrenal medullary cells [12]. On the other hand, leptin (3–30 nM) caused a significant increase in the synthesis of [^{14}C]catecholamines from [^{14}C]tyrosine in a concentration-dependent manner (Fig. 3). After submitting our present paper, Takekoshi *et al.* [13] reported that leptin at high concentrations (50–100 nM) stimulates

TABLE 1. Effect of leptin on catecholamine secretion in cultured adrenal medullary cells

	Catecholamine secretion (μg/4 × 10^6 cells/20 min)
Control	0.164 ± 0.025
Leptin	
1 nM	0.224 ± 0.041
3 nM	0.186 ± 0.040
10 nM	0.176 ± 0.032
30 nM	0.214 ± 0.077

Cells were incubated with or without leptin (1–30 nM) at 37° for 20 min. The catecholamines secreted were measured. Data are means ± SD of 4–6 separate experiments carried out in duplicate.

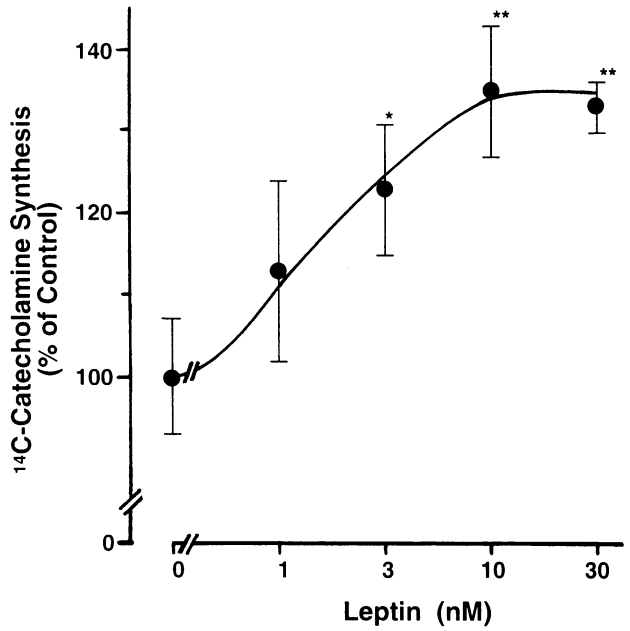

FIG. 3. Effect of leptin on the synthesis of [^{14}C]catecholamines from [^{14}C]tyrosine in cultured adrenal medullary cells. Cells were incubated with or without leptin (1–30 nM) in the presence of l-[U-^{14}C]tyrosine (20 μM) at 37° for 20 min. Data are means ± SD of 4–6 separate experiments carried out in duplicate and are expressed as percentage of control. Control synthesis of [^{14}C]catecholamines was 19,700 ± 1,300 dpm/4 × 10^6 cells/20 min. Key: (*) $P < 0.05$ and (**) $P < 0.01$, compared with control.

catecholamine secretion from cultured porcine adrenal medullary cells, although the human serum level of leptin is reported to be around 0.2 to 0.7 nM [19]. Furthermore, they also found that long treatment of the cells with leptin (1–100 nM) for 8 hr increased the level of tyrosine hydroxylase mRNA. Taking together with these data, it is likely that leptin stimulates catecholamine synthesis, at least, in adrenal medullary cells. Since leptin is well known to have a significant role in the regulation of energy expenditure or fat mass [1, 2], the present result gives rise to the possibility that the action of leptin on the adrenal medulla is involved in the anti-obesity effect of leptin through activation of β-adrenoceptors in adipose or other tissues.

It remains to be determined which type of ObR acts to regulate catecholamine synthesis. ObRb is homologous to members of the cytokine receptor superfamily and contains sites for activation of both JAK and STAT. Vaisse *et al.* [20], however, reported that leptin fails to induce STAT activation in the mouse adrenal gland. Their result may support our RT–PCR finding regarding ObRb mRNA. On the other hand, ObRa has the binding site of JAK that is capable of tyrosine phosphorylation [21]. Furthermore, in a Chinese hamster ovary cell line stably expressing ObRa, leptin can activate MAPK and jun-B mRNA induction [22]. It is reported that tyrosine hydroxylase, a rate-limiting step of catecholamine biosynthesis [23], can be phosphorylated and activated by MAPK [24]. Leptin also increased the phosphorylation and activity of tyrosine hydroxylase in cultured bovine adrenal medullary cells (unpublished observations), suggesting an involvement of a protein kinase/phosphatase pathway. Although it is possible that leptin may stimulate catecholamine synthesis through an ObRa (or ObRb) - MAPK pathway, more work is required to clarify this possibility. The stimulation of ObR by leptin and its cellular signal transduction in the regulation of catecholamine synthesis are currently under study in our laboratory.

In conclusion, the present findings suggest that leptin stimulates catecholamine synthesis through its specific receptors in adrenal medullary cells. This information adds to our understanding of a new mechanism by which leptin acts on metabolic homeostatic processes such as fat regulation and energy expenditure.

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