Anti-leptin receptor antibody mimics the stimulation of lipolysis induced by leptin in isolated mouse fat pads

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Abstract **An anti-leptin receptor polyclonal antibody (receptor antibody), as well as leptin, stimulated the release of free fatty acids from isolated mouse fat pads in a time-dependent manner. Following a 90-min incubation, maximal lipolysis was observed at 6 μg/ml receptor antibody and 0.1 nM leptin. The receptor antibody did not show any additive effect to the stimulation of lipolysis induced by leptin, suggesting that they exert their actions through a similar mechanism involving the leptin receptor. *N*-[2-(*p*-bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H-89), quin 2-AM, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7), and neomycin sulfate (neomycin) all potently inhibited the stimulation of lipolysis by the receptor antibody and leptin. Short-term incubation of the fat pads with the receptor antibody or leptin showed a transient increase in the cellular content of cAMP and *myo*-inositol 1,4,5-trisphosphate (IP_3) in similar concentrations to the free fatty acid release. Quin 2-AM and W-7 also inhibited the increase in cAMP content, suggesting that a Ca^{2+}/calmodulin-dependent process may be involved in a part of the mechanism in which the receptor antibody and leptin exert their effects. The increase in cellular IP_3 content via phosphoinositide-specific phospholipase C (PLC) sensitive to neomycin appears to be a primary step to initiate intracellular events. Both the receptor antibody and leptin may stimulate the lipolysis through mechanisms involving a transient increase in the cellular IP_3 content followed by cAMP production, which leads to the activation of cAMP-dependent protein kinase.**—Kawaji, N., A. Yoshida, T. Motoyashiki, T. Morita, and H. Ueki. **Anti-leptin receptor antibody mimics the stimulation of lipolysis induced by leptin in isolated mouse fat pads.** ***J. Lipid Res.*** **2001.** 42: **1671–1677.**

Supplementary key words Ca^{2+} • *myo*-inositol 1,4,5-trisphosphate • cAMP

Leptin is the product of the *obese (ob)* genes (1). It is exclusively synthesized in adipose tissue and secreted into the circulation to show various physiological actions, including the regulation of feeding behavior, energy expenditure, and adiposity (2–4). When the fat cells from different types of mice were incubated with leptin, the lipolysis stimulation was observed in fat cells of lean and *ob/ob* mice, but not in those from *diabetic (db/db)* mice, which have a mutation in the leptin receptor gene (5).

The leptin receptor isolated from mouse choroid plexus has been reported to have a single membrane-spanning receptor domain with a long intracellular domain (6, 7). The receptor mRNAs have been detected in several spliced forms in tissues including hypothalamus, lung, heart, kidney, spleen, small intestine, adipose tissue, and testis (8). A mutation in the human leptin receptor gene has been reported to cause obesity and pituitary dysfunction (9). Thus, a part of these mutant receptors may cause a divergence between the ability to bind leptin and the signal transduction of its intracellular effects. To date there has not yet been any information on the production of an anti-leptin receptor antibody (receptor antibody) and its physiological actions. It would be of great advantage to the investigations of mutant receptors if we knew the intracellular effects of the receptor antibody.

In the present study, we show that the receptor antibody mimics the stimulation of lipolysis in isolated mouse fat pads induced by leptin. Both cause a transient increase in the cellular content of *myo*-inositol 1,4,5-trisphosphate (IP_3) followed by the cAMP production, leading to the activation of cAMP-dependent protein kinase (PKA).

MATERIALS AND METHODS

Animals

Male ddy mice (6 to 8 weeks old, 30–32 g) were fed on a commercial laboratory chow ad libitum for 1 week in accordance with the Guide for Care and Use of Laboratory Animals established by Fukuyama University. For each experiment mice were used without fasting, due to fed mice showing much higher lipolysis than fasted mice.

Abbreviations: *db*, diabetic; GH, growth hormone; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; IP_3, *myo*-inositol 1,4,5-trisphosphate; KRBGA, Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5mM glucose and 2% BSA; neomycin, neomycin sulfate; *ob*, obese; PKA, cAMP-dependent protein kinase; PLC, phosphoinositide-specific phospholipase C; receptor antibody, anti-leptin receptor antibody; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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Chemicals

Recombinant murine leptin was purchased from R & D System, Inc. (Minneapolis, MN). Polyclonal (rabbit) anti-leptin receptor was from Affinity Bioreagents, Inc. (Golden, CO). This antibody cross-reacts with short and long forms of the leptin receptor in various tissues, including mouse adipose. Quin 2-AM was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Neomycin sulfate (neomycin) was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA). *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) was from Seikagaku Co. (Tokyo, Japan). *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7) was from Alexis Biochemicals Co. (San Diego, CA). The cAMP EIA system and IP_3 assay system were from Amersham Pharmacia Biotech Ltd. (Little Chalfont, England). All other chemicals used were of analytical grade.

Preparation of fat pads and determination of lipolysis

Epididymal adipose tissues were quickly removed from mice killed under ether anesthesia. They were then cut into small pieces (30–40 mg) with scissors in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 5 mM glucose and 2% BSA (KRBGA) at 37°C. The fat pads (150 mg) were incubated with the receptor antibody or leptin in 1.5 ml KRBGA at 37°C for the desired periods (10). The incubated fat pads were removed by centrifugation, and the FFA in the resultant supernatants were determined by a colorimetric method (11). The lipolytic activity is expressed in terms of μmol FFA/h/g fat pads.

Determination of cAMP content in fat pads

The fat pads (100 mg) were incubated with the receptor antibody or leptin in 1 ml KRBGA at 37°C for 0 to10 min. The incubated fat pads were quickly separated, frozen in dry ice-acetone to terminate the reaction, and then homogenized in 1 ml chilled 6% TCA for 1 min with a Physcotron (Nition Medical and Scientific homogenizer, NS-310E). After centrifugation at 1,500 *g* for 10 min, the infranatant was extracted four times with chilled H_2O-saturated diethyl ether to remove TCA. The cAMP content in the residual solution was determined using a commercially available cAMP EIA system (12).

Determination of IP_3 content in fat pads

The fat pads (100 mg) were incubated with the receptor antibody or leptin in 1 ml KRBGA at 37°C for 0–120 s. The incubated fat pads were quickly frozen to terminate the reaction and homogenized in chilled 12.5% TCA. After centrifugation at 16,000 *g* for 10 min, the infranatant was extracted with chilled H_2O-saturated diethyl ether to remove TCA. The IP_3 content in the residual solution was determined using a commercially available IP_3 assay system (13, 14).

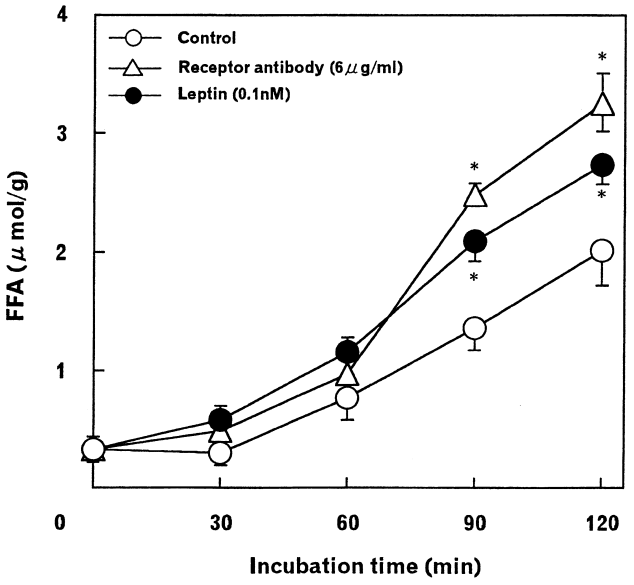

Fig. 1. Time course of lipolysis stimulation by the receptor antibody or leptin. The fat pads (150 mg) were incubated for up to 120 min with 6 μg/ml receptor antibody or 0.1 nM leptin, while the control was incubated with buffer alone. Similar results were obtained in three separate experiments. Significant differences compared with the control group: * $P < 0.05$.

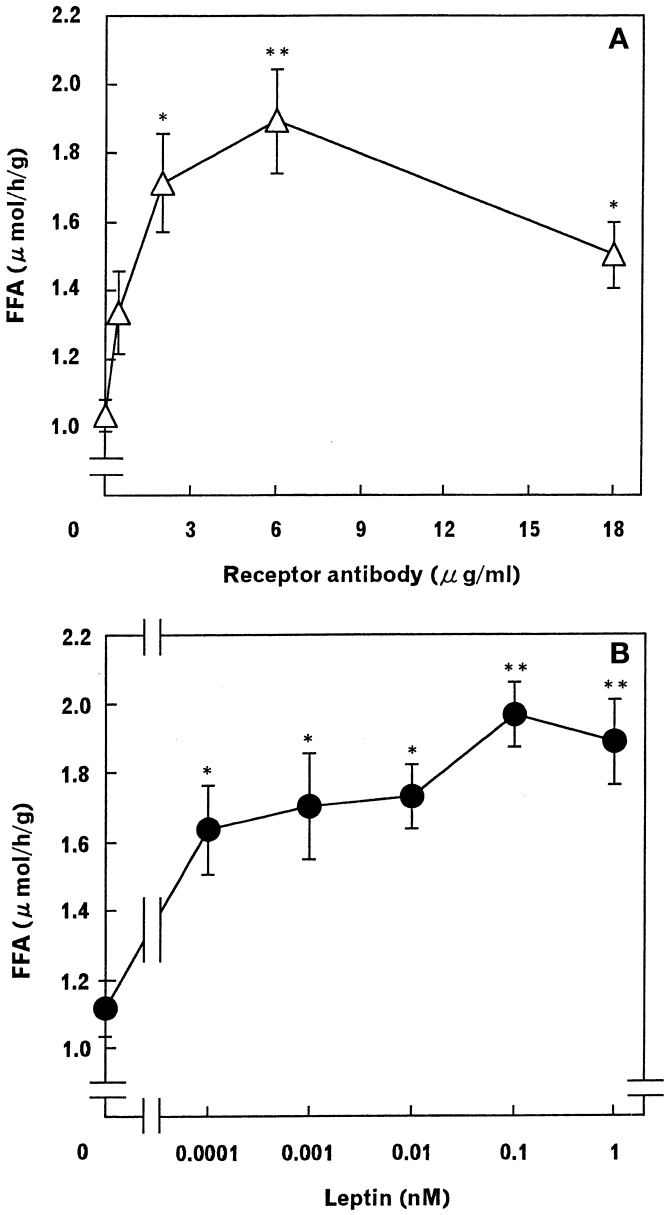

Fig. 2. Dose-response curve for stimulation of lipolysis by the receptor antibody or leptin. The fat pads (150 mg) were incubated with receptor antibody (A) or leptin (B) in KRBGA for 90 min at the indicated concentrations. Similar results were obtained in three separate experiments. Significant differences compared with nontreated group: * $P < 0.05$ and ** $P < 0.01$.

Statistical analysis

All results are expressed as the mean ± SE of four observations. Similar results were obtained in at least two separate experiments. The data were analyzed by Student's *t*-, Dunnett's, or Bonferroni tests.

RESULTS

Stimulation of lipolysis by the receptor antibody or leptin

Figure 1 shows the stimulation of lipolysis by the receptor antibody or leptin over a 120-min incubation period. A significant stimulation of lipolysis was observed at the 90- and 120-min incubations with both the receptor antibody and leptin, although spontaneous lipolysis also progressively increased with the longer incubation times. When the fat pads were incubated for 90 min with varying concentrations of the receptor antibody or leptin, the maximal effects were observed at 6 μg/ml receptor antibody or 0.1 nM leptin (**Fig. 2A** and **B**). Although a 90-min incubation of fat cells from mice with 0.1 nM leptin increased the lipolysis to only 129% over the control (5), in fat pads of lean mice, 0.1 nM leptin or 6 μg/ml receptor antibody increased it to 177% and 190%, respectively, with a similar incubation time. Therefore, lean mouse fat pads were used throughout this study.

Table 1 shows the effect of the receptor antibody on the stimulation of lipolysis induced by leptin. No additive effect on the leptin-stimulated lipolysis was observed with the receptor antibody with concentrations up to 6 μg/ml.

Effects of various inhibitors on the stimulated lipolysis

Figure 3 (A–D) show the inhibitory effects of various inhibitors on the lipolysis stimulation by the receptor antibody or leptin. H-89, a potent inhibitor of PKA (15); quin 2-AM, an intracellular Ca^{2+} chelator (16); W-7, a calmodulin antagonist (17); and neomycin, an inhibitor of phosphoinositide-specific phospholipase C (PLC) (18, 19), all inhibited the stimulation of lipolysis by both the receptor antibody and leptin. These results suggest that the receptor antibody, as well as leptin, may stimulate the lipolysis via Ca^{2+}/calmodulin- and cAMP-dependent processes.

Increase in cellular cAMP content by the receptor antibody or leptin

Figure 4 shows the time course of the increased cAMP content in fat pads produced by the receptor antibody or leptin. Similar profiles for the increases in the cellular cAMP content were observed with 6 μg/ml antibody or 0.1 nM leptin. The maximal increases were reached following a short-term incubation of 2 min, but they had fallen dramatically by 5 min. Quin 2-AM and W-7 markedly inhibited the increases by both the receptor antibody and leptin, suggesting that both compounds activate adenylyl cyclase via Ca^{2+}/calmodulin-dependent processes (**Figs. 5** and **6**).

TABLE 1. Effects of the receptor antibody at increasing concentrations on the leptin-induced lipolysis

	Lipolysis	
Receptor Antibody	With Leptin	Without Leptin
μg/ml	*μmol FFA/h/g*	
0	2.8 ± 0.2	1.5 ± 0.1
0.6	2.9 ± 0.3	1.9 ± 0.2
2.0	2.7 ± 0.3	2.4 ± 0.3
6.0	2.9 ± 0.2	2.7 ± 0.2

The fat pads (150 mg) were incubated with 0.1 nM leptin in KRBGA for 90 min in the presence of receptor antibody at the indicated concentrations.

Increase in cellular IP_3 content by the receptor antibody or leptin

The time dependence of an increase in the IP_3 content in fat pads was investigated with 6 μg/ml receptor antibody or 0.1 nM leptin over a 120-s incubation period (**Fig. 7**). The maximal increases were observed with a 60-s incubation. Neomycin completely inhibited the increases by both compounds, suggesting that the receptor antibody, as well as leptin, increases the cellular IP_3 content through stimulation of a process that produces IP_3 (**Fig. 8**).

DISCUSSION

The results presented here show that both the receptor antibody and leptin stimulate lipolysis in the fat pads through a transient increase in the cellular IP_3 content followed by cAMP production. The combined incubation of the receptor antibody and leptin did not show any additive effect, suggesting that they exert their effects through a similar mechanism involving the leptin receptor. It is well known that lipolysis progresses with the increase in cellular cAMP content, accompanied by the activation of PKA. Lipolysis stimulation by the receptor antibody or leptin was almost completely inhibited by H-89, suggesting that PKA activation is involved in the mechanism of their action. The inhibitory effects of quin 2-AM on the stimulated lipolysis and an increase in cellular cAMP content by the receptor antibody and leptin suggest that they exert their effects by raising the intracellular Ca^{2+} concentration.

Adenylyl cyclases catalyze the production of cAMP, an important intracellular second messenger in eukaryotic cells, and now are classified into eight to ten isoforms, based on the diversity of mRNA expression patterns (20, 21). Most of the adenylyl cyclases are regulated by stimulatory and inhibitory receptors coupled to the catalytic subunit through the regulatory G proteins Gs and Gi, although certain adenylyl cyclases are also activated by Ca^{2+}/calmodulin or phosphorylation. W-7 at concentrations that inhibited the receptor antibody- or leptin-stimulated lipolysis also inhibited the stimulation of cAMP production, suggesting that a Ca^{2+}/calmodulin-dependent process may be involved in a part of the adenylyl cyclase activation by the receptor antibody or leptin.

IP_3, a product of inositol phospholipid that is cleaved by PLC, is an important intracellular messenger for Ca^{2+} release from internal stores (22, 23). Hormones and neurotransmitters stimulate an increase in the cellular IP_3 content through activation of an agonist-sensitive PLC (24). In rat adipocytes, IP_3 stimulates Ca^{2+} release from the

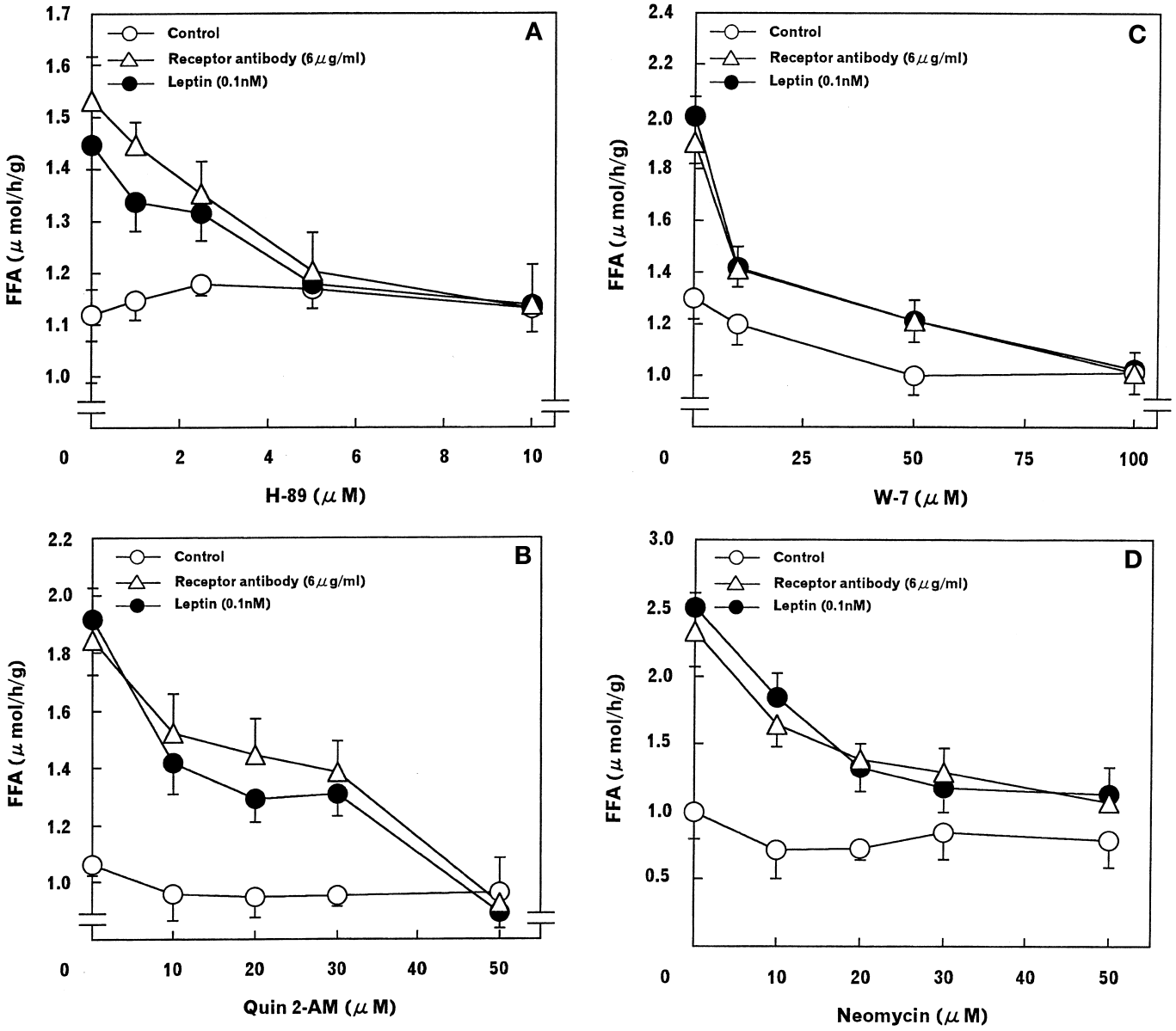

Fig. 3. Effects of various inhibitors on stimulation of lipolysis by the receptor antibody or leptin. The fat pads (150 mg) were preincubated with H-89 (A), quin 2-AM (B), W-7 (C), or neomycin (D) at the indicated concentrations in KRBGA for 15 min at 37°C. They were then further incubated for 90 min with 6 μg/ml receptor antibody or 0.1 nM leptin, while the control was incubated with buffer alone. A similar tendency was seen in two separate experiments.

endoplasmic reticulum, but not from plasma membranes (25). In rat fat pads, vanadate induces an increase in the cellular IP_3 content, which is inhibited by neomycin (26). Leptin enhances the PLC-mediated insulin secretion from pancreatic islets of *ob/ob* mice (27). Our results showed that neomycin at the same concentration that inhibited the receptor antibody- or leptin-stimulated lipolysis also inhibited the stimulation of IP_3 production by both compounds. Binding of the receptor antibody or leptin to the leptin receptor also seems to stimulate Ca^{2+} release from calcium stores, probably from the endoplasmic reticulum, by stimulating the IP_3 production through the activation of a neomycin-inhibited PLC. The stimulation of IP_3 production appears to be the primary step for initiation of the downstream cellular events.

An anti-insulin receptor antibody shows the insulin-mimetic actions, such as the stimulation of glucose incorporation into lipid, glucose transport, and amino acids uptake, without activation of the receptor tyrosine kinase activity (28–30). These data indicate that at least some of insulin's actions may not require the activation of receptor tyrosine kinase activity. Autophosphorylation of the insulin receptor β subunit induces a conformational change in the protein (31). Therefore, binding of the antibody to the receptor has been thought to convert the structure to an active form, which is similar to the conformation change induced by insulin-mediated autophosphorylation, which generates the intracellular signals.

The leptin receptor is a member of the class I cytokine receptor superfamily (32–34). Receptors of this class lack

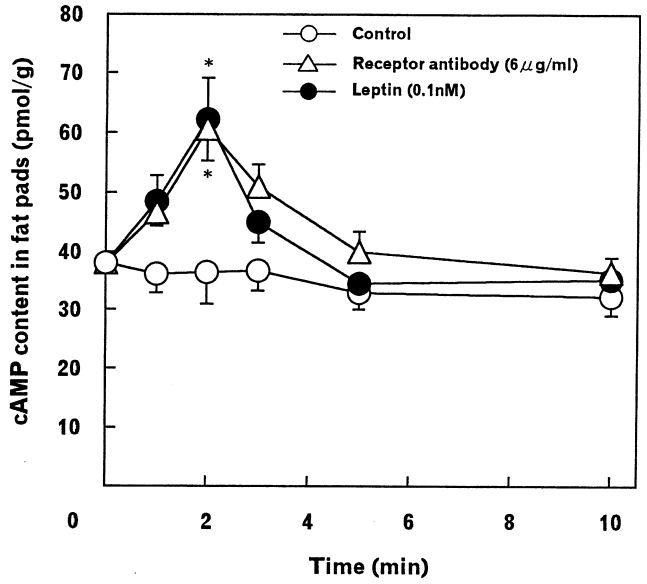

Fig. 4. Time course of the increase in cellular cAMP content induced by the receptor antibody or leptin. The fat pads (150 mg) were incubated for up to 10 min with 6 μg/ml receptor antibody or 0.1 nM leptin, while the control was incubated with buffer alone. Similar results were obtained in three separate experiments. Significant differences compared with the control group: * $P < 0.05$.

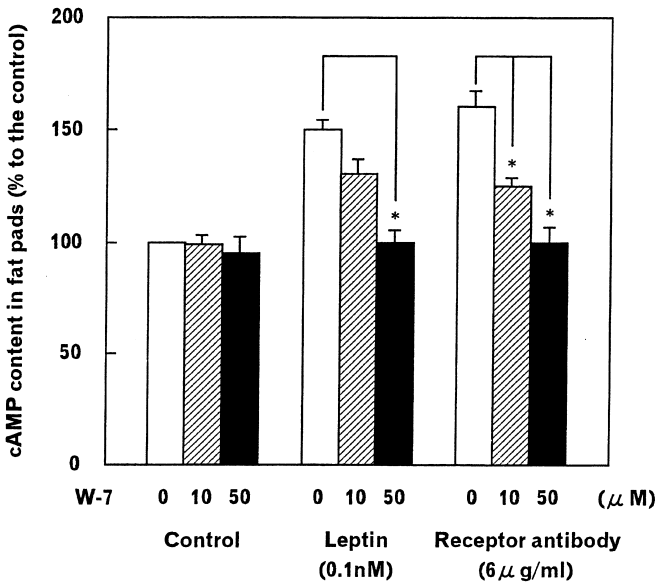

Fig. 6. Inhibitory effect of W-7 on the increase in cellular cAMP content by the receptor antibody or leptin. The fat pads (150 mg) were preincubated with or without 10 and 50 μM W-7 in KRBGA for 15 min at 37°C. They were then further incubated for 2 min with 6 μg/ml receptor antibody or 0.1 nM leptin, while the control was incubated with buffer alone. Similar results were obtained in three separate experiments. Significant differences compared with the receptor antibody- or leptin-treated group without W-7: * $P < 0.05$.

intrinsic tyrosine kinase activity and are activated by ligand-induced receptor homo- or heterodimerization, accompanied by the activation of receptor-associated kinases of the Janus family. When leptin binds to the receptor it dimerizes two receptor extracellular chains and this leads to aggregation of two receptor intracellular domains, which triggers the downstream signaling events (34). Dimerization of growth hormone (GH) receptor, one of the class I

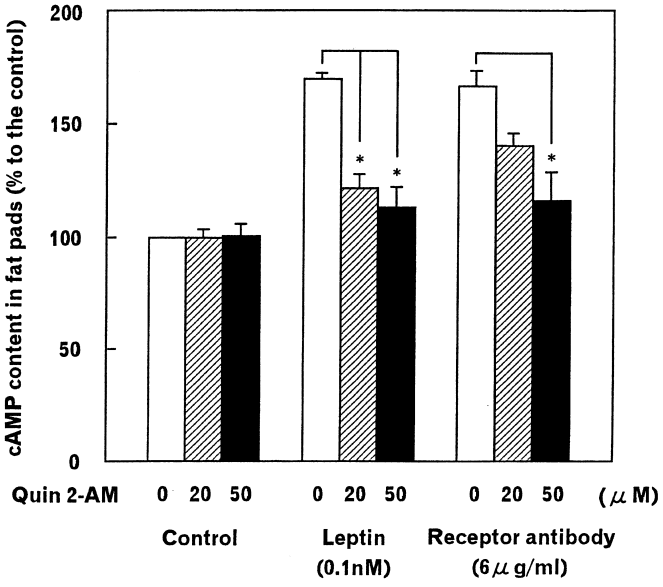

Fig. 5. Inhibitory effect of quin 2-AM on the increase in cellular cAMP content induced by the receptor antibody or leptin. The fat pads (150 mg) were preincubated with or without 20 and 50 μM quin 2-AM in KRBGA for 15 min at 37°C. They were then further incubated for 2 min with 6 μg/ml receptor antibody or 0.1 nM leptin, while the control was incubated with buffer alone. Similar results were obtained in three separate experiments. Significant differences compared with the receptor antibody- or leptin-treated group without quin 2-AM: * $P < 0.05$.

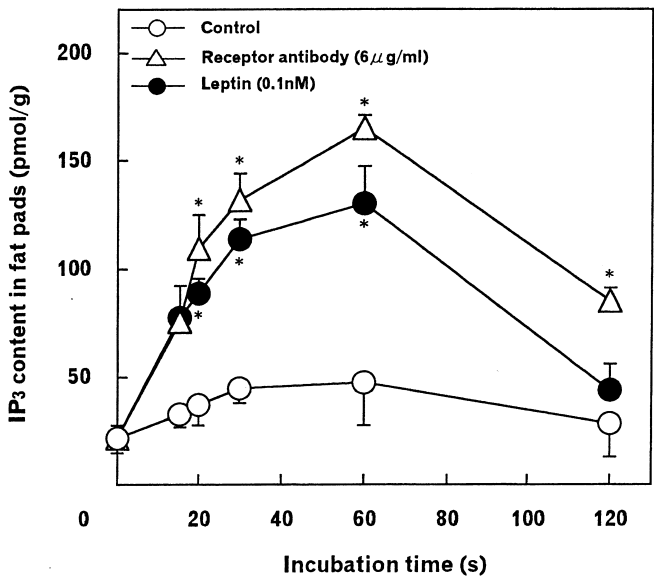

Fig. 7. Time course of increase in the cellular IP_3 content by the receptor antibody or leptin. The fat pads (150 mg) were incubated for up to 120 s with 6 μg/ml receptor antibody or 0.1 nM leptin, while the control was incubated with buffer alone. Similar results were obtained in three separate experiments. Significant differences compared with the control group: * $P < 0.05$.

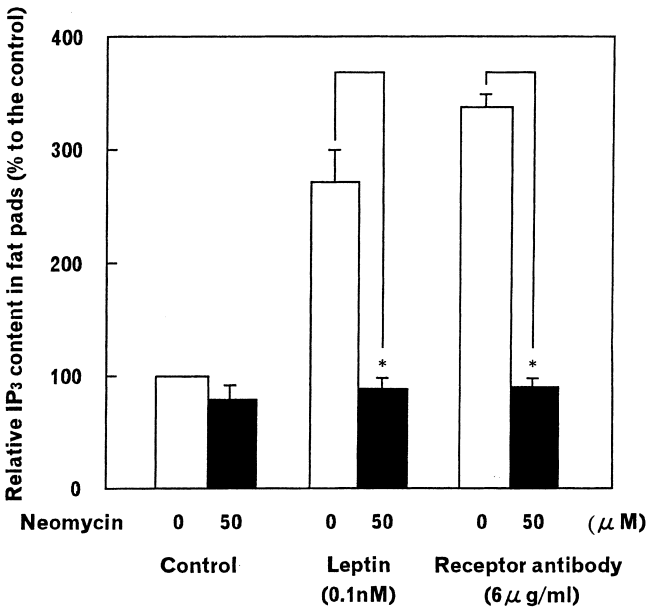

Fig. 8. Inhibitory effect of neomycin on the increase in cellular IP_3 content by the receptor antibody or leptin. The fat pads (150 mg) were preincubated with or without 50 μM neomycin in KRBGA for 15 min at 37°C. They were then further incubated for 60 s with 6 μg/ml receptor antibody or 0.1 nM leptin, while the control was incubated with buffer alone. Similar results were obtained in three separate experiments. Significant differences compared with the receptor antibody- or leptin-treated group without neomycin: * $P < 0.01$.

cytokine receptors, is necessary for GH-induced lipolysis. An anti-GH receptor antibody dimerized the receptor and induced GH-like biological actions (35). Therefore, the binding of the receptor antibody to the leptin receptor may induce homodimerization of the receptor, allowing it to mimic the actions of leptin, including the stimulation of lipolysis. However, more detailed studies are necessary for further elucidation on this. The receptor antibody appears to be a useful biological tool for studying abnormal reactivities of mutant leptin receptors.

In conclusion, both the receptor antibody and leptin appear to induce the stimulation of lipolysis through mechanisms involving a transient increase in the cellular IP_3 content, followed by the cAMP production, which leads to the activation of PKA.

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