Leptin Induces Mitogen-activated Protein Kinase-dependent Proliferation of C3H10T1/2 Cells*

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Leptin, secreted by adipocytes, regulates satiety and energy expenditure. Several forms of leptin receptors produced by alternative mRNA splicing are found in many tissues, including the hypothalamus, liver, lung, kidney, hematopoietic cells, and gonads, suggesting that leptin exerts effects in these tissues. In accordance with the distribution of leptin receptors, there is accumulating evidence that leptin plays various roles in reproduction, hematopoiesis, and the immune systems in addition to the regulation of food intake and energy expenditure. In the present study, we examined the in vitro effects of leptin on proliferation of a mouse embryonic cell line, C3H10T1/2, and its mechanism of action. Leptin caused a dose- and time-dependent increase in mitogen-activated protein kinase (MAPK) activity that was accompanied by an increase in C3H10T1/2 cell number. The MAPK kinase-1-specific inhibitor PD98059 completely blocked the increases in both MAPK activity and cell proliferation caused by leptin. These findings indicate that leptin stimulates the proliferation of C3H10T1/2 cells via the MAPK cascade.

The ob gene has been cloned as a genetic factor responsible for obesity in genetically obese rodents, ob/ob mice (1). The ob gene product (leptin), mutated in ob/ob mice, serves as a satiety factor secreted from adipose tissue and plays an important role in regulating body weight through its receptor in the hypothalamus (1–4). In addition to regulating body weight, leptin also influences reproductive, hematopoietic, and immune systems in which its receptors are expressed (5–10), suggesting that leptin also has extrahypothalamic actions. Furthermore, leptin receptors are expressed in various tissues including lung, kidney, testis, and adipose tissue (11, 12). These findings suggest that leptin plays diverse roles in many systems. However, the mechanism of its signal transduction in these organs remains unclear.

Recently, STATs* have been suggested to be involved in the signal transduction mechanism of leptin. It was reported that leptin activated STAT-1, -3, and -5 (13) and STAT-3, -5, and -6 (14) in artificial reconstruction systems using COS cells transfected with leptin receptors and STATs. Furthermore, activation of STAT-3 by leptin was observed in the hypothalamus in vitro (15). We recently found that leptin induced tyrosine phosphorylation of several cellular proteins including STAT-1 in ACHN cells (cloned human renal carcinoma cells) and suggested that ACHN cells were useful for analyzing the signal transduction mechanism of leptin (16).

On the other hand, pathways other than STATs may be involved in leptin signal transduction system. Because leptin is considered to be a member of the cytokine family from the results of structural analysis (17), its signal transduction system may be similar to those of other cytokines. Many cytokines stimulate a molecular cascade coupled with Ras activation (18–20). p21ras plays a key role in the phosphorylation and activation of mitogen-activated protein kinases (MAPKs) (21, 22), which in turn phosphorylate a number of nuclear transcription factors and cytoplasmic proteins involved in the regulation of macromolecular synthesis and mitogenesis (23–25). Thus, we examined the involvement of MAPKs in the leptin signal transduction and the effects of MAPK activation caused by leptin on cell growth using the mouse embryonic cell line C3H10T1/2.

Experimental Procedures

Materials—C3H10T1/2 cells were kindly provided by Dr. Tatsuya Kobayashi (Kobe University). The sources of materials used in this study were as follows: basal minimum Eagle’s medium (BME) was from Life Technologies, Inc.; fetal calf serum (FCS) was from Bio Whittaker; PD98059, anti-phospho-MAPK antibody, Elk1 fusion protein, and anti-phospho-Elk1 were from New England Biolabs; [3H]thymidine, polyvinylidene difluoride membranes, and the ECL chemiluminescence kit were from Amersham Corp.

Production of Recombinant Leptin—A human leptin cDNA fragment spanning nucleotides +64 to +501, encoding the full-length leptin protein, was cloned from human adipose tissue by reverse transcription and polymerase chain reaction. The cDNA was cloned into the expression vector pET29b to produce S-peptide-tagged leptin and expressed in Escherichia coli, BL21 (DE3), as described previously (16). Recombinant leptin protein was purified using an S-Tag purification kit according to the manufacturer’s protocol. After cleavage of S peptide by thrombin, the recombinant leptin was purified and confirmed to migrate as a single band on a SDS-polyacrylamide gel. The bioactivity of the recombinant leptin was confirmed by Western blotting to detect tyrosine phosphorylation of cellular proteins of ACHN cells as described previously (16).

Cell Culture—C3H10T1/2 cells were cultured in BME supplemented with 8% FCS in an atmosphere of 5% CO2 at 37 °C as described previously (26).

Cell Proliferation Assays—Approximately 48 h before the assay, C3H10T1/2 cells were seeded into 24-well plates at 1–3 × 105 cells/well. When cells reached 60–70% confluence, the medium was replaced with BME containing 0.1% bovine serum albumin (BSA) and both were added at the indicated concentrations. The cultures were incubated in a CO2 incubator for 72 h at 37 °C unless otherwise indicated. Cells were washed with phosphate-buffered saline (PBS) and stripped with 0.05% trypsin-0.53 mM EDTA. The cell number was then determined using a cell counter. All samples were assayed in duplicate, and each experiment was repeated at least three times. Representative data are presented.

Cell Death Assays—Cells were treated as described under “Cell Proliferation Assays.” Live and dead cells from each subculture were
counted by trypan blue at sequential time points during incubation, and the ratio of dead/(dead + live) cells was calculated. The mean values of these ratios and the S.E. were calculated at each time point.

**Determination of Thymidine Incorporation**—Following starvation for 24 h, C3H10T1/2 cells were incubated with leptin at the indicated concentrations for 20 h. The cell number at higher concentrations (200 ng/ml) was less than that at 100 ng/ml leptin. Leptin stimulation was achieved at 100 ng/ml. The dose-response relationship of thymidine uptake in C3H10T1/2 cells dose-dependently increased by approximately 2.5-fold following a 72-h incubation correlated well with that of the increase in cell number evoked by leptin.

**Mapk in Vivo Kinase Assays**—C3H10T1/2 cells were cultured in 60-mm dishes in BME with 5% FCS. When approximately 70–80% confluent, the cells were starved for 24 h, washed with PBS, and then incubated in cell assay medium, comprising of BME containing 0.1% BSA and leptin. Leptin at 100 ng/ml was used for time course experiments, and the incubation time was 15 min for dose-response experiments. PD98059 was added 1 h before leptin stimulation. After incubation, MAPK activity of C3H10T1/2 cells was measured as described previously (27–29) using a MAP Kinase Assay Kit (New England Biolabs, Beverly, MA). Briefly, the cells were washed with PBS, lysed in lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), scraped off the dishes, transferred to microcentrifuge tubes, sonicated, incubated overnight at 4 °C, and microcentrifuged for 20 min at 4 °C. The supernatant was used for immunoprecipitation of activated MAPKs. The supernatant was incubated with anti-phospho-MAPK antibody (1:100 dilution) for 4 h. Protein A-agarose beads were then added to the tubes and incubated for another 3 h. The pellets were washed twice with ice-cold lysis buffer and twice with kinase buffer (25 mM Tris (pH 7.5), 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). The pellets were incubated with 100 mM ATP, and 20 mg/ml Elk1 fusion protein, a substrate of MAPK, for 30 min at 30 °C. The reaction was terminated by adding Laemmli buffer. Samples were boiled, separated by electrophoresis through a 10% SDS-polyacrylamide gel, and transferred to polyvinylidene difluoride membranes. The membranes were incubated for 1 h at room temperature in blocking buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 5% skimmed milk, 0.1% Tween 20) and then probed with 1:1000 anti-phospho-Elk1 antibody. The membranes were washed three times in 20 mM Tris (pH 7.5), 150 mM NaCl, 0.1% Triton-X and incubated with a 1:1000 dilution of horse- radish peroxidase-conjugated anti-rabbit immunoglobulin in antibody dilution buffer. The immunoreactivity of phosphorylated Elk1 was determined using an ECL chemiluminescence kit.

**Statistics**—Statistical analysis was performed by analysis of variance, followed by Scheffe’s test. Data are expressed as the means ± S.E.

**RESULTS**

**Leptin-dependent Proliferation of C3H10T1/2 Cells**—After screening a number of cell lines to find leptin-responsive cells, we found that leptin stimulated proliferation of the mouse embryonic cell line C3H10T1/2 in a time- and dose-dependent manner (Fig. 1 A and B). The number of C3H10T1/2 cells was increased by approximately 2.5-fold following a 72-h incubation with 100 ng/ml leptin (Fig. 1A). The phenotypic features of the C3H10T1/2 cells were not changed. To exclude the possibility that leptin acted as a survival factor and suppressed the cell death of C3H10T1/2 cells, we counted the numbers of live and dead cells in time course experiments. The ratio of dead to total cell number of leptin-treated cells was not significantly different from that in untreated cells (Table I). Leptin-induced cell proliferation was dose-dependent over the range of 0–100 ng/ml (Fig. 1D). The cell number at higher concentrations (200 ng/ml) was less than that at 100 ng/ml leptin. Leptin stimulated thymidine uptake in C3H10T1/2 cells dose-dependently (Fig. 1C). The lowest stimulatory dose was 50 ng/ml, and maximal stimulation was achieved at 100 ng/ml. The dose-response relationship of thymidine uptake in C3H10T1/2 cells correlated well with that of the increase in cell number evoked by leptin.

**Leptin Stimulated MAPK Activation**—Various cytokines and growth factors stimulate MAPK activation and cell prolifera-

**FIG. 1.** Leptin-induced proliferation of C3H10T1/2 cells. A, when incubated with 100 ng/ml leptin, the number of C3H10T1/2 cells increased time-dependently. Significant differences (versus the initial cell number) are shown by asterisks, *p < 0.05; **p < 0.01. B, leptin induced cell proliferation in a dose-dependent manner. The numbers of C3H10T1/2 cells were counted after 72 h of incubation with leptin at the indicated concentrations. Significant differences (versus the control) are shown by asterisks. *, p < 0.05; **, p < 0.01. C, leptin stimulated thymidine incorporation in a dose-dependent manner. Significant differences (versus the control) are shown by asterisks. *, p < 0.05; **, p < 0.01.

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tein. Leptin stimulated Elk 1 phosphorylation in a dose- and time-dependent manner, and the phosphorylation of Elk 1 appeared to plateau with the addition of 40–60 ng/ml leptin (Fig. 2, A and B).

**PD98059 Inhibited MAPK Activity in C3H10T1/2 Cells**—To determine if MAPK activation is responsible for leptin-induced proliferation of C3H10T1/2 cells, we used the MEK1 inhibitor PD98059, because it has been shown to act as a highly selective inhibitor of MEK1 and the MAPK cascade (31–34). At 10 μM, PD98059 inhibited leptin-induced MAPK activation, and 50 μM PD98059 inhibited leptin-induced MAPK activation completely (Fig. 3).

**PD98059 Inhibited Leptin-induced Proliferation of C3H10T1/2 Cells**—To clarify the involvement of MAPKs in leptin-induced cell growth, we examined the effects of PD98059 on leptin-induced proliferation of C3H10T1/2 cells. Time course experiments showed that PD98059 dose-dependently inhibited cell proliferation induced by leptin (Fig. 4A). The IC₅₀ of PD98059 for suppression of leptin-induced cell proliferation was approximately 25 μM (Fig. 4B), and 50 μM PD98059 showed complete inhibition of both leptin-induced and insulin-induced cell proliferation (Fig. 4C).

**DISCUSSION**

In the present study, we demonstrated that leptin induced cell proliferation in the mouse embryonic cell line C3H10T1/2. Leptin increased cell number and thymidine uptake of C3H10T1/2 cells but did not inhibit cell death. These findings indicated that leptin exerted its action as a mitogen but not as a survival factor. Furthermore, the mitogenic effect of leptin was dose-dependent and was observed over the physiological concentration range (35). C3H10T1/2 cells are mouse embryonic fibroblasts and have the potency to differentiate to muscle cells, adipocytes, and chondrocytes following 5-aza-CR treatment (36–38). Recently, Bennett et al. reported that leptin and its cognate receptor constituted a novel hematopoietic pathway that was required for normal lymphopoiesis (7), and Gainsford et al. noted that leptin was able to induce proliferation, differentiation, and functional activation of hematopoietic cells (8).
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Taken together, these observations suggest that leptin may have a role in the proliferation and differentiation of mesenchymal cells.

In addition, we found that leptin-induced activation of MAPKs in C3H10T1/2 cells. It is not surprising that leptin induced cell proliferation, because its structure resembles that of cytokines such as IL-2, IL-6, and growth hormone (17), and leptin receptor is related to gp130, which is the signal-transducing component of IL-6, ciliary neurotrophic factor, leukemia inhibitory factor, oncostatin-M, IL-11, and cardiotrophin-1 receptors (11). These cytokines, as well as other diverse extracellular stimuli including osmotic shock, stress, and elevated temperature, cause activation of phosphorylation cascades involving MAPKs (39–46). The sustained activation of MAPKs is sufficient to induce the proliferation or differentiation of several types of cells (47).

To clarify whether the MAPK pathway is involved in leptin-induced cell proliferation, PD98059, a specific inhibitor of MAPK kinase-1 (31), was added to the culture medium of C3H10T1/2 cells. PD98059 specifically inhibits the action of MAPK kinase-1 both in vitro and in vivo (33); e.g. in nerve growth factor-induced differentiation of PC12 cells (50) and insulin-induced MAPK activation in 3T3L1 cells and L6 cells (34). In the present study, PD98059 significantly suppressed leptin-induced MAPK activation in C3H10T1/2 cells. Moreover, PD98059 completely inhibited leptin-induced proliferation of C3H10T1/2 cells. These findings demonstrated that MAPKs were essential in C3H10T1/2 cell proliferation caused by leptin.

Several findings have suggested that STATs proteins are involved in the mechanism of leptin signal transduction. Ghilardi et al. and Baumann et al. reported that leptin activated STAT-1, -3, and -5 (13) and STAT-3, -5, and -6 (14), respectively, in artificial reconstitution systems using Cos cells transfected with leptin receptors and STATs. Vaisse et al. showed that leptin activated STAT-3 in the hypothalamus in vivo (15), although it is not known which hypothalamic cells were activated. We recently reported that in ACHN cells, which are derived from human renal cell carcinoma cells, at least five proteins have undergone tyrosine-phosphorylation 15 min after leptin stimulation (16). The estimated molecular masses of these proteins were approximately 180, 130, 91, 44, and 42 kDa. The 44- and 42-kDa proteins phosphorylated after treatment with leptin were MAPKs (2). These findings suggested that MAPKs in addition to STATs are involved in the signal transduction by leptin in ACHN cells. MAPKs regulate a Janus kinase-STAT signaling cascade in various steps (48, 49). Therefore, activation of MAPKs might modify the STATs signaling pathway in leptin signal transduction.

The targets of MAPKs and STATs remain unknown. Wood and Stock reported that intravenous administration of leptin induced c-Fos expression in the hypothalamus where the leptin receptors are present (51). Thus, c-Fos expression may be one of the targets of MAPKs and STATs pathways activated by leptin as shown in the stimulation by many cytokines. However, further studies are needed to clarify whether the c-Fos gene is the target of MAPKs or STATs activated by leptin. In conclusion, MAPKs are involved in leptin signal transduction and play an essential role in the proliferation of C3H10T1/2 cells induced by leptin.

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