Leptin Induces Apoptosis via ERK/cPLA2/Cytochrome c Pathway in Human Bone Marrow Stromal Cells*

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Leptin, the Ob gene product, has emerged recently as a key regulator of bone mass. However, the mechanism mediating leptin effect remains controversial. Because the action of leptin is dependent on its receptors, we analyzed their expression in osteoblast-lineage primary human bone marrow stromal cells (hBMSC). Both the short and long forms of leptin receptors were detected in hBMSC. Leptin significantly decreased the viability of hBMSC. This cytotoxic effect was prevented by Z-Val-Ala-Asp-fluoromethylketone, a pan-caspase inhibitor, implicating that leptin-induced hBMSC death was caspase-dependent. Further investigation demonstrated that leptin activated caspase-3 and caspase-9, but not caspase-8, and increased the cleavage of poly(ADP-ribose) polymerase and cytochrome c release into cytosol. Leptin activated ERK, but not p38 and JNK, and up-regulated cPLA2 activity; the latter was abolished by pre-treatment of cells with the MEK inhibitor (PD98059 or U0126) or cPLA2 inhibitor (AACOCF3). PD98059, U0126, and AACOCF3 also diminished the leptin-induced cytochrome c release into cytosol, cell death, and caspase-3 activation. These data indicated that leptin induced hBMSC apoptosis via ERK/cPLA2/cytochrome c pathway with activation of caspase-9 and caspase-3, and cleavage of poly(ADP-ribose) polymerase. To our knowledge, this is the first study demonstrating the direct detrimental effect of leptin on bone cells.

Leptin, the protein product encoded by ob gene, is a circulating hormone produced primarily by the adipose tissue and is a multifunctional hormone that plays important roles in body weight homeostasis, neuroendocrine function, fertility, immune function, and angiogenesis (1–3). The biological actions of leptin on target tissues are carried out through interaction with its specific receptor, Ob-R (4). A hallmark of Ob-R expression is the presence of several receptor variants (Ob-Ra through Ob-Rf) that are generated by alternative splicing; they share the same extracellular domain but differ in the length of the transmembrane/cyttoplasmic coding regions (2). The long Ob-Rb subtype (Ob-Rb) appears as the functional, signal-transducing isoform, responsible for the action of leptin. The roles of the shorter Ob-R isoforms (Ob-Rs) remain to be characterized. Although leptin’s precise sites of action are not known, its effect is thought largely mediated via hypothalamus. However, the wide expression of Ob-Rs throughout the body suggests that leptin may also operate directly in peripheral tissues (5). There is now a significant amount of evidence implicating that leptin is active in the periphery (6).

Recently, leptin has emerged as a key element in the regulation of bone mass. However, the mechanism by which leptin acts upon the bone remains unclear. Thomas et al. (7) reported that leptin enhanced the differentiation of hBMSC,1 and Stepan et al. (8) reported that the femurs of leptin-deficient ob/ob mice were shorter than those in the normal mice, and intraperitoneal injection of leptin significantly increased bone area. In addition, a recent study (9) suggested that leptin inhibited osteoclast generation in cultures of human peripheral blood mononuclear cells and murine spleen cells. These data suggest that leptin has a favoring effect on bone formation. However, using ob/ob and leptin receptor-deficient db/db mice, Ducy et al. (10) demonstrated that leptin could function as a strong inhibitor of bone formation. Both ob/ob and db/db mice have increased trabecular bone mass. Because neither a direct effect of leptin nor leptin receptor was detected in osteosteoblasts, and intracerebroventricular injections of leptin resulted in a decreased bone mass, the inhibition of bone formation by leptin was thought to be most likely mediated via a hypothalamic relay (10). Recent reports, however, suggest that leptin may have a direct effect on osteoblasts (7, 8, 11). Thus, the aims of this study were to document the following: 1) whether leptin directly acts on cells of osteoblast lineage, and 2) which signal pathways mediate this effect. In this report, we demonstrated that both Ob-Rs and Ob-Rb were expressed in hBMSC, and leptin induced apoptosis of

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EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Recombinant human leptin was purchased from either Calbiochem or BioGenex, (Palo Alto, CA). The purity of leptin from both sources was 95% or greater, and endotoxin level was less than 0.1 ng/ml of leptin. Because the same results were obtained using leptin from either source, only representative data were presented. PD98059, U0126, and pan-caspase inhibitor Z-VaL-Ala-Asp-fluoromethylketone (Z-VAD-fmk) were purchased from Calbiochem. AACC1357, AG490, and bromoenol lactone (BEL) were purchased from Biomol. Antibodies against ERK (ERK1 and ERK2), phospho-ERK, cytochrome c, JAK, and Ob-R (corresponding to amino acids 541–840 of Ob-R; sc-8325) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against caspase-3, caspase-8, caspase-9, poly(ADP-ribose) polymerase (PARP), p53, phospho-p53, JNK, phospho-JNK, STAT1, phospho-STAT1, STAT3, phospho-STAT3, and phospho-tyrosine (P-Tyr-100) were obtained from Cell Signaling Technology (Beverly, MA). Hoechst 33342 was purchased from Molecular Probes (Eugene, OR). [5,6,8,9,11,12,14,15-3H]-Arachidonic acid (150–230 Ci/mmol) was purchased from Amersham Biosciences. [α-32P]dCTP was from PerkinElmer Life Sciences. All other reagents were of analytical or research grade or better.

hBMSC Culture—hBMSC were isolated from ribs that were discarded at the time of open thoracotomy in patients without metabolic bone disease as described previously (12). Briefly, the ribs were excised aseptically, cleaned of soft tissue, and opened longitudinally. The exposed bone marrow was flushed out using several washes of serum-free α-MEM (Sigma) and centrifuged at 1400 rpm for 10 min. Cell pellets were resuspended in culture medium, and the hBMSC fraction was obtained by Ficoll/Hypaque (specific gravity 1.077; Nycomed, Oslo, Norway) gradient centrifugation. The cells were seeded into a 75-cm² plastic culture flask at a density of 3 × 10⁵ cells/75-cm² and cultured in α-MEM containing 10% bovine serum (FBS; Invitrogen) and penicillin, streptomycin (100 units/ml and 100 μg/ml, respectively, Sigma). The medium was changed twice weekly from the second week onward, and when the cells were grown to 80–90% confluence, they were subcultured using 0.01% trypsin and 0.05% EDTA. The second-passage cells were used in the experiments. To reduce background growth factor response, cells were incubated with α-MEM containing 0.1% FBS for 24 h before leptin treatment. To study the effect of inhibitors, cells were pretreated with each inhibitor 2 h prior to leptin treatment.

Previous studies (12–14) have shown that when cells were cultured to confluence in the presence of serum, these cells possess many of the phenotypic characteristics of differentiated osteoblasts, including production of type 1 collagen and formation of bone nodules. We also confirmed that these cells deposited calcium into the extracellular matrix and expressed mRNAs characteristic of osteoblastic cells such as alkaline phosphatase, α1(1)collagen, and osteopontin. The absence of monocytic cells was confirmed by staining cultures for nonspecific esterase.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)—Total cellular RNA was isolated from hBMSC using an RNaseasy mini kit (Qiagen) following the manufacturer’s instructions. cDNA was synthesized from 1 μg of RNA using oligo(dT)₁₂−₁₈ primer and a first and standard cDNA synthesis kit (MBI Fermentas). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified as a control for RNA loading and variations in cDNA synthesis efficiency. Primer sequences for the common region of all the OB-R isoforms and the variant region of Ob-R have been reported previously (7); these are as follows: sense, 5'-TGTGTTGAATGTCTTGGCC-3' and antisense, 5'-TACTCCAGTCACTCCAGATTC-3', which encodes a 394-bp fragment in a region common to all Ob-R variants; and sense, 5'-ATAGTTGATCGCTACGATTGTC-3' and antisense, 5'-CCTGGAGGACACTGTTCC-3', which encodes a 338-bp fragment specific for Ob-R1.

Oil Red O Staining—Oil Red O staining for lipid droplets was performed as described previously (15). Briefly, hBMSC were fixed with 10% formalin for 15 min followed by rinsing with distilled water. After soaking in 100% propylene glycol for 2 min, cells were stained with 0.3% Oil Red O for 10 min. Subsequently, they were rinsed with 100% propylene glycol for 1 min, rinsed with distilled water, and counterstained with Harris hematoxylin. Murine 3T3L1 preadipocytes (ATCC) were induced into mature adipocytes by incubation of the cells with Dulbecco’s modified Eagle’s medium containing 25 mM glucose, 10% FBS, 1 μg/ml insulin, 1 mM dexamethasone, and 0.5 mM isobutyl-l-methylxanthine as described previously (16) and were used as the positive control in Oil Red O staining.

Northern Blot Analysis for PPARγ mRNA—Northern blot analysis for PPARγ mRNA was performed as described previously (17). Briefly, total cellular RNA was isolated from hBMSC using a RNaseasy mini kit following the manufacturer’s instructions. Human fat tissues discarded at the time of abdominal plastic surgery were used as the positive control. Of total RNA, 20 μg was electrophoresed through a denaturing formaldehyde 1% agarose gel and transferred to a nylon membrane (Schleicher & Schuell). Hybridization with [α-32P]dCTP-labeled cDNA probes was carried out at 65 °C according to the manufacturer’s instructions. To remove nonspecific binding, membranes were washed twice at room temperature with 2× SSC, 0.1% SDS buffer and then once with 0.1× SSC and 0.1% SDS buffer.

Cell Viability Assay—A colorimetric assay based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) by mitochondrial dehydrogenase of viable cells to a formazan dye was used following the manufacturer’s instructions (Roche Applied Science). Briefly, hBMSC (5 × 10⁴ cells/well) in 100 μl of α-MEM containing 10% FBS were plated in a 96-well plate for 2 days. hBMSC were then incubated in 100 μl α-MEM containing 0.1% FBS for 24 h followed by treatment with various concentrations of leptin for the indicated period of time. After the incubation period, 10 μl of cell proliferation reagent (WST-1) was added to each well, and incubation was continued for 2 h in an atmosphere of 37 °C, 5% CO₂. Cell proliferation was assessed by measuring the absorbance at 450 nm using a microtiter plate (enzyme-linked immunosorbent assay) reader (SPECTRAMax 340 PC; Molecular Devices, Palo Alto, CA) with a reference wavelength at 650 nm. The effect of leptin on cell viability was expressed as percent of cells survived in comparison with the untreated control.

Alkaline Phosphatase Activity and Osteocalcin Assay—Cells were seeded into 6-well plates at a density of 1 × 10⁵/well and cultured for 2 days in α-MEM containing 10% FBS. Cells were then incubated without or with various concentrations (10⁻⁴, 10⁻³, 10⁻², and 10⁻¹ M) of leptin in α-MEM containing 0.1% bovine serum albumin and 50 mMol/liter L-glutamine for 3 days. Then, conditioned medium was harvested and stored at −80 °C until osteocalcin measurement. The cell layer was washed with PBS, and alkaline phosphatase activity was measured using the p-nitrophenyl phosphate hydrolysis method (18). For osteocalcin assay, the conditioned medium was centrifuged free of cellular debris, and the concentration of osteocalcin in the supernatant was measured by immunoassay using a Novocheck commercial kit (Metra Biosystems, Mountain View, CA). Both alkaline phosphatase activity and osteocalcin concentration were normalized with total cellular protein content, which was determined by the Lowry method.

FACS Analysis—Cells were stained using an Annexin V-FITC kit (R & D systems) following the manufacturer’s instructions. Briefly, cells (2 × 10⁵/well) were cultured in a 6-well plate for 24 h. After overnight incubation in α-MEM containing 0.1% FBS, cells were treated without or with various concentrations (10⁻⁴, 10⁻³, and 10⁻² M) of leptin for 24 h. Both floating and adherent (released by trypsin) cells were collected and washed with ice-cold phosphate-buffered saline (PBS). Cells were resuspended in 100 μl of binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, and 1.8 mM CaCl₂) containing 0.25 μg/ml Annexin V-FITC and 5 μg/ml propidium iodide (PI). After 15 min of incubation at room temperature in the dark, 400 μl of binding buffer was added, and samples were processed by flow cytometry. Leptin-untreated cells were used as control. Data acquisition and analysis were performed in a BD Biosciences FACSscan flow cytometer using CellQuest software (BD Biosciences, Mountain View, CA). Annexin V is a cell surface receptor for the 25-kDa plasma membrane associated phospholipid-binding protein with a high affinity for phosphatidylserine. Phosphatidylserine is normally present in the inner lipid bilayer but becomes exposed on the cell surface within the first few hours of the onset of apoptosis (19). PI is a red DNA-binding dye that can only enter cells whose membranes are disrupted such as in cells undergoing necrosis. Apoptotic cells are stained positively with Annexin V-FITC but are resistant to PI staining.

Hoechst 33342 Staining—Cells (3 × 10⁴/well) grown on a 4-well chamber slide (Lab-Tek 174747) were incubated with or without 10⁻⁶ M leptin for 48 h. Control (not treated with leptin) and leptin-treated cells were fixed in 100% cold methanol for 20 min, washed with ice-cold PBS, and then stained for 1 h with Hoechst 33342 dye (Molecular Probes, Eugene, OR). Fluorescent nuclei were visualized in fluorescence microscope (Olympus). Under these conditions, nuclei from living, apoptotic, and necrotic cells could be clearly distinguished. Cells with highly fluorescent, condensed nuclei that showed patches of com-

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**RESULTS**

**Leptin Receptors (Ob-R) Were Expressed in hBMSC**—Using RT-PCR and immunoblotting, we clearly demonstrated that hBMSC expressed leptin receptor mRNA and protein. By RT-PCR, we confirmed a 394-bp fragment common to all Ob-R isoforms (Ob-Rs and Ob-Rl) and the specific long cytoplasmic domain of Ob-Rl by RT-PCR. The PCR products (394 bp for the common extracellular domain of both Ob-Rs and Ob-Rl and 338 bp specific for the long cytoplasmic domain of Ob-Rl) were visualized on a 2% agarose gel containing 0.1% (w/v) ethidium bromide. GAPDH expression was used as internal control for RT-PCR. B, total membrane proteins of hBMSCs were examined by immunoblot analysis using a rabbit polyclonal antibody directed against the common region of all Ob-R. Antibody-protein complexes were visualized by a chemiluminescence detection system. The upper arrow indicates the position of Ob-Rs (130 kDa), and the lower arrow indicates the position of Ob-Rl (100 kDa). Total protein extracts of mouse hypothalamus were used as the positive control. C, Oil Red O staining was performed on hBMSCs and adipocytes differentiated from murine ST311 preadipocytes. Oil Red O staining is observed as red spots in cytoplasm. This figure shows that adipocytes were diffusely stained with Oil Red O. In contrast, no stained cells were observed in hBMSC. D, Northern blot analysis reveals that expression of PPARγ mRNA was observed in fat tissue but not in hBMSC. GAPDH expression was used as internal control for Northern blot analysis.

**Fig 1. hBMSCs express both the long (Ob-Rl) and short (Ob-Rs) forms of Ob-R. A, aliquots of cDNA synthesized from 1 µg of total RNA of hBMSC were amplified using specific primers for the common extracellular region of all the Ob-R isoforms (Ob-Rs and Ob-Rl) and the specific long cytoplasmic domain of Ob-Rl by RT-PCR. The PCR products (394 bp for the common extracellular domain of both Ob-Rs and Ob-Rl and 338 bp specific for the long cytoplasmic domain of Ob-Rl) were visualized on a 2% agarose gel containing 0.1% (w/v) ethidium bromide. GAPDH expression was used as internal control for RT-PCR. B, total membrane proteins of hBMSCs were examined by immunoblot analysis using a rabbit polyclonal antibody directed against the common region of all Ob-R. Antibody-protein complexes were visualized by a chemiluminescence detection system. The upper arrow indicates the position of Ob-Rs (130 kDa), and the lower arrow indicates the position of Ob-Rl (100 kDa). Total protein extracts of mouse hypothalamus were used as the positive control. C, Oil Red O staining was performed on hBMSCs and adipocytes differentiated from murine ST311 preadipocytes. Oil Red O staining is observed as red spots in cytoplasm. This figure shows that adipocytes were diffusely stained with Oil Red O. In contrast, no stained cells were observed in hBMSC. D, Northern blot analysis reveals that expression of PPARγ mRNA was observed in fat tissue but not in hBMSC. GAPDH expression was used as internal control for Northern blot analysis.
found to express both short and long forms of leptin receptors (data not shown).

It has been well documented that bone marrow stromal cells can differentiate into both adipocytes and osteoblasts (23). Moreover, leptin receptors have been shown to be expressed by preadipocytes (24). To ensure that the presence of leptin receptors in our hBMSC preparations was not derived from contaminating adipocytes, the hBMSC cells were tested with Oil Red O staining for lipid droplets in the cytoplasm and analyzed for PPARγ mRNA expression. PPARγ is a nuclear receptor essential in adipocyte cell fate determination from stem cells (23). No Oil Red O-stained cells could be detected in our hBMSC cultures (Fig. 1C), and these cells did not express PPARγ mRNA (Fig. 1D). These data indicate that our hBMSC preparations are not contaminated with adipocytes and exclude adipocytes as the source of leptin receptors in these cells.

Leptin Decreased the Cell Viability, Alkaline Phosphatase Activity, and Osteocalcin Secretion of hBMSC—To examine the direct effect of leptin on hBMSC, we measured the cell viability using WST-1 assay. Leptin decreased hBMSC viability in a dose-dependent manner (Fig. 2A, p < 0.01). Incubation of hBMSC with 10^{-9}, 10^{-8}, 10^{-7}, and 10^{-6} M leptin for 24 h reduced cell viability to 95.3 ± 2.6, 84.3 ± 3.1, 75.0 ± 3.3, and 67.0 ± 4.1%, respectively, of the control level. To confirm that the toxic effect on hBMSC was leptin-specific, but not because of contaminants in the products, hBMSC were cultured in α-MEM containing 0.1 μg/ml of rabbit anti-Ob-R antibody (sc-8325) with or without 10^{-7} M leptin for 24 h. For controls, hBMSC were treated with α-MEM containing the same concentration of normal rabbit IgG (sc-2027; Santa Cruz Biotechnology, Inc.) with or without the same concentration of leptin. In the presence of anti-Ob-R antibody, leptin failed to induce any cytotoxic effect on hBMSC, because the cell survival rate was the same as those treated with anti-Ob-R or normal IgG alone (Fig. 2B). These data suggest that the cytotoxic effect induced by the commercially available leptin preparations is leptin-specific and that this leptin-induced cytotoxic effect on hBMSC is solely mediated via Ob-R.

Furthermore, hBMSC viability was decreased by leptin in a time-dependent manner (Fig. 2C, p < 0.01). Treatment of hBMSC with 10^{-6} M leptin for 4–8 h did not affect cell viability (Fig. 2C). However, leptin significantly reduced cell viability after 12, 24, 48, and 72 h to 83.1 ± 2.4, 62.5 ± 2.0, 51.2 ± 1.1, and 41.8 ± 2.2% of the control level, respectively (Fig. 2C, white bars). The detrimental effect of leptin on osteoblastic cells was not limited to bone marrow stromal cells derived from ribs; the cell viability of hBMSC isolated from femurs and that of hOB cells were also decreased by leptin in a dose-dependent and time-dependent manner (data not shown). Because caspases are known to be important enzymes in cell death, we analyzed the role of caspases in leptin-induced hBMSC death. Incubation of hBMSC with 50 μM Z-VAD-fmk, a pan-caspase inhibitor, nearly completely blocked leptin-induced cytotoxic effect (Fig. 2C, black bars). This data suggest that the leptin-induced cytotoxic effect in hBMSC is very likely derived from a caspase-dependent apoptosis.

Because leptin has been reported to either stimulate or inhibit bone formation (7, 10, 16), we also investigated the effects of leptin on both early and late osteoblast differentiation markers such as alkaline phosphatase activity and osteocalcin production, respectively, in hBMSC. Leptin significantly reduced alkaline phosphatase activity in a dose-dependent manner from 52.6 ± 1.1 nmol/mg protein/min (untreated control) to 45.6 ± 1.6 nmol/mg protein/min (10^{-3} M), 40.4 ± 1.6 nmol/mg protein/min (10^{-2} M), and 37.0 ± 1.5 nmol/mg protein/min (10^{-1} M), (Fig. 2D, p < 0.01). In the same fashion, leptin significantly decreased osteocalcin production from 8.21 ± 0.18 ng/mg protein (untreated control) to 7.46 ± 0.16 ng/mg protein (10^{-3} M), 6.03 ± 0.19 ng/mg protein (10^{-2} M), 4.29 ± 0.20 ng/mg protein (10^{-1} M), and 3.46 ± 0.12 ng/mg protein (10^{-0} M) (Fig. 2E, p < 0.01). These data further substantiate that leptin has a detrimental effect on hBMSC.

Leptin Induced Apoptosis in hBMSC—To confirm that leptin induced hBMSC apoptosis, FACS analysis and Hoechst staining were performed. Cells were incubated with 10^{-8}, 10^{-7}, and...
10^{-6} \text{M} \text{ leptin for 24 h, and the number of apoptotic cells was determined by FACS after annexin V binding and PI staining. Leptin dose-dependently increased the percentage of apoptotic cells (annexin V-positive but PI-negative) from 5.21 ± 3.32% (control) to 15.31 ± 4.33% (10^{-6} \text{M}, 29.24 ± 3.24% (10^{-7} \text{M}), and 40.16 ± 2.56% (10^{-6} \text{M}) (Fig. 3A, p < 0.01). Additionally, staining with Hoechst 33342 demonstrated that highly fluorescent condensed nuclei, which are hallmarks of apoptotic cells, were detected in leptin (10^{-6} \text{M} for 48 h)-treated cells but not in the control cells (Fig. 3B, arrows). These combined data clearly demonstrate that leptin directly induces apoptosis in hBMSC.

**Leptin Activated Caspase-9 and Caspase-3 but Not Caspase-8**—Because several caspases are involved in apoptosis, we analyzed the regulation of the activities of caspase-3, caspase-8, and caspase-9 in hBMSC by leptin. Fig. 4A shows that leptin (10^{-6} \text{M}) increased caspase-9 activity 1.8-fold after 8 h of incubation and 3.4-fold by 24 h. Leptin also elevated caspase-3 activity 2.5-fold after 12 h and 3.1-fold by 24 h (Fig. 4B). In contrast, the activity of caspase-8, a key enzyme in the cytochrome c-independent apoptosis pathway (death receptor path)way), was not altered by leptin (Fig. 4C). Thus, leptin induction of hBMSC apoptosis appears to be via a cytochrome c-dependent pathway.

**Leptin Enhanced Cytochrome c Release into Cytoplasm, Stimulated the Conversion of Procaspase-3 and Procaspase-9 into Their Respective Active Forms, and Increased PARP Cleavage**—It is known that translocation of cytochrome c from mitochondria to cytosol is an important step in the apoptotic signaling pathway, linking mitochondrial changes to the activation of caspases (26). Once located into cytosol, cytochrome c, together with Apaf-1 and procaspase-9, forms a multiprotein complex, which initiates the activation of caspase-3 leading to cell apoptosis (27). To investigate whether this signaling pathway is induced by leptin in hBMSC, we analyzed the translocation of cytochrome c into cytosol, the generation of activated caspase-9 (M_9 38,000), caspase-8 (M_8 55,000), and caspase-3 (M_3 19,000) from their respective proenzyme, and the cleavage of a M_6 89,000 fragment from the full-length PARP (M_6 116,000) following incubation of these cells with 10^{-6} \text{M} leptin using immunoblot analysis. As shown in Fig. 5, leptin increased the release of cytochrome c from mitochondria into cytoplasm at 8 h with further increment observed at 12 and
24 h. In the same fashion, activated caspase-9 and activated caspase-3 were generated beginning at 12 h after incubation with leptin with further increments noted at 24 h. In contrast, no activated caspase-8 was detected in leptin-treated hBMSC at all the time points analyzed (Fig. 5). Consistent with the activation of caspase-8, the degradation of PARP, which is a caspase-3 substrate, was also clearly demonstrated at 12 and 24 h (Fig. 5). These data suggest that cytochrome c is a key factor in leptin-induced apoptosis in hBMSC and that its release may relate to the activation of caspase-9 and caspase-3, which subsequently triggers the cleavage of PARP and the appearance of apoptosis.

**Leptin Activated ERK and cPLA2—MAPK cascade** is well known to play an essential role in controlling cellular proliferation, differentiation, and apoptosis (28, 29). Therefore, to elucidate the mechanism mediating the apoptotic effect of leptin in hBMSC, we examined MAPK signalings induced by leptin. Leptin-stimulated ERK activity in hBMSC after 0.5 h of incubation as demonstrated by the increase in the phosphorylated ERK (p-ERK) levels (6.3 ± 1.1-fold of untreated control; see Fig. 6A). The peak activation of ERK occurred at 2 h (11.8 ± 0.6% of untreated control), which tapered off afterward, and by 24 h no p-ERK could be detected. In contrast, leptin has no
effect on the activities of p38 and JNK, because none of their phosphorylated forms were detected (Fig. 6A). Because MEK1 and MEK2 are known to be the upstream effectors of ERK, we analyzed their role in leptin-induced activation of ERK. PD98059, which inhibits MEK1, and U0126, which inhibits both MEK1 and MEK2, completely prevented this effect (Fig. 6B). AACOCF3, which inhibits cPLA2 activity, had little effect on leptin-induced ERK activation (Fig. 6B). Because ERK has been shown to activate cPLA2 (30), we analyzed the effect of leptin on cPLA2 activity as determined by the release of arachidonic acid from cells into medium. Incubation of hBMSC with leptin resulted in an increased activity of cPLA2 beginning at 0.5 h (126.4 ± 9.1%), peaked at 1 h (295.3 ± 5.8%), and tapered off afterward (Fig. 6C). The activation of cPLA2 by leptin was inhibited by both PD98059 and U0126, implicating that MEK/ERK activation by leptin was essential for the activation of cPLA2 (Fig. 6C). As expected, AACOCF3, which inhibits cPLA2 activity, prevented the stimulation of cPLA2 by leptin (Fig. 6C). These data indicate that leptin stimulates the MEK/ERK/cPLA2 signal transduction pathway in hBMSC.

MEK/ERK/cPLA2/Cytochrome c Signaling Pathway Mediated Leptin-induced Apoptosis and Activation of Caspase-3—With the demonstration that leptin activated caspases, enhanced cytochrome c release, and stimulated ERK/cPLA2 in hBMSC, we examined the hierarchial relationship among these signaling molecules. Preincubation of cells with MEK inhibitors (PD98059 and U0126) or cPLA2 inhibitor (AACOCF3) increased cell viability in the presence of leptin (Fig. 7A) and inhibited the activation of caspase-3 by leptin (Fig. 7B). In contrast, BEL (an iPLA2 inhibitor) did not affect leptin-induced cell death and caspase-3 activity (Fig. 7, A and B). These data indicate that the MEK/ERK/cPLA2 signal transduction pathway mediates the apoptotic effect of leptin on hBMSC and that the activation of caspase-3 by leptin requires intact MEK/ERK/cPLA2 signaling. Because cytochrome c release into cytosol preceded the activation of caspase-3, we examined the role of MEK/ERK/cPLA2 signal transduction in leptin-induced cytochrome c release. Preincubation of hBMSC with PD98059, U0126, or AACOCF3, but not BEL, drastically reduced the release of cytochrome c into cytosol by leptin (Fig. 7C). These data provide evidence to support that cytochrome c release is downstream of ERK/cPLA2.

Leptin Activated JAK/STAT1 Signaling Pathway in hBMSC—In addition to the MAPK/ERK signaling, it has been well established that leptin also activates JAK/STAT pathway in many cell systems and that STAT signaling mediates apoptosis in A431 and HeLa cells (31). We wished to examine whether the JAK/STAT signal transduction pathway was also activated by leptin and the role of this signaling in leptin-induced apoptosis in hBMSC. As shown in Fig. 8A, leptin activated STAT1, because the level of phosphorylated STAT1 was increased, beginning at 0.5 h (3.6 ± 0.5-fold of untreated control), peaked at 1 h (7.3 ± 0.9-fold of untreated control), and tapered off afterward (p < 0.05 and p < 0.01, respectively, compared with the untreated control). In contrast, leptin had no effect on the activation of STAT3 in hBMSC, because no phosphorylated STAT3 was detected. The activation of STAT1, but not STAT3, by leptin was confirmed by immunoprecipitation using anti-phosphotyrosine antibody followed by Western blotting for STAT1 and STAT3 (Fig. 8B). Phosphorylation of JAK was also increased by leptin dose-dependently (Fig. 8B, p < 0.01). Preincubation of hBMSC with JAK inhibitor, AG490, substantially reversed leptin-induced cell death (Fig. 8C). These data implicate that leptin activates the JAK/STAT1 signal transduction pathway in hBMSC and that this signaling may play an important role in leptin-induced hBMSC apoptosis. Although the relationship between ERK/cPLA2/cytosolic c and JAK/STAT1 signal transduction pathways in hBMSC has not been explored at present, several studies have shown that there were numerous potential cross-talks between these two signal transduction pathways (32). By combining our data and the reports of others, we propose in Fig. 9 the likely signal transduction pathway mediating leptin-induced apoptosis in hBMSC.

DISCUSSION

Recently, it has been suggested that leptin is a key element in bone mass regulation. However, the mechanism by which leptin acts upon the bone remains unclear. We now demonstrate that the precursor cells of the osteoblast-lineage, hBMSC, can be direct targets of leptin, because they express a functional leptin receptor, Ob-Rb, and leptin induces their
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Leptin exerts its function by binding to Ob-R. Several splice variants of OB-R have been detected in mouse, human, and rat cells that encode isoforms of OB-R with various lengths of cytoplasmic domain (2, 33). It has been well established that upon binding of leptin to Ob-R, JAK2 is activated that subsequently phosphorylates tyrosine residues in the intracellular domain of OB-R and initiates the downstream signaling (34).

Because the longest isoform of the leptin receptor (Ob-Rb/H11005 Ob-RL) is the only isoform that contains tyrosine residues in the intracellular domain (35), it is considered as the only functionally active leptin receptor. Previous studies have arrived at contradictory conclusions about the presence of Ob-Rb in cells of the osteoblast-lineage. Although Ducy et al. (10) did not observe any Ob-R in primary osteoblasts obtained from wild-type mice, several studies reported the presence of leptin receptor in human and rodent osteoblasts (7, 8, 11, 36). Our data substantiate the expression of Ob-Rb in cells of the osteoblast lineage in human.

In our experiment, leptin exerts significant toxic effects on cell viability in hBMSC. This is in conflict with the report by Thomas et al. (7), who demonstrated that leptin had no effect on the proliferation of the bone marrow stromal cell line. The grounds of this disparity are not clear but could be because of differences in the cell line used or other unknown variables. Although primary hBMSC were used in our study, Thomas et al. employed a hBMSC cell line that was immortalized with a temperature-sensitive mutant of a Simian virus 40 large T antigen. The presence of this antigen may alter cell property and masks the apoptotic effect of leptin. While this paper was in preparation, Gordeladze et al. (37) reported that leptin stimulates cell proliferation and significantly protects cells from apoptosis in hOB. The mechanism leading to this opposing apoptotic effect of leptin is not clear. The sources of hBMSC or the degree of differentiation does not seem to matter, because we also observed the decreased cell viability with leptin in hOB isolated from ribs, as well as in hBMSC isolated from femurs (data not shown). One possible explanation may reside in the cell culture condition; our cells were pretreated with or without AG490 (30 μM) for 2 h followed by treatment with or without leptin (10 μM) for 24 h. *, p < 0.01 when compared with leptin-treated control.

![Figure 8](http://www.jbc.org/)

**Fig. 8.** Leptin activates JAK/STAT1 but not STAT3, and leptin-induced cell death is inhibited by JAK inhibitor, AG490. Cells were treated as described for Fig. 6. A. 30 μg of each cytosolic protein was subjected to immunoblot analysis using anti-rabbit polyclonal antibodies against STAT1, phosphorylated STAT1 (p-STAT1), STAT3, and phosphorylated STAT3 (p-STAT3). C, control without leptin treatment. Shown are representative immunoblots for p-STAT1 and p-STAT3. The band intensity was quantified by densitometry, and is represented in the corresponding bar graph. The density of the untreated control is designated as 1.0. B, after treatment with the indicated concentration of leptin for 2 h, the cells were lysed, and lysates were immunoprecipitated (IP) with anti-phospho tyrosine (anti-pY) antibody. The immune complexes were separated in SDS-PAGE, and immunoblot analysis was performed using anti-STAT1, anti-STAT3, and anti-JAK antibody. Shown are representative immunoblots for STAT1, STAT3, and JNK and the corresponding bar graph depicting the relative intensity of each band. C, cells were pretreated with or without AG490 (30 μM) for 2 h followed by treatment with or without leptin (10 μM) for 24 h. *, p < 0.01 when compared with leptin-treated control.

![Figure 9](http://www.jbc.org/)

**Fig. 9.** Schematic of the signaling pathway in leptin-induced apoptosis in hBMSC. Dashed lines indicate the signaling not yet confirmed in hBMSC.
Apoptosis is a tightly regulated physiological process, triggered by a variety of metabolic or cytokine-dependent stimuli that leads to cell death. Although leptin displays proliferative and anti-apoptotic activities in a variety of cell types including hOB (38–43), evidence for a link between leptin and apoptosis has been shown in a few studies. Qian et al. (44) observed apoptosis of adipocytes after intracerebroventricular administration of leptin in rats. Cohen et al. (45) reported that leptin induces apoptosis in adipose vascular endothelial cells, although others reported a protective effect of leptin from apoptosis in another vascular endothelial cells, such as human umbilical vein endothelial cells (43). We have demonstrated by several criteria such as flow cytometry, Hoechst 33342 staining, and caspase activation that leptin induces hBMSC apoptosis. To our knowledge, this is the first study that shows a direct linkage between leptin and the development of apoptosis in osteoblast precursor cells. With the increase in hBMSC death, the number of osteoblast precursor cells available to differentiate into mature osteoblasts will be limited, which may lead to decreased bone formation. In fact, the rate of apoptosis of osteoblast-lineage cells has been shown to be a critical determinant of bone formation in vivo (46). Moreover, with the advancement of fatty marrow formation as we age, leptin produced by these marrow adipocytes would have direct detrimental effects on the surrounding marrow stromal cells, which may contribute to aging-associated osteoporosis. It would be of interest in the future to verify the apoptotic effect of leptin on cells of osteoblast-lineage in vivo and to determine the relevance of this mechanism in mediating the detrimental effect of leptin on bone mass in comparison with the hypothalamic relay pathway as proposed by Ducy et al. (10).

Apoptosis manifests in two major execution programs downstream of the death signal: the caspase pathway (death receptor signaling pathway) and the mitochondrial dysfunction pathway (drug-induced pathway) (47, 48). Caspase-8 is thought to be the key upstream regulatory caspase in the caspase pathway, in which two caspase-3 activation pathways are evolved based on whether cytochrome c is required. One is the direct activation of caspase-3 by caspase-8 without cytochrome c release, and the other one is the indirect activation of caspase-3 through cytochrome c release induced by caspase-8. In mitochondrial dysfunction pathway, cytochrome c is released from mitochondria into cytosol where it forms an essential part of the vertebrate “apoptosome” with Apaf-1 and procaspase-9. The result of apoptosome formation is the activation of caspase-9, which then processes and activates other caspases to orchestrate the biochemical execution of cells. The enhanced cytochrome c release into cytosol and the activation of caspase-9 and caspase-3 but not caspase-8 in hBMSC suggests that leptin induces apoptosis in cells of the osteoblast lineage by mitochondrial dysfunction mechanisms.

PARP is a chromatin-associated enzyme that catalyzes the transfer of successive units of ADP-ribose moiety from NAD+ covalently to itself and other nuclear acceptor proteins. The catalytic activity of PARP is strictly dependent on the number of nicks in DNA; therefore, only about 1% of the total poly ADP-ribose molecules would be active under physiological conditions (49). PARP is known to be important for many cellular processes that require DNA cleavage and rejoicing reactions such as DNA replication, recombination, and repair, cell cycle regulation, cell differentiation, and neoplastic transformation (50, 51). In addition, recent studies (52, 53) have shown that PARP is involved in the execution of apoptosis. It has been well recognized that limited proteolysis of PARP by the caspase family of cysteine proteases is an early event or prerequisite for the execution of apoptosis (52, 54, 55). Cleavage of PARP by caspase results in the separation of the two zinc-finger DNA-binding motifs in the amino terminus and the catalytic domains in the carboxyl terminus of the enzyme (54). Consequently, this cleavage excludes the catalytic domain from being recruited to the sites of DNA fragmentation during apoptosis and presumably disables PARP from coordinating subsequent repair of genome maintenance events (56). Our data clearly showed that leptin triggers the cleavage of PARP following the activation of caspase-9 and caspase-3, further confirming the evidence of leptin-induced apoptosis in hBMSC.

To gain further insight into the mechanisms by which leptin promotes apoptosis in hBMSC, we have evaluated the early signaling events. It is known that interaction of leptin with the Ob-Rb, a member of the gp130 interleukin-6 receptor family, leads to activation of STAT1, 3, and 5 and ERK1/2 depending on the cell models used (57–60). However, leptin-induced signaling has not been reported in bone cells. Herein, we report that leptin induces activation of ERK1/2 in hBMSC. More importantly, inhibition of ERK activation by MEK inhibitors greatly reduces leptin-induced caspase-3 activation and increases the hBMSC survival rate in the presence of leptin. These data suggest that ERK signaling mediates, at least in part, leptin-induced apoptosis in cells of osteoblast lineage. We have also demonstrated that cPLA2 is a downstream effector of ERK in leptin signalings. cPLA2 belongs to the phospholipase A2 superfamily that hydrolyzes the sn-2 ester bond in phospholipids. cPLA2 isoform is a Ca2+-dependent cytosolic enzyme that is specific for the hydrolysis of arachidonylated phospholipids (61). The role of cPLA2 in apoptosis is dependent on the stimulants and the cell type being targeted. For example, cPLA2 does not have any role in Fas-induced apoptosis in human leukemic cells (62) but is needed for tumor necrosis factor α-induced apoptosis in several cell types (63, 64). Our data revealed that the induction of apoptosis in hBMSC by leptin is dependent on the activation of cPLA2.

In addition to the activation of ERK signaling, we demonstrated that leptin also stimulated JAK/STAT1 in hBMSC. The stimulation of both MAPK and STAT pathways has been observed in many cells after exposure to many growth factors and cytokines, including interferons and epidermal growth factor. JAK/STAT signaling plays essential roles in cell differentiation, cell cycle control, and development. Interestingly, Chin et al. (31) reported that activation of STAT1 is critical for interferon-γ and epidermal growth factor-induced apoptosis. Kumar et al. (65) demonstrated a defective apoptosis in STAT1-null cells. Therefore, STAT1 appears to play a critical role in apoptosis depending on the cell type and the stimulants used. Our data indicate that JAK/STAT1 may also contribute to the development of leptin-induced apoptosis in hBMSC.

In summary, our results demonstrate that leptin promotes apoptosis in hBMSC via MEK/ERK/cPLA2/cytochrome c and possibly JAK/STAT1 pathways. Although the significance of the leptin-induced apoptosis in terms of functional outcomes in bone in vivo is not clear at present, an improved understanding of this pathway could ultimately lead to identification of new therapeutic targets for osteoporosis.

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