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Leptin Stimulates Rat Aortic Smooth Muscle Cell Proliferation and Migration

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Leptin, a peptide secreted from adipose tissue, plays an important role in the regulation of food intake and energy expenditure. In obese patients, plasma leptin levels are elevated and obesity is one of the major risk factors for cardiovascular diseases. Therefore, in this study, we investigated the effect of leptin on vascular smooth muscle cell (VSMC) functions. Cultured rat aortic VSMC expressed 130-kDa short form of leptin receptor. Leptin stimulated both proliferation and migration of VSMC. Leptin stimulated phosphorylation and activation of mitogen-activated protein (MAP) kinases, and also increased phosphatidylinositol (PI) 3-kinase activity. Further, two distinct PI 3-kinase inhibitors, wortmannin and LY294002 inhibited the migratory effect of leptin. These results demonstrate that leptin is a proliferative and migratory factor for VSMC, implying that leptin may play a role in the formation and development of vascular lesions.

Arterial intimal thickening observed in atherosclerosis and after balloon angioplasty has been attributed to proliferation of vascular smooth muscle cells (VSMC) that migrate into the intima from the media in response to a number of factors [1]. Several biological substances, such as growth factors, cytokines, and vasoactive peptides promote VSMC proliferation and migration, contributing the formation and development of vascular lesions.

Obesity is one of the major risk factors for cardiovascular diseases and the evidences from prospective studies have demonstrated the significant association between regional body fat distribution and cardiovascular diseases [2, 3, 4, 5, 6]. However the mechanisms responsible for this relationship has not been established. The *ob* gene product, leptin has been defined as a regulator of food intake and energy expenditure [7]. Leptin acts through it's receptors (OB-R) being homologous to the class I cytokine-receptor family [8] and having at least 5 splice variants [9]. It has been shown that the effect of leptin on food intake is mediated through the long form of OB-R in hypothalamus [10, 11]. On the other hand, short forms of OB-R are widely expressed throughout the body [12][13]. This receptor expression pattern suggests that, in addition to the control of food intake and body weight, leptin may have other important physiological functions.

Proliferative effect of leptin has been reported on hematopoietic, embryonic fibroblast, and pancreatic ß cell lines [14, 15, 16, 17, 18]. In vascular cells, endothelial cell proliferation and promotion of angiogenesis, which involve tyrosine kinase-dependent intracellular pathways, by leptin are reported [19, 20]. However, to date, it is unknown whether leptin has biological effects on VSMC, or even if OB-R are expressed in VSMC. In the present report, we examined the expression of OB-R protein in VSMC and the effects of leptin on VSMC

functions. This report shows that short form of OB-R is expressed in VSMC and that leptin stimulates VSMC proliferation and migration by stimulating mitogen-activated protein (MAP) kinases and phosphatidylinositol (PI) 3-kinase activation. These results suggest a role of leptin in the formation and development of vascular diseases.

MATERIALS AND METHODS

Materials

Human recombinant leptin was kindly provided by Dr. Yasuhiko Okimura, Faculty of Health Science, Kobe University School of Medicine. Myelin basic protein (MBP) and wortmannin were obtained from Sigma (St. Louis, MO). The anti-OB-R goat polyclonal antibodies, and anti-phosphotyrosine monoclonal antibody PY-20 were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA), and Transduction Laboratories (Lexington, KY), respectively. Anti-phosphotyrosine monoclonal antibody 4G10 was obtained from Upstate Biochemicals Inc (Lake Placid, NY). Streptavidin-biotinylated horseradish peroxidase complex was from Amersham Life Science (Tokyo, Japan). Anti-MAP kinase mouse monoclonal antibody, biotin conjugated rabbit anti-goat IgG (H+L) was from Zymed Laboratories (San Francisco, CA). [γ-^{32}P]ATP (4,500 Ci/mmol) was from ICN (Costa Mesa, CA). ITS™ Premix (insulin, transferrin, selenium) was from Collaborative Biomedical Products (Bedford, MA). Protein A-sepharose CL-4B was from Pharmacia Biotech (Uppsala, Sweden). Phosphatidylinositol was from Avanti Polar Lipids (Alabaster, AL). Silica Gel 60 plates were from MCB reagents (Merck, Rahway, NJ). LY294002 was from Alexis corporation (San Diego, CA). Other materials and chemicals were obtained from commercial sources.

Cell Culture

VSMC were isolated from rat thoracic aorta by enzymatic dissociation as described previously [21]. Cells were grown and passaged as described previously [22]. For experiments, cells between passage levels 8 and 16 were made quiescent by incubation with serum-free Dulbecco's modified Eagle's medium (DMEM) for 48 h before use.

Immunoblot analysis with anti OB-R antibody

VSMC were lysed into a buffer containing 50 mM Tris-HCl, pH 7.5, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4 and 10 μg/ml leupeptin. After removing insoluble materials by centrifugation at 15,000 rpm for 20 min, protein concentrations in the supernatants were normalized using Bio Rad protein assay (Bio Rad), and lysates were directly added to a 1/4 vol. of 5x sample buffer of SDS-polyacrylamide gel electrophoresis and boiled at 100°C for 5 min. Samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and the separated proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore). Blots were incubated with the anti-OB-R goat polyclonal antibody following the incubation with biotin conjugated rabbit anti-goat IgG (H+L) and then incubated with streptavidin-biotinylated horseradish peroxidase complex. Peroxidase-labeled proteins were visualized by enhanced chemiluminescence reagents (Amersham Life Science).

Quantification of cell number

Cell number was measured using the crystal violet staining method described before [23]. Briefly, VSMC were suspended in DMEM containing 0.5% fetal bovine serum (FBS) and seeded into each well of a 96-well plate (Falcon, Becton Dickinson, NJ) at a density of

$4x10^3$/well. After cell attachment, the medium was replaced with DMEM containing either 2% FBS and ITS™ Premix (insulin, transferrin, selenium) in the presence or absence of leptin. After 3 days of incubation, the cells were fixed with 10 µl of 10% glutaraldehyde, stained with 0.1% crystal violet solution at pH 6.0, and dissolved with 10% acetic acid. The wells were read at 590 nm in a Microplate Reader (model 3550, Bio-Rad). A calibration curve was drawn based on a known number of cells.

Cell migration assay

Chemotactic response was assayed by using a 48-well microchemotaxis chamber (Neuro Probe Inc. Cabin John, MD). The cells were treated with 0.25% trypsin and 0.01% EDTA, and then resuspended in DMEM containing 0.5% bovine serum albumin (BSA) at $5x10^5$ cells/ml. A polycarbonate membrane(polyvinylpyrrolidone-free, pore size 8.0 µm) was coated with 1% gelatin. Chemoattractant solution (28 µl) containing leptin (100 ng/ml) was placed in the lower chamber, and the cell suspension ($2.5x10^4$ cells in 52 µl) was placed in the upper chamber. The assembled chamber was incubated in a CO_2 incubator for 6 h. The membrane was stained with Diff-Quick (International Reagents Corp., Kobe, Japan), and the number of cells that had migrated to the lower surface of the membrane was counted.

Immunoblot analysis with anti phosphotyrosine and anti-MAP kinase antibodies

Cells were starved for 48 h, and stimulated with 100 ng/ml of leptin at 37°C for 10 min. Samples were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and transferred to a PVDF membrane on a semi-dry transfer unit. Blots were incubated with the indicated monoclonal antibodies and primary antibodies were detected using horseradish peroxidase-labeled donkey anti-mouse IgG, followed by enhanced chemiluminescence (Amersham Life Science).

MAP kinase assay in polyacrylamide gel containing MBP

MAP kinase activity was measured by a MAP kinase renaturation assay in MBP-containing polyacrylamide gels as described previously [24]. Briefly, the cell lysates from VSMC stimulated with leptin were electrophoresed on 10% SDS-polyacrylamide gel containing 0.5 mg/ml MBP. After washing the gel with 50 mM Tris-Cl, pH 8.0 and 20% 2-propanol, and then with 50 mM Tris-Cl, pH 8.0 and 5 mM 2-mercaptoethanol (buffer A) to remove SDS, the enzymes were denatured by treating the gel with 6 M guanidine HCl and then renatured with buffer A containing 0.04% Tween 40. The gel was incubated at 22°C for 1 h with 40 mM HEPES, pH 8.0, 2 mM 1, 4-dithiothreitol, 0.1 mM EGTA, 5 mM $MgCl_2$, and 25 mM [γ-^{32}P]ATP ($1x10^5$ cpm/nmol) for kinase reactions. The gel was extensively washed with 5% trichloroacetic acid containing 1% sodium pyrophosphate, dried, and the radioactivity was analyzed using a Fuji BAS 2000 bioimaging analyzer.

PI 3-kinase assay

PI 3-kinase was measured in a manner similar to that described by Endemann et al. [25]. Cells were starved for 48 h, and stimulated with 100 ng/ml of leptin at 37°C for 0-20 min. Cells were lysed in a buffer containing 1% NP-40, 137 mM NaCl, 20 mM Tris, pH 8.0, 10% glycerol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 2 mM Na_3VO_4, 25 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF), vortexed and subsequently cleared by centrifugation at 14,000 rpm for 15 min at 4°C. Cleared lysates (750 µg of protein) were incubated with 2 µg/ml of anti-phosphotyrosine monoclonal antibody for 2 h at 4°C. Thirty µl of a 50% slurry protein A-sepharose CL-4B was added and incubated for another 1 h at 4°C.

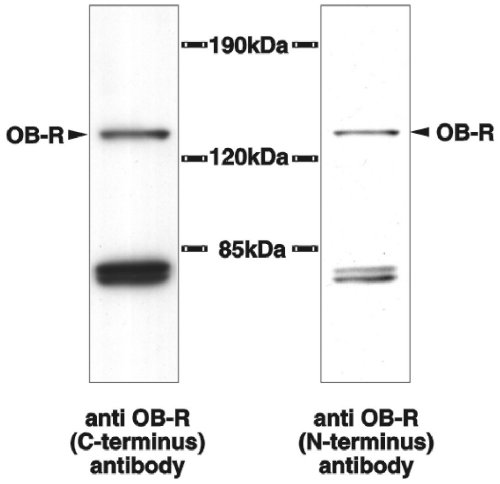
Immunoprecipitates were spin down and washed twice with each of the following solutions: PBS containing 1% NP-40; 100 mM Tris-Cl pH 7.5 and 500 mM LiCl; 10 mM Tris-Cl pH 7.2, 100 mM NaCl and 1 mM EDTA. After the final wash, immunoprecipitates were incubated with 10 µg of sonicated PI and [γ-^{32}P]ATP (5 µCi/sample) for 6 min at room temperature. The phosphorylation reaction was stopped by adding 15 µl of 4 N HCl and 130 µl of chloroform:methanol (1:1), vortexed, and the biphasic mixture was microcentrifuged to extract lipids. The bottom organic layer was carefully collected, and ^{32}P-labeled phospholipids were resolved by thin-layer chromatography (TLC) using Silica Gel 60 plates with a chloroform:methanol:water:ammonium hydroxide (60:47:11.3:2) solvent system. The radioactivities in the PI-3-phosphate fraction were determined using a Fuji BAS 2000 bioimaging analyzer.

Statistical analysis

Where applicable, results were expressed as the mean ± standard error of the mean (S.E.). Statistical analysis was performed with unpaired Student's t test or ANOVA when appropriate, and differences were considered to be significant when the probability value was < 0.05.

RESULTS

At first, we investigated whether OB-R was expressed in VSMC by immunoblotting with anti-OB-R antibodies which recognize either N- or C-terminal region of OB-R. We detected three bands with their apparent molecular sizes of 78-, 80- and 130-kDa (Fig. 1). The 130 kDa band was thought to be a short intracellular domain form of OB-R, referred to here as a short form of OB-R [26, 27].



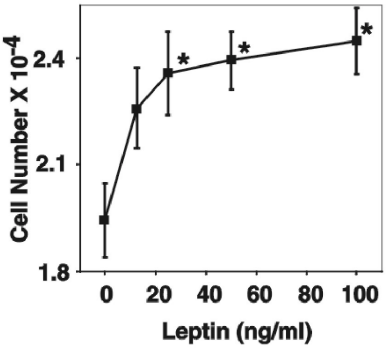
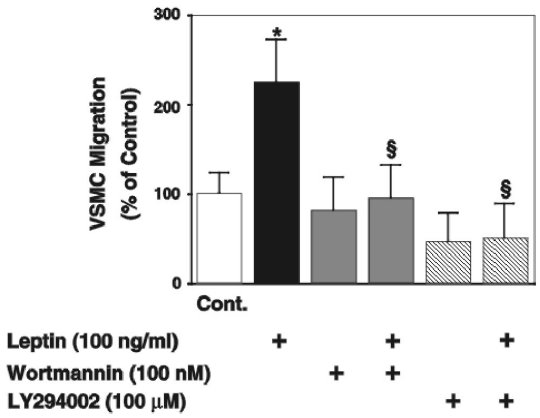


Fig. 1. (Left) Expression of OB-R protein in VSMC. Total cell lysates were separated on 10% SDS-PAGE gels and transferred onto PVDF filters. Blots were incubated with anti-rat OB-R antibodies (1:50,000) and the primary antibody was detected using biotin-conjugated anti-goat IgG (1:10,000) and streptavidin-biotinylated horseradish peroxidase (1:10,000), followed by enhanced chemiluminescence. Bars indicate molecular weight standards. Leptin receptors were detected as three bands with their approximate molecular sizes of 78-, 80- and 130-kDa.

Fig. 2. (Right) Leptin-stimulated VSMC proliferation. After three days incubation with the indicated amounts of leptin in a medium containing 2% FBS and ITS (insulin, transferrin, selenium), cell number was evaluated by crystal violet staining. Leptin stimulated VSMC proliferation in a dose dependent

manner. Maximal effect was observed at 100 ng/ml of leptin, and half maximal effect was 15 ng/ml. 100 ng/ml leptin increased cell number by approximately 20%. Values represent the mean ± S.E. of six wells in a representative experiment. The experiment was repeated three times. *, $p < 0.05$ vs. control.

To investigate the physiological role of leptin for VSMC, we examined the effect of leptin on VSMC proliferation and migration. VSMC were treated with leptin for 3 days, and cell number was measured by crystal violet staining method. Leptin increased the cell number in a dose dependent manner. Maximal and half-maximal effect was at 100 and 15 ng/ml, respectively; the maximal effect was approximately 20% increase in cell number (Fig. 2). Migration experiments were performed using a modified Boyden's chamber method. Leptin (100 ng/ml) induced VSMC migration by approximately 3-fold compared to the control (0.5% FBS containing medium) (Fig. 3).



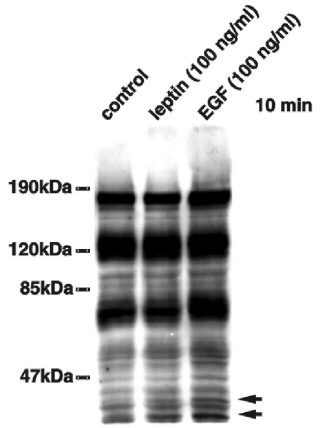


Fig. 3. (Left) Leptin-stimulated VSMC migration. Migratory response of VSMC to 0.5% fetal bovine serum (FBS) and leptin (100 ng/ml) as measured in a 48-well microchemotaxis chamber. Migration of VSMC was quantitated and plotted as the percentage of the response to 0.5% FBS. Leptin stimulated VSMC migration by approximately 3-fold compared to 0.5% FBS. Values represent the mean ± S.E. of eight independent assays. *, $p < 0.05$ vs. control. §, $p < 0.05$ vs. leptin-stimulated. Wortmannin (100 nM) and LY294002 (100 μM) were added both in the upper and lower compartments of microchemotaxis chamber.

Fig. 4. (Right) Detection of tyrosine phosphorylation of cellular proteins in VSMC by anti-phosphotyrosine immunoblotting. VSMC were incubated for 10 min with leptin (100 ng/ml). Whole cell extracts were analyzed by immunoblotting with anti-phosphotyrosine antibody. Epidermal growth factor (EGF) was used as a positive control. Arrowheads indicate tyrosine-phosphorylated proteins stimulated by leptin. The molecular weights of protein standards are indicated in the left side.

To determine the mechanism of VSMC proliferation and migration by leptin, we examined the signal transduction pathways induced by leptin. VSMC was tested for tyrosine phosphorylation of cellular proteins by immunoblot analysis with anti-phosphotyrosine antibody. In VSMC, at least two proteins were significantly phosphorylated in their tyrosine residues 10 min after the addition of leptin. The estimated molecular weights of these proteins were 44- and 42-kDa (Fig. 4). Epidermal growth factor (EGF) also stimulated tyrosine phosphorylation of protein migrating at the same position on SDS-PAGE as leptin-responsive 44- and 42-kDa protein. As the 44- and 42-kDa proteins phosphorylated after treatment with EGF has been shown to be MAP kinases [28], we assessed if these tyrosine phosphorylated proteins in response to leptin were MAP kinases by blotting the same membrane with anti-

MAP kinase antibody. The used monoclonal antibody to MAP kinase can recognize both erk-1 and erk-2 gene products. Activation of MAP kinase requires phosphorylation, which change the electrophoretic mobility. As shown in Fig. 5A, treatment of VSMC for 10 min with 100 ng/ml of leptin caused the immunoreactive MAP kinases to shift to bands with higher molecular sizes (compare lane 1 with lane 4). Further, kinase activities of MAP kinases were assayed by an in gel kinase assay using MBP-containing gels. Leptin stimulated MAP kinase activity by 3-fold at 10 min and declined at 20 min after leptin administration (Fig. 5B).

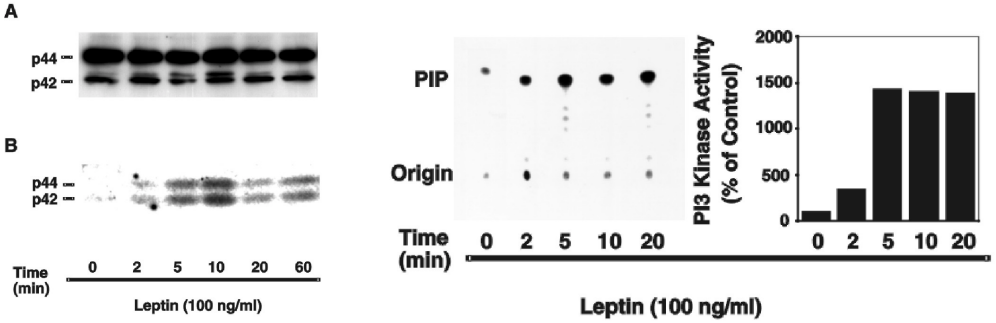


Fig. 5. (Left) Leptin-stimulated phosphorylation and activation of MAP kinases. A, Starved cells were stimulated with leptin (100 ng/ml). Whole cell extracts were analyzed by immunoblotting with anti-MAP kinase antibody. Vascular smooth muscle cells possessed two kind of MAP kinase of different molecular size p44 and p42. When activated, these MAP kinases were phosphorylated and their mobilities reduced on SDS-gel. B, Kinase activities of MAP Kinase were determined by in-gel kinase assays.

Fig. 6. (Right) Leptin-stimulated PI 3-Kinase activity in VSMC. Starved cells were stimulated with leptin (100 ng/ml) for the indicated time. PI 3-kinase activities were measured in anti-phosphotyrosine immunoprecipitates. The origin and the spot corresponding to PI-3-phosphate are indicated.

Next, we measured PI 3-kinase activity in anti-phosphotyrosine antibody immunoprecipitates using phosphatidylinositol as a substrate. Leptin (100 ng/ml) increased the PI 3-kinase activity by 14-fold at 5 min, and this effect was sustained for up to 20 min after stimulation (Fig. 6). To address whether PI 3-kinase was involved in the migratory effect of leptin, we used two structurally unrelated PI 3-kinase inhibitors, wortmannin and LY294002. As shown in Fig. 3, not only wortmannin (100 nM) but also LY294002 (100 μM) completely inhibited VSMC migration with the concentrations of inhibitors which are considered to be specific for PI 3-kinase (Fig. 3)[29, 30]. Moreover, these inhibitors seemed to reduce the basal migratory activity of VSMC, although inhibitory effects were not statistically significant.

DISCUSSION

The present study demonstrates that rat VSMC express functionally active leptin receptor (OB-R) and that leptin has a potential role for VSMC proliferation and migration. Apparent molecular size of this OB-R was 130 kDa. This was confirmed by using two antibodies either against N- or C-terminal region of OB-R. OB-R shows sequence homology to the members of the cytokine receptor superfamily, and at least five different isoforms are generated by alternative mRNA splicing [9], but little is currently known about the signaling capacity and functions of these multiple forms. The long intracellular domain form of OB-R (OB-Rb) is expressed primarily in hypothalamus, but has also been found in peripheral tissues [9]. OB-Rb has a cytoplasmic domain that transduces the leptin signal via JAK/STAT pathway [31, 32]. On the other hand, short form of OB-R (OB-Ra) is abundant in various peripheral tissues

including lung, kidney and liver, as well as choroid plexus [9, 12, 13]. It is hypothesized for short form of OB-R to play a role in the transport of leptin across the blood-CSF or the blood-brain barrier, and might mediate leptin clearance at one or more tissues. The present study is the first observation of expression of functional OB-R in vascular smooth muscle cells. The function of OB-R of VSMC was demonstrated by the induction of VSMC migration and proliferation by leptin. Results indicated that leptin was able to stimulate an increase in cell number, which was observed with increasing leptin concentrations up to 100 ng/ml. Moreover, leptin showed a migratory effect at a concentration of 100 ng/ml. These data suggest the possible involvement of this newly discovered peptide in the regulation of VSMC proliferation and migration. In these experiments, we used ranging from 12.5 ng/ml to 100 ng/ml of recombinant human leptin, it is comparable to the serum leptin levels reported in obese animals and human obese patients [33].

In vasculature, OB-R, whose molecular size is 170-kDa, is expressed in vascular endothelial cells [19, 20]. Leptin increased the number of human umbilical vein endothelial cells in a dose-dependent manner and stimulated angiogenesis via tyrosine kinase-dependent pathway [19, 20]. In this study, tyrosine phosphorylation of VSMC proteins were observed and 44-and 42-kDa phosphorylated proteins were identified as MAP kinases by immunoblotting with monoclonal antibody recognizing both 44-and 42-kDa MAP kinase (ERK1/2) and by in gel kinase assay. Leptin stimulated MAP Kinases activity by 3-fold. Recent data have also shown the proliferative effects of leptin with an activation of MAP kinase in COS cells expressing OB-Rb or OB-Ra, mouse embryonic cells, and pancreatic ß cells [16, 34, 35]. Originally, short form of OB-R was considered to be inactive for signal transduction, because it comprises the conserved box 1 motif without having the box 2 motif. These two motifs have been shown to be required for interaction and activation of tyrosine kinases of JAK family and for receptor signaling function [36]. However, recent report has revealed that short forms of OB-R are capable of mediating signal transduction [26, 34, 37]. A direct link between MAP kinase and proliferation was demonstrated in fibroblasts by expression of a dominant-negative mutant and antisense cDNA [38]. These results suggest the involvement of MAP kinase activation in leptin-induced VSMC proliferation.

It has been suggested that migration of VSMC from media to intima is prerequisite to subsequent VSMC proliferation and intimal thickening in atherosclerosis and after balloon angioplasty. Several growth factors such as PDGF, IGF-1 and NGF are involved in this process [39, 40] and induce VSMC migration via PI 3-kinase pathway [41]. Our results, which PI 3-kinase inhibitors, wortmannin and LY294002 completely inhibited leptin-induced VSMC migration, support this latter finding.

In this study, we present the first evidence for a potential role of the leptin in the regulation of VSMC proliferation and migration. These results imply that leptin may play an important role in vascular lesion formation such as atherosclerosis and neointimal formation after balloon angioplasty in obese state. Approaches designed to inhibit this pathway, therefore, may be useful to prevent or inhibit the vascular lesion formation.

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