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Original article

Leptin stimulates interleukin-6 production *via* janus kinase 2/signal transducer and activator of transcription 3 in rheumatoid synovial fibroblasts

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Leptin stimulates interleukin-6 production

Abstract

Objective: The aim of this study was to determine the influence of leptin on the production of proinflammatory cytokines by rheumatoid synovial fibroblasts (RSFs).

Methods: Synovial tissue was obtained from patients with rheumatoid arthritis (RA). Leptin receptor mRNAs were detected by reverse transcription–polymerase chain reaction (RT-PCR). Productions of mRNA and protein of interleukin (IL)-1β, tumor necrosis factor-α (TNF-α), and IL-6 in the culture medium were detected by real-time PCR and ELISA kit, respectively. Small interfering RNA (siRNA) was transfected into RSF to down-regulate the expression of leptin receptor. Effects of inhibitors of janus kinase 2 (JAK2), phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) on IL-6 production were evaluated. Phosphorylation of signal transducer and activator of transcription 3 (STAT3) in RSF were determined by Western blot analysis.

Results: We detected leptin receptor mRNAs in RSFs. Expression of IL-1β and IL-6 mRNA was enhanced in a concentration-dependent manner by addition of leptin to RSFs. IL-6 secretion by RSFs showed an increase after leptin stimulation. Leptin-induced production of IL-6 by RSFs was decreased after exposure to siRNA targeting leptin receptor (Ob-Rb). A JAK2 inhibitor, but not PI3K and MAPK inhibitors, decreased leptin-induced IL-6 production. Enhanced phosphorylation of STAT3 was observed in RSFs after stimulation by leptin.

Conclusions: Leptin may be one of the proinflammatory cytokines that up-regulates IL-6 production in RSFs *via* activation of JAK2/STAT3. Leptin and JAK/STAT pathway may represent a new alternative therapeutic target in the treatment of RA.

Key words: leptin, rheumatoid arthritis, IL-6, JAK2, STAT3

Introduction

Adipose tissue is a structural component of many organs and a site for energy storage. In addition, recent studies have demonstrated that the major cellular component of adipose tissue, the adipocyte, has the ability to synthesize and release physiologically active molecules, such as leptin, adiponectin, and resistin, as well as cytokines like interleukin (IL)-6 and tumor necrosis factor-α (TNF-α) [1]. These molecules are called adipokines or adipocytokines. Some adipokines may have a central role in the regulation of insulin resistance [2], as well as being involved in many aspects of inflammation and immunity [3]. Leptin is the product of the *ob* gene, and is a 16-kDa nonglycosylated peptide hormone synthesized almost exclusively by adipocytes that regulates appetite and energy expenditure centrally at the hypothalamic level [4]. It is also suggested that leptin may contribute to inflammation and autoimmunity [5]. This is not explained by gene background such as polymorphism of LEP rs2167270 (19 G>A) [6].

Rheumatoid arthritis (RA) is characterized by extensive inflammation and proliferation of the synovium that affects multiple joints. Since proinflammatory cytokines, including TNF-α, IL-1β, and IL-6, play a central role in the pathophysiologic mechanisms of RA, novel methods of neutralizing these cytokines with monoclonal antibodies or soluble receptors have recently been developed as new treatments for this disease [7]. Although blockade of the above-mentioned cytokines is beneficial, it is not curative and the effect is only partial, with many patients failing to respond. Therefore, it seems possible that other

proinflammatory cytokines may also contribute to inflammation in RA. We previously reported that adiponectin (one of the adipokines) stimulates the production of IL-8 [8] and prostaglandin E_2 [9] by rheumatoid synovial fibroblasts (RSFs). We also reported that the serum levels of leptin and adiponectin were elevated in patients with RA [10]. Moreover, leptin levels are increased in synovial fluid of RA patients [11]. These findings suggest that some adipokines may contribute to synovial inflammation in RA. Accordingly, we examined the direct effects of leptin on cultured RSFs in the present study.

Materials and methods

Materials

Recombinant human leptin was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in 15 mM HCl and 7.5 mM NaOH at a pH of approximately 5.2 in accordance with the manufacturer's instructions. Recombinant human IL-1β, recombinant human TNF-α, and recombinant human IL-6 were purchased from R&D Systems, Inc. (Minneapolis, MN) and were dissolved in sterile phosphate-buffered saline (PBS) containing 0.1% (volume/volume) bovine serum albumin to prepare stock solutions. Mouse anti-human IL-6 antibody was obtained from R&D Systems, Inc. Rabbit anti-human signal transducer and activator of transcription (STAT) 3 polyclonal antibody and rabbit anti-human phosphorylated STAT

(p-STAT) 3 (Tyr705) polyclonal antibody were sourced from Cell Signaling Technology (Beverly, MA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). ECL Western blotting detection reagent was purchased from GE Healthcare UK Ltd. (Buckinghamshire, UK), and polyvinylidene difluoride membranes (Immobilon-P) were obtained from Millipore Corp. (Billerica, MA). AG490 (2-cyano-3-(3,4-dihydroxyphenyl)-*N*-(phenylmethyl)-2-propenamide), a janus kinase (JAK) 2 inhibitor came from Merck KGaA (Darmstadt, Germany), while LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), a phosphatidylinositol 3-kinase (PI3K) inhibitor and PD98059 (2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), a mitogen-activated protein kinase (MAPK) inhibitor for extracellular signal-regulated kinase (ERK) were from Sigma-Aldrich. RPMI 1640 medium, penicillin/streptomycin solution, fetal bovine serum (FBS), and 0.25% trypsin/EDTA were sourced from Invitrogen Corp. (Carlsbad, CA). PBS was purchased from Takara Shuzo Co., Ltd. (Shiga, Japan), and all other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Cell culture

RSFs were prepared from synovial tissue as described previously [12]. RA tissue specimens were obtained from patients undergoing total knee replacement who fulfilled the

revised criteria [13] for the classification of RA. The protocol for this study was approved by the Toho University Ethics Committee (approval number: 19021), and all patients gave written consent to the use of their tissue for the research. Synovial tissue was digested for 2 hours with 0.25% (weight/volume) bacterial collagenase (ImmunoBiological Laboratories, Gunma, Japan) and then was suspended in RPMI 1640 medium with 10% (v/v) FBS, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. The cells were incubated at 37°C under 5% CO_2 for several days, after which nonadherent cells were removed. Fibroblast-like adherent cells from the third or fourth passages were used as RSFs at a concentration of 2.5×10^6 cells/75 cm^2 flask.

Reverse transcription– polymerase chain reaction (RT-PCR)

Cells were seeded in culture medium containing 10% (v/v) FBS, and total RNA was extracted with an RNeasy mini kit (Qiagen GmbH., Hilden, Germany) in accordance with the manufacturer's instructions. Reverse transcription was performed with a SuperScript first-strand synthesis system for RT-PCR (Invitrogen Corp.) was employed according to the manufacturer's instructions, using 2 μg of total RNA from the cells as a template. Equal amounts of each reverse-transcribed product were amplified by PCR with HotStar Taq polymerase (Qiagen GmbH). The primer sequences and number of cycles were

5’-TTCAGGTGCGCTGTAAGAGGCT (sense) and

5’-AGGCTCCAAAAGAAGAGGACCACA (antisense) with 38 cycles for Ob-Rb (920 bp),

5’-TCCCATATCTGAGCCCAAAG and 5’-CATCAGGGGCTTCCAAAGTA with 32 cycles for Ob-Re (565 bp), and 5’-CCTCGCCTTTGCCGATCC and 5’-GGATCTTCATGAGGTAGTCAGTC with 28 cycles for β-actin (626 bp). After initial denaturation for 15 minutes at 95°C, PCR involved amplification for a variable number of cycles of 30 seconds at 95°C (β-actin and Ob-Re) or 94°C (Ob-Rb), 30 seconds at 56°C (β-actin) or 59°C (Ob-Re) or 55°C (Ob-Rb), and 45 seconds (β-actin and Ob-Re) or 30 seconds (Ob-Rb) at 72°C, followed by elongation for 5 minutes at 72°C. The amplified complementary DNA (cDNA) fragments were resolved by electrophoresis on 2% (w/v) agarose gel, and were detected under ultraviolet light using an LAS-3000 (Fujifilm Corp. Tokyo, Japan) after the gel was stained with ethidium bromide.

Real-time PCR

To semi-quantitatively evaluate the expression of messenger RNA (mRNA) for IL-6, IL-1β, and TNF-α, real-time PCR was performed using real-time TaqMan technology with a Sequence Detection System model 7000 according to the manufacturer’s recommendations (Applied Biosystems, Foster City, CA). Cells were cultured under various conditions in medium containing 1% (v/v) FBS, after which extraction of total RNA and synthesis of cDNA were performed as described above. Specific probes for IL-6, IL-1β, and TNF-α were obtained from TaqMan Gene Expression Assay (Applied Biosystems), with the ID numbers of the products

being Hs99999032_m1 for IL-6, Hs99999029_m1 for IL-1β and Hs00174128_m1 for TNF-α. The threshold cycle was calculated from a standard curve and expression of the target mRNA was normalized for the expression of β-actin mRNA.

Western blot analysis

Cells were cultured under various conditions at a density of $5\times10^4/cm^2$ in medium containing 1% (v/v) FBS. Subsequently, the cells were lysed in mammalian protein extraction reagent containing Halt™ phosphatase inhibitor cocktail (Pierce Biotechnology, Rockford, IL). The protein content of the lysates was determined with bicinchoninic acid protein assay reagent (Pierce Biotechnology), using bovine serum albumin as the standard. Then cell lysates were adjusted to 10 μg of protein and were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel (10-15% (w/v)) electrophoresis. Next, the proteins were electroblotted onto Immobilon-P poly (vinylidene difluoride) membranes with a semidry blotter (Atto Corp., Tokyo, Japan). After the membranes had been blocked in 10 mM Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20 (TBST) and 5% (w/v) skim milk, the primary antibody (anti-human STAT3 antibody or anti-human phospho-STAT3 antibody) was added at a dilution of 1:1000 in TBST, and incubation was done for 18 hours at 4°C. After the membranes had been washed with TBST, the secondary antibody (HRP-conjugated goat anti-rabbit antibody) was added at a dilution of 1:10,000 in TBST and incubation was performed for 1 hour. After further washing

with TBST, protein bands were detected with an enhanced ECL Western blotting detection reagent (GE Healthcare UK Ltd.) using LAS-3000 (Fujifilm Corp.).

Measurement of cytokines in the culture medium

Cells were seeded in 24-well plastic plates (1×10^5/well) and cultured for 24 hours under various conditions in medium containing 1% (v/v) FBS under an atmosphere of 5% CO_2. Then the concentrations of IL-6, IL-1β, and TNF-α in the medium were measured with an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's recommendations (Invitrogen Corp.). Experiments using RSFs were done in triplicate wells, and the concentrations of IL-6, IL-1β, and TNF-α was measured in triplicate.

RNA interference assay with Ob-Rb

An RNA interference assay was performed to assess the effect on RSFs of down-regulating Ob-Rb expression. Small interfering RNA (siRNA) for Ob-Rb (Stealth™ RNAi) and negative control siRNA were purchased from Invitrogen Corp. For gene knockdown experiments, RSFs were plated in 10 cm plastic dishes (3×10^5/dish) in RPMI 1640 medium with 10% (v/v) FBS and cultured for 18 hours. Then the medium was changed to serum-free RPMI 1640 medium, and the cells were transfected with siRNA (10 pmol/ml) for Ob-Rb or with control siRNA (10 pmol/ml) using Lipofectamine™ RNAiMAX (Invitrogen Corp.) according to the manufacturer's recommendations (Invitrogen Corp.). After 72 hours, the cells were replated

into 35-mm plastic dishes for PCR or into 96-well plastic plates for measurement of IL-6 by ELISA.

Statistical analysis

Data are expressed as the mean ± SEM. Statistical analysis was performed with Prism ver. 5.0 software (Graphpad Software, San Diego, CA). To compare two groups, Student's *t*-test was employed. Groups (≥ 3) were compared by using one-way analysis of variance (ANOVA). One-way ANOVA with Bonferroni's post hoc test was used to determine differences among all the groups. One-way ANOVA with Dunnett's post hoc test was used for comparison to control (no treatment). In all analyses, $P<0.05$ was considered significant.

Results

Effect of leptin on production of inflammatory cytokines by RSFs

To determine whether leptin increased the production of IL-1β, IL-6, and TNF-α by RSFs, real-time PCR was performed. The results showed that leptin significantly increased the expression of IL-1β and IL-6 mRNA by RSFs in a concentration-dependent manner (Figure 1). In contrast, expression of TNF-α mRNA was not increased by leptin (data not shown). To confirm the production of IL-1β and IL-6 proteins, we measured the concentrations of these cytokines in the culture medium of RSFs incubated with leptin (Figure 2). We found that leptin

significantly increased IL-6 production by RSFs in a concentration-dependent manner. In contrast, concentrations of IL-1β and TNF-α were not detectable ever after stimulation with leptin.

Effect of siRNA for leptin receptor on IL-6 production by RSFs

mRNAs for both leptin receptors (Ob-Rb and Ob-Re) were expressed by cells from 3 patients with RA (data not shown). RSFs were transfected with siRNA targeting Ob-Rb (the leptin receptor) or with negative control siRNA, and then expression of Ob-Rb and Ob-Re mRNA was detected by RT-PCR. This showed that Ob-Rb mRNA expression by RSFs was decreased after exposure to the siRNA for Ob-Rb (Figure 3A). When cells were seeded in 96-well plates and incubated with leptin for 18 hours, IL-6 production by RSFs transfected with the siRNA targeting Ob-Rb was significantly lower than that by RSFs transfected with negative control siRNA (Figure 3B).

Effect of leptin on STAT3 phosphorylation in RSFs

We then examined more details of the signal transduction involved in these effects of leptin. To determine whether leptin induced STAT3 phosphorylation in RSFs, Western blotting was performed. This revealed that leptin increased STAT3 phosphorylation in a concentration-dependent manner (Figure 4A). To investigate whether phosphorylation of STAT3 was related to the induction of IL-6 production by leptin, RSFs were incubated with an anti-IL-6

antibody (Figure 4B). Phosphorylation of STAT3 in response to leptin was not inhibited by addition of the anti-IL-6 antibody, but STAT3 phosphorylation in response to IL-6 was inhibited by the antibody.

Effects of signaling pathway inhibitors on leptin-induced IL-6 production by RSFs

We examined the effects of inhibitors of major signaling pathways on leptin-induced IL-6 upregulation in RSFs. As a result, leptin-induced IL-6 production was significantly inhibited by addition of AG490, a JAK2 inhibitor (Figure 5), but not by LY294002, a PI3K inhibitor (Figure 6A) or PD98059, a MAPK inhibitor for ERK (Figure 6B). These findings suggested that leptin induces IL-6 production in RSFs *via* the JAK2/STAT3 pathway.

Discussion

In the present study, we demonstrated that leptin induced the expression of IL-6 mRNA and protein in RSFs *via* the JAK2/STAT3 pathway. This finding is supported by data obtained in leptin-deficient *ob/ob* mice by Busso et al. [14], who reported that leptin-deficient mice were partly protected against antigen-induced arthritis, showing less synovial tissue proliferation and a weaker humoral response to the injected antigen. Moreover, Sugioka et al. [15] reported that acquired leptin resistance by high-fat feeding reduces inflammation from collagen antibody-induced arthritis in mice.

Harigai et al. [16] reported that TNF-α induced IL-6 production by synovial fibroblasts in a dose dependent manner. On the other hand, the present study showed that leptin stimulated IL-6 production. Gonzalez-Gay et al. [17] reported that leptin concentration was not changed by administration of anti-TNF-α-blocker infliximab. Therefore, TNF-α-induced IL-6 production might not be mediated by leptin.

IL-6 is a pleiotropic cytokine that is overexpressed in the synovial tissue of RA patients, who have elevated concentrations of IL-6 in both serum and synovial fluid [18]. IL-6 influences the function of neutrophils, T cells, B cells, monocytes, and osteoclasts. It is a major inducer of the hepatic acute phase response, which is also a key feature of RA that is correlated with disease activity and joint destruction. Thus, IL-6 is thought to play a pivotal role in RA. Tocilizumab is a humanized anti-IL-6 receptor monoclonal antibody that has shown efficacy for treating RA in clinical trials [19]. The average levels of IL-6 in serum and synovial fluid of RA patients were 14 pg/mL and 4 ng/mL, respectively [20]. In our study, 20-283 ng/mL of IL-6 was produced by 25-300 nM of leptin (Figure 2). Thus, our present results suggest a contribution of leptin to the pathogenesis of RA *via* its influence on IL-6.

Although IL-1β mRNA in RSFs was increased by leptin, IL-1β protein was not detectable in culture medium of RSFs. In general, synovial fibroblasts are not the principal sources of inflammasome-mediated IL-1β production in the synovium [21]. This might be one

of the reasons of the discrepancy between changes in mRNA and protein of IL-1β in our study.

Six isoforms of the leptin receptor have been identified [22]. The Ob-Re isoform is a soluble receptor that lacks the transmembrane and cytoplasmic domains, while Ob-Rb is a long form that has an intracellular signaling domain and is thought to be involved in intracellular signaling. In the present study, we found that both Ob-Rb and Ob-Re mRNAs were expressed by RSFs. In addition, the response of IL-6 to leptin was reduced when RSFs were transfected with siRNA targeting Ob-Rb. Therefore, the induction of IL-6 production by leptin was mediated by Ob-Rb.

It is known that JAK/STAT pathway is activated by leptin in several kinds of human cells, that is hepatocellular carcinoma [23], peripheral blood mononuclear cells [24], colorectal adenoma [25]. However, these report have not shown upregulation of IL-6 by leptin. In addition, this is the first report that leptin stimulates IL-6 production *via* JAK2/STAT3 in RSFs. Although leptin has been shown to stimulate IL-6 production in human osteoarthritic cartilage [26], this was mediated by the nuclear factor κB and MAPK pathway rather than the JAK2/STAT3 pathway. Since Migita et al. [27] reported that IL-6 induced acute-phase serum amyloid A genes *via* JAK2/STAT3 activation in RSFs, we determined whether STAT3 phosphorylation was affected by the leptin-induced upregulation of IL-6. Phosphorylation of STAT3 in response to leptin was not inhibited by the anti-IL-6 antibody, suggesting that STAT3 phosphorylation might

be due to a direct effect of leptin on RSFs.

A previous study demonstrated that leptin activated two signaling pathways (PI3K and MAPK) in RSFs and human peripheral blood mononuclear cells [28, 29]. Therefore, we investigated the effect of LY294002 (a PI3K inhibitor) and PD98059 (a MAPK inhibitor for ERK) on RSFs incubated with leptin. As a result, leptin-induced IL-6 production was not mediated by signaling of PI3K and/or MAPK.

The serum leptin level in RA patients was reported to be in the 1-30 nM range [10], so the concentration of leptin used in this study was higher, but it might be possible that leptin stimulates a vicious cycle of inflammation by a paracrine effect in the articular cavity [30]. Further studies will be necessary to confirm the mechanism by which leptin influences RA.

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Figure legends

Figure 1. Effect of leptin on mRNA expressions of inflammatory cytokines by RSFs.

Rheumatoid synovial fibroblasts (RSFs) were incubated with leptin for 6 hours and the expression of interleukin (IL)-1β and IL-6 mRNA by RSFs was assessed by real-time polymerase chain reaction. The results showed that leptin significantly increased the expression of IL-1β and IL-6 mRNA by RSFs in a concentration-dependent manner. Expression of the target mRNA was normalized for the expression of β-actin mRNA, and fold induction was determined relative to expression by cells incubated without leptin. Bars show the mean and SEM (n=3). $^{*}P<0.05$; $^{**}P<0.01$ *vs.* no treatment. Significance was evaluated by one-way analysis of variance with Dunnett's post hoc test.

Figure 2. Effect of leptin on the production of IL-6 by RSFs. Rheumatoid synovial fibroblasts (RSFs) were incubated with leptin at the indicated concentrations for 24 hours and the interleukin (IL)-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin significantly increased IL-6 production by RSFs in a concentration-dependent manner. Bars show the mean and SEM (n=3). *$P<0.05$; **$P<0.01$ *vs.* no treatment. Significance was evaluated by one-way analysis of variance with Dunnett's post hoc test.

Figure 3. Effect of siRNA targeting the leptin receptor on IL-6 production by RSFs. (**A**) Rheumatoid synovial fibroblasts (RSFs) were transfected with small interfering RNA (siRNA)

for Ob-Rb or negative control (NC) siRNA, and Ob-Rb and Ob-Re mRNA levels were analyzed by reverse transcription–polymerase chain reaction. Representative results obtained with fibroblasts from 3 patients are shown. Ob-Rb mRNA expression by RSFs was decreased after exposure to the siRNA for Ob-Rb when compared with exposure for NC siRNA. (**B**) After transfection with siRNA, RSFs were treated with 300 nM leptin or phosphate-buffered saline for 18 hours, and the interleukin (IL)-6 concentration in the culture medium were measured by enzyme-linked immunosorbent assay. IL-6 production by RSFs transfected with the siRNA targeting Ob-Rb was significantly lower than that by RSFs transfected with NC siRNA. Bars show the mean and SEM (n=3). $^{*}P<0.05$; $^{**}P<0.01$. Significance was evaluated by one-way analysis of variance with Bonferroni's post hoc test.

Figure 4. Effect of leptin on phosphorylation of STAT3 in RSFs. (**A**, **B**) Rheumatoid synovial fibroblasts (RSFs) were incubated with leptin, interleukin (IL)-6, or anti-IL-6 antibody (at the indicated concentrations), and then Western blot analysis was performed. Leptin increased signal transducer and activator of transcription (STAT3) phosphorylation in a concentration-dependent manner (A). Phosphorylation of STAT3 in response to leptin was not inhibited by addition of the anti-IL-6 antibody, but STAT3 phosphorylation in response to IL-6 was inhibited by the antibody (B). Representative results obtained with fibroblasts from 3 patients are shown.

Figure 5. Effect of a JAK2 inhibitor on IL-6 production by RSFs. Interleukin (IL)-6 level in culture medium of rheumatoid synovial fibroblasts incubated for 18 hours with/without leptin and AG490. The IL-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin-induced IL-6 production was significantly inhibited by addition of AG490, a janus kinase 2 inhibitor. Bars show the mean and SEM (n=3). $^{*}P<0.05$; $^{**}P<0.01$. Significance was evaluated by one-way analysis of variance with Bonferroni's post hoc test.

Figure 6. Effect of signaling pathway inhibitors on IL-6 production by RSFs. (**A, B**) Interleukin (IL)-6 level in culture medium of rheumatoid synovial fibroblasts incubated for 18 hours with/without leptin, LY294002, and PD98059. The IL-6 concentration in the culture medium was measured by enzyme-linked immunosorbent assay. Leptin-induced IL-6 production was not inhibited by LY294002, a phosphatidylinositol 3-kinase inhibitor (A) or PD98059, a mitogen-activated protein kinase inhibitor for extracellular signal-regulated kinase (B). Bars show the mean and SEM (n=3). $^{*}P<0.05$; $^{**}P<0.01$. Significance was evaluated by one-way analysis of variance with Bonferroni's post hoc test.

Figure 1.
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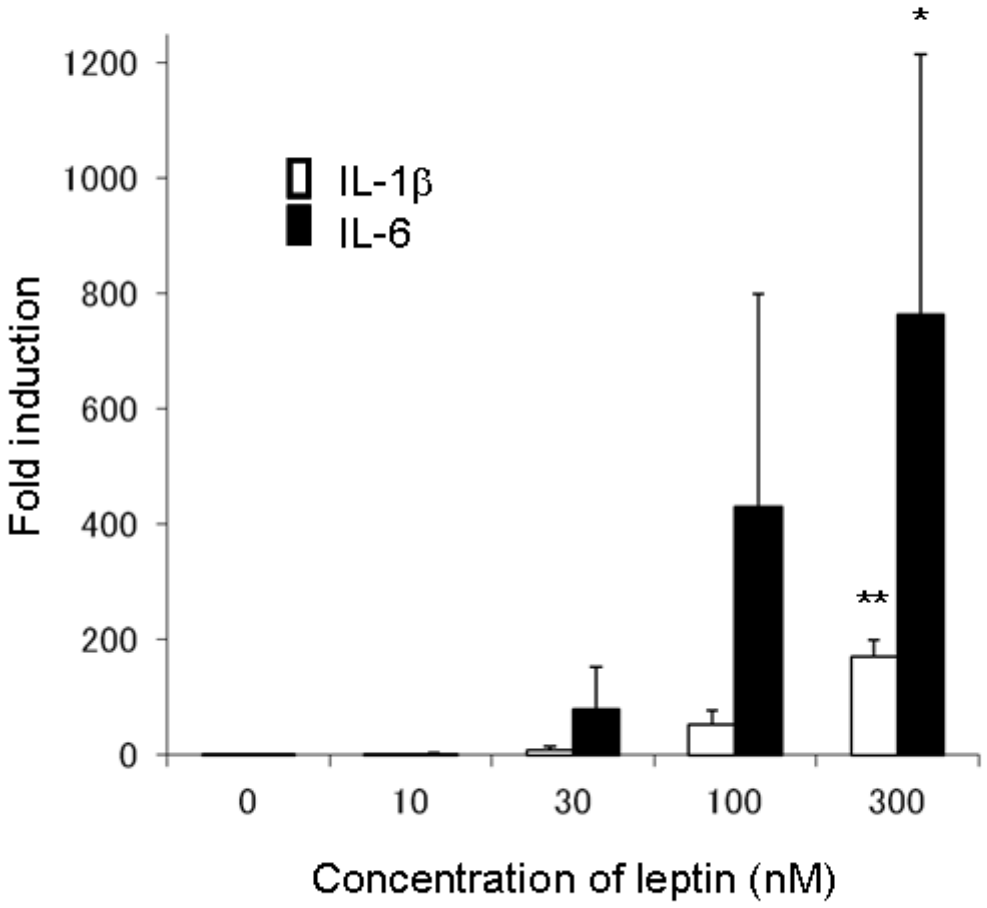

Figure 2.
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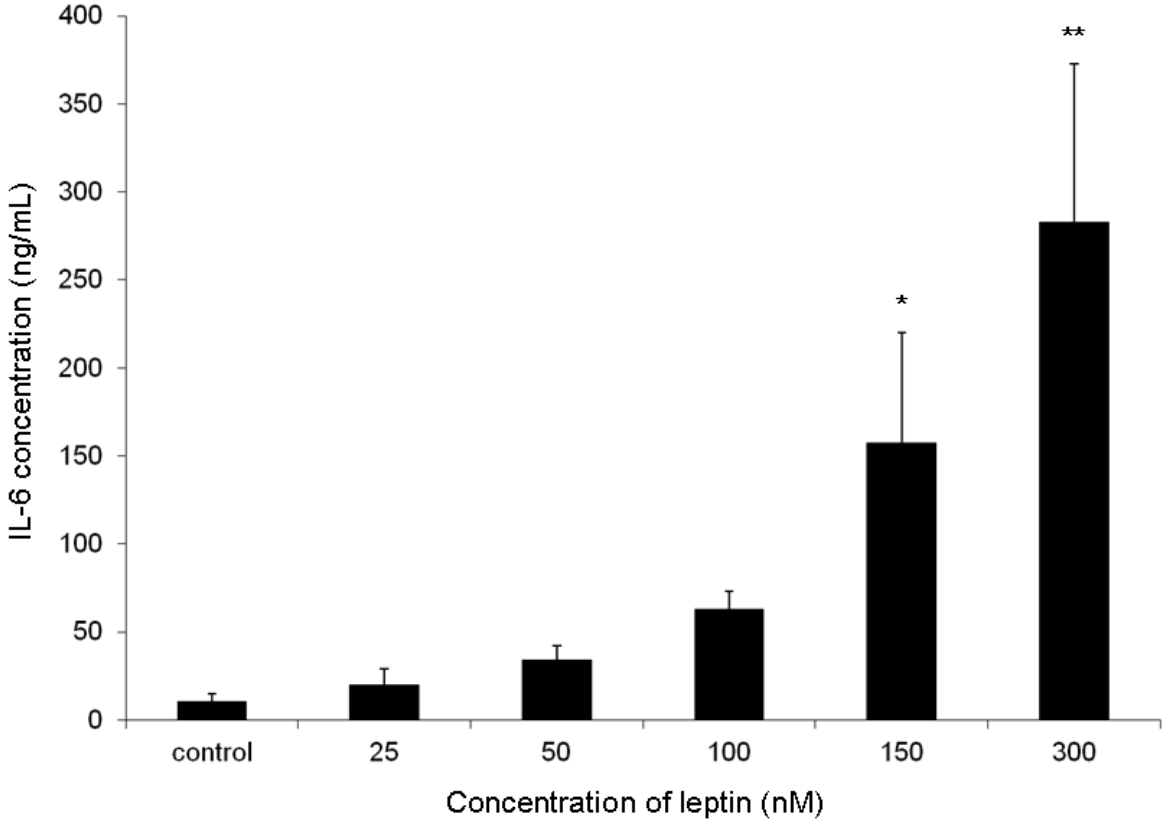

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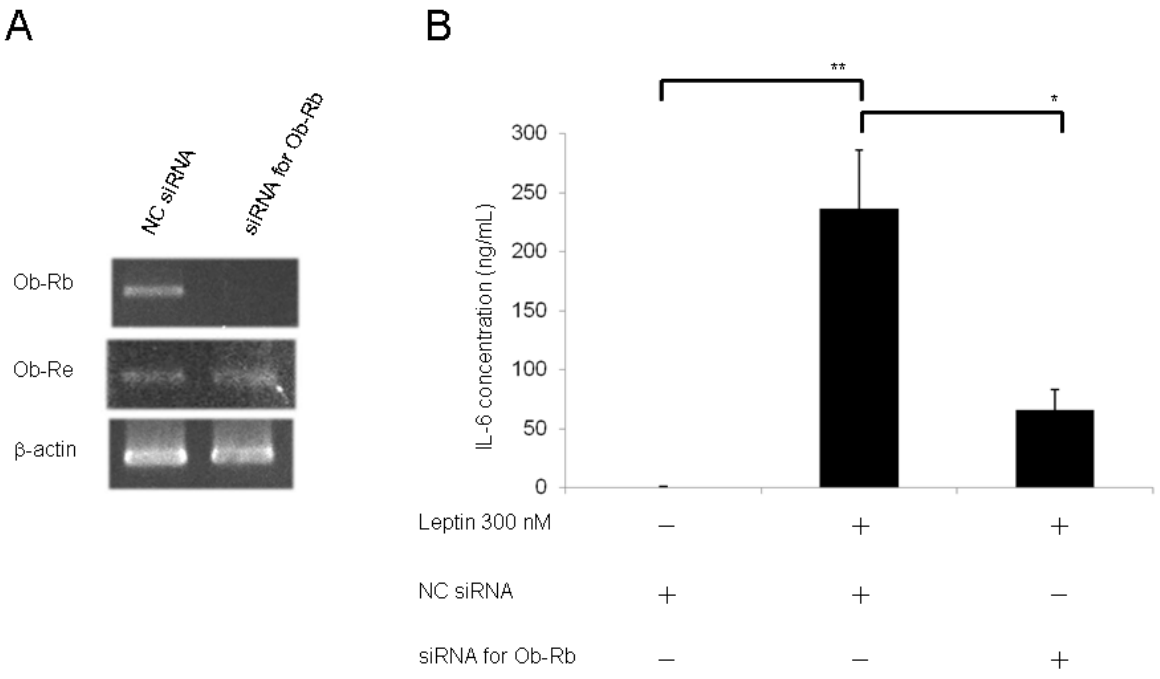

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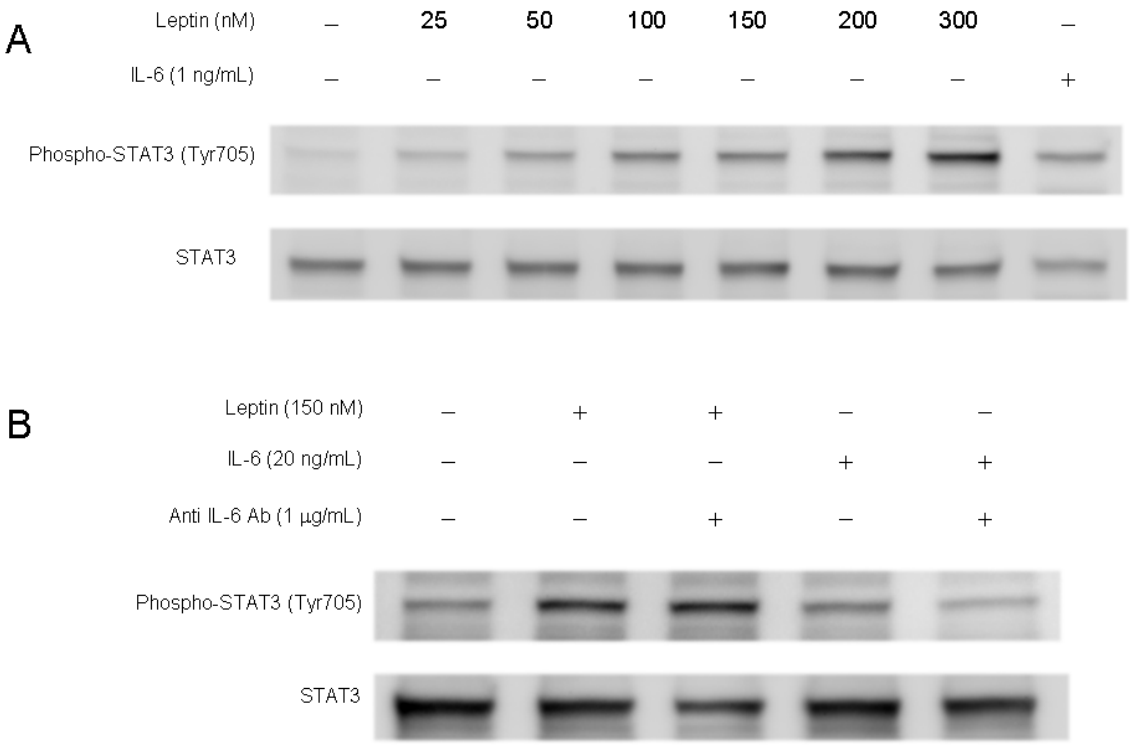

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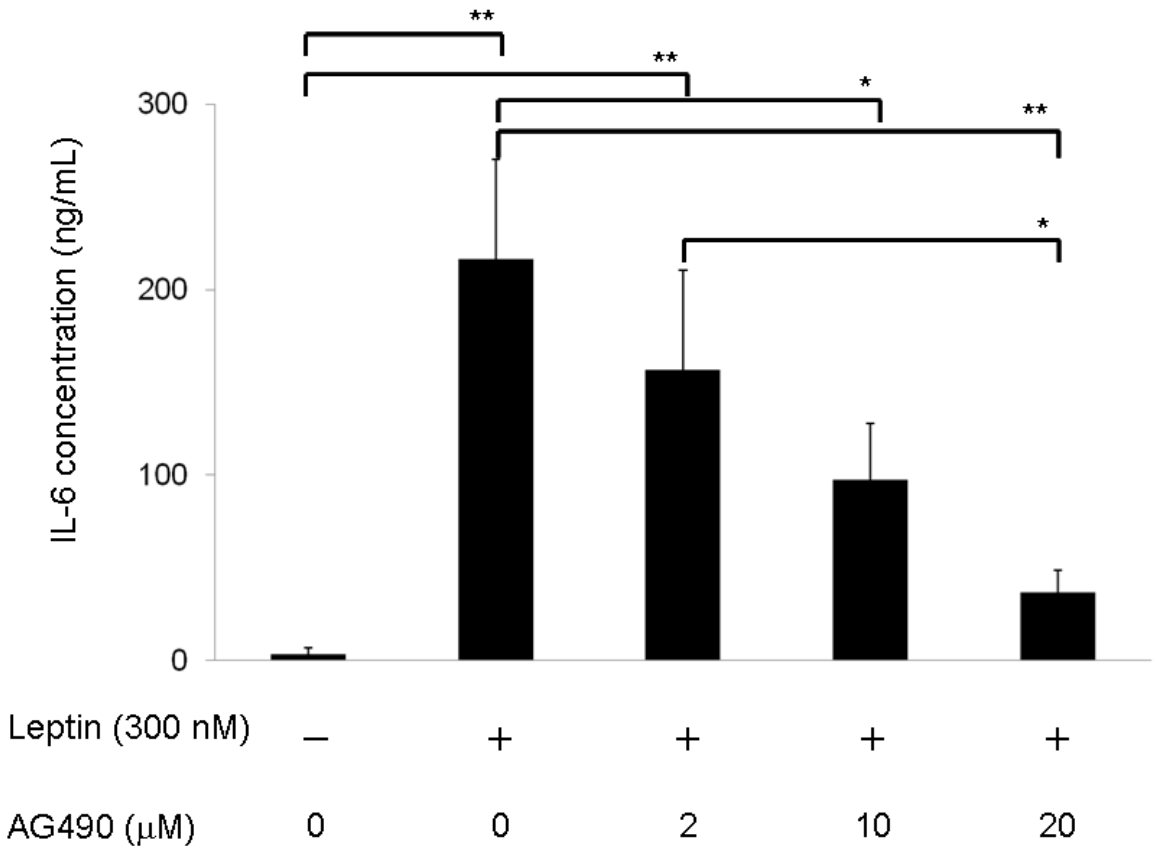

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