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Citation	Journal of Veterinary Medical Science, 69(5), 509-514 https://doi.org/10.1292/jvms.69.509
Issue Date	2007-05
Doc URL	http://hdl.handle.net/2115/27992
Type	article (author version)
File Information	JVMS69-5.pdf



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免疫学 (原著)

Induction of Proinflammatory Cytokines and Caspase-1 by Leptin in Monocyte/Macrophages from Holstein Cows.

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Running title: Leptin induces cytokines and caspase-1

Abbreviations used: Con A, concanavalin A; FBS, fetal bovine serum; IFN, interferon; IL, interleukin; Ob-Rb, long isoform of leptin receptor; PBMC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; TNF- α , tumor necrosis factor- α .

ABSTRACT

The proliferation of peripheral blood mononuclear cells (PBMC) containing both monocyte/macrophages and T lymphocytes increased after treatment with T-cell mitogen (concanavalin A: ConA). PBMC treated with either leptin alone or combination of leptin and ConA showed enhanced proliferative activity by 10-40%, compared with those treated with ConA alone. In contrast, isolated T lymphocytes treated with leptin and ConA showed lowered proliferative activity than the ConA-treated alone, indicating that leptin induced production of some cytokines from monocyte/macrophages, that subsequently resulted in enhancement of T lymphocytes proliferation in PBMC. Among the cytokines examined, monocyte/macrophages constitutively expressed interleukin (IL)-1 β , IL-12p35, IL-18 mRNA, and faintly expressed tumor necrosis factor (TNF)- α and IL-12p40 mRNA. Leptin treatment augmented the monocyte/macrophages mRNA expression of only TNF- α and IL-12p40 to comparable levels of cells treated with lipopolysaccharide (LPS). However, leptin treatment increased monocyte/macrophages production of IL-1 β as well as TNF- α induced the mRNA expression of caspase-1, which is shown to mediate the conversion of latent pro-IL-1 β and pro-IL-18 to active forms. These results suggest that leptin directly acts on monocyte/macrophages to produce factors that induce T lymphocytes proliferation such as IL-12p35/p40 complex through IL-12p40 induction and IL-1 β /IL-18 production through caspase-1 induction.

KEYWORD: caspase-1, IL-1, IL-12, IL-18, leptin.

INTRODUCTION

Leptin is an adipocyte-derived hormone that regulates food intake as well as metabolic and endocrine functions [1, 9]. Leptin also plays regulatory roles in immunity, inflammation, and hematopoiesis [7]. In experimental animal models, defect in leptin production (*ob/ob* mice) or a deletion in the long isoform of the leptin receptor (Ob-Rb; *db/db* mice) causes profound obese phenotype with hyperphagia and impairs immune functions including the reduction of peripheral T and B lymphocytes and of responsiveness of T lymphocytes to mitogen [3, 12]. It is also reported that human congenital leptin deficiency is associated with reduced circulating CD4⁺ T lymphocytes, impaired T cell proliferation and cytokine release, all of which could be reversed by recombinant human leptin administration [8].

Leptin acts on splenocytes from wild-type mouse and T lymphocytes from normal human to enhance alloproliferative mixed-lymphocyte reaction [15] and mitogen-induced proliferation [18]. However, Lord et al. show that leptin enhances anti-CD3-induced proliferation of human naïve T lymphocytes, but inhibits that of human memory T lymphocytes [16]. The apparent difference in responsiveness of T lymphocytes to leptin is due to subpopulation of T lymphocyte and probably also the presence of the other type of cells such as macrophages. Indeed, leptin affects monocyte/macrophage functions as evidenced by the findings that impaired ability of phagocytosis and cytokine production of monocyte/macrophages in *ob/ob* mice could be normalized by exogenous leptin administration [13].

Recently, we have demonstrated that leptin inhibits anti-CD3-induced proliferation of bovine T lymphocytes (Ahmed et al., unpublished results) like in human memory T lymphocytes [15]. However, we wondered whether the responsiveness of bovine T lymphocytes to leptin in the presence of monocyte/macrophages was altered. In the present study, we thus tested the effects of leptin on

proliferative response of bovine peripheral blood mononuclear cells (PBMC) containing both monocyte/macrophages and T lymphocytes, and on cytokine expression and production of monocyte/macrophages. We found that leptin enhanced concanavalin A (ConA)-induced proliferation of PBMC and increased expression of inflammatory cytokine-related genes, including caspase-1 that converts pro-interleukin (IL)-1 β and pro-IL-18 into active forms [11].

MATERIALS AND METHODS

Preparation of peripheral blood mononuclear cells (PBMC) and monocyte/macrophage

fraction: Experimental procedures and care of animals were in accordance with the Guidelines of the Animal Care and Use of Hokkaido University, Japan, and the study was approved by the Committee for the Care and Use of Laboratory Animals in the Graduate School of Veterinary Medicine, Hokkaido University. Six Holstein cows (age 2-4 years) without being milked for more than one year and without having any clinical symptoms were used for multiple blood sampling. Blood was collected from jugular vein into heparinized tubes and PBMC were separated by density gradient centrifugation of blood over Ficoll-Paque PLUS (Amersham Biosciences Co., Piscataway, NJ, USA) as previously described [2, 26]. PBMC were washed twice in phosphate-buffered saline (PBS) containing 1 mM EDTA and resuspended in RPMI1640 medium (Sigma–Aldrich Co., St. Louis, MO, USA) containing 10% fetal bovine serum (FBS, Trace Scientific Ltd, Melbourne, Australia), 100 U/ml penicillin and 100 µg/ml streptomycin, 2.38 mg/ml HEPES, and 110 µg/ml sodium pyruvate. PBMC were further incubated at 37 °C for 2 h on dishes coated with heat-inactivated FBS, and the adherent cells were washed twice with PBS and used as a monocyte/macrophage fraction. The non-adherent cells were passed through a nylon wool column (Wako Pure Chemical, Osaka, Japan), and the pass-through fraction was collected as a T lymphocyte fraction.

Proliferation assay: PBMC and T lymphocytes were cultured in triplicates or quadruplicates at a concentration of 10^4 cells per well of 96-well plates (Nunc, Tokyo, Japan) in a total volume of 200 µl RPMI1640 medium containing 10 % FBS and indicated concentrations of Con A (Sigma) as a mitogen either in the absence or presence of indicated concentrations of human recombinant leptin (Pepro TechEC Ltd., London, UK). After 72 h culture at 37 °C in 5% CO₂ humidified atmosphere, the rates of cell proliferation were assessed by CellTiter-Glo

luminescent viability assay kit (Promega Co., Madison, WI, USA) according to the instruction provided.

Analysis of gene expression: To examine the presence of the Ob-Rb, the predominant isoform of leptin receptor, in bovine leukocytes, total RNA was extracted from PBMC and monocyte/macrophages with TRIzol (Invitrogen, Carlsbad, CA, USA), and 2 µg of RNA were reverse-transcribed with oligo-dT primer and M-MLV reverse transcriptase (Invitrogen). The primers for Ob-Rb were designed based on the sequence reported (GenBank accession number U62385) as shown in Table 1. PCR amplification was conducted for 35 cycles each consisted of denaturation at 94 °C for 30 sec, annealing at 58 °C for 1 min and DNA extension at 72 °C for 1 min. The PCR product was analyzed in 1.5 % agarose gel with ethidium bromide staining. To confirm the amplification of OB-Rb mRNA, the PCR product after electrophoresis was excised, ligated to pGEM-T Easy Vector (Promega), cloned in DH5α competent cells (Invitrogen), and sequenced using an ABI PRISM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan).

To examine the effects of leptin on mRNA expression of inflammatory cytokines in monocyte/macrophages, the cells were cultured in the absence or presence of either increasing concentration of leptin (0.1-10 nM) or lipopolysaccharide (LPS, 10 ng/ml) for 24 h, and cellular total RNA was prepared. The mRNA expression of IL-1β, IL-12p40, IL-12p35, IL-18, tumor necrosis factor (TNF)-α, caspase-1, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were detected by a conventional RT-PCR method using 0.5 µM respective primer sets specific for each gene (Table1). The PCR conditions are also summarized in Table 1. The PCR products were electrophoresed and intensities of bands were analyzed densitometrically using NIH Image program (<http://rsb.info.nih.gov/nih-image/>).

Detection of cytokines: TNF-α and IL-1β in the medium of bovine monocyte/macrophages culture

were determined by bovine TNF- α and IL-1 β ELISA systems using reagents and antibodies provided from Endogen (PIERCE, Rockford, IL, USA). In brief, each well of a 96-well microplate (ReactiBindTM, Endogen) was incubated with 100 μ l of either anti-bovine TNF- α (5 μ g/ml) or IL-1 β (3 μ g/ml) antibodies in PBS overnight at room temperature. All the subsequent reactions were performed at room temperature and the wells were washed three times with 300 μ l of PBS containing 0.05% Tween20 (PBST) before each reaction. The wells were incubated with 300 μ l of 4% BSA and 5% sucrose in PBS for 1h, then, with a 100 μ l of culture medium or respective recombinant proteins in PBS as standard for 1h. Subsequently, the wells were incubated with 100 μ l of biotin-labeled anti-bovine TNF- α (2 μ g/ml) or IL-1 β (2 μ g/ml) antibodies for 1 h, and then a 100 μ l of streptavidin-conjugated horseradish peroxidase solution for 30 min. Finally, the wells were incubated with 100 μ l of tetramethyl benzidine solution for 3-15 min in dark, and the color development was stopped by adding a 100 μ l of 0.18M sulfuric acid. Spectrophotometric measurements were performed at 450nm and 550nm as a reference.

Statistical analysis: Results are expressed as means \pm S.E. Statistical analysis was performed using student's *t*-test or analysis of variance (ANOVA) and Fischer's protected least –significant difference test, with $p < 0.05$ as statistically significant.

RESULTS

To confirm the presence of a long-isoform of leptin receptor (Ob-Rb) in bovine monocyte/macrophages, first, we examined mRNA expression of Ob-Rb by RT-PCR. As shown in Fig. 1, a 288 bp PCR product from Ob-Rb mRNA was expressed in monocyte/macrophages prepared from five different cows as well as bovine brain.

Next we tested whether leptin affects mitogen-induced T lymphocyte proliferation in the presence of monocyte/macrophages, PBMC were treated with increasing concentration of Con A, a mitogen for T lymphocytes. As shown in Fig. 2A, PBMC showed enhanced proliferative activity in response to ConA dose-dependently. PBMC treated with leptin alone also increased the proliferation marginally but significantly by 20% (Fig. 2B, see columns with Con A 0 $\mu\text{g/ml}$). PBMC treated simultaneously with leptin and ConA showed augmented proliferation by 10-40% compared with that of ConA alone (Fig.2 B).

To examine factors that enhance T lymphocyte proliferation in PBMC in response to leptin, mRNA expression of four cytokines in monocyte/macrophages were examined. Among these, monocyte/macrophages constitutively expressed IL-1 β , IL-12p35, and IL-18 mRNA, but faintly expressed TNF- α and IL-12p40 mRNA (Fig. 3). Monocyte/macrophages treated with leptin significantly showed increased mRNA expression of TNF- α (1.75-fold of control at 10 nM, $p < 0.05$ vs. control) and IL-12p40 (2.19-fold of control at 10 nM, $p < 0.05$ vs. control) to comparable levels with the cells treated with LPS. In contrast, monocyte/macrophages treated with leptin failed to show increased expression of constitutively expressed IL-1 β , IL-12p35, and IL-18 mRNA (Fig.3).

To confirm the enhancement of cytokine production by leptin, concentrations of TNF- α $\square\square\square$ IL-1 β in the culture medium were measured by respective bovine ELISA systems. Treatment with leptin significantly increased TNF- α in the medium by 55 %, and coincidentally IL-1 β by 79%

(Fig.4). We, therefore, examined the mRNA expression of caspase-1, which is shown to activate IL-1 β and IL-18 by proteolysis [11]. As shown in Fig. 5, leptin treatment dramatically induced expression of caspase-1 mRNA in monocyte/macrophages.

DISCUSSION

In the present study, we demonstrated that leptin alone dose-dependently induced PBMC proliferation and enhanced ConA-induced proliferation (Fig. 2B). Conversely, it is reported that leptin inhibits anti-CD3-induced proliferation and lowers IL-2 production of human memory T lymphocytes [16] as well as of bovine purified T lymphocytes (Ahmed et al., manuscript in submission). Therefore, the leptin-induced PBMC proliferation might be explained by the fact that leptin stimulates proliferation of monocyte/macrophages [14, 22]. However, it is unlikely that the major proliferating cells in bovine PBMC are monocyte/macrophages in the presence of both T-cell mitogen and leptin, since leptin enhances proliferation of T lymphocytes in human PBMC in the presence of ConA [18]. Therefore, it is presumably that in PBMC, leptin activates monocyte/macrophages to produce some cytokines that stimulate proliferation of T lymphocytes, resulting in overcoming the direct suppressive effect of leptin on T lymphocytes.

In the present study, we tested the effect of leptin on mRNA expression of four kinds of cytokines that could enhance bovine PBMC proliferation either in the presence or absence of ConA [4, 5, 24, 27]. Among these, TNF- α mRNA was faintly expressed in monocyte/macrophages from Holstein cows and induced by leptin treatment, accompanying increase of TNF- α in the culture medium. This is in accord with the previous findings that leptin enhances the secretion of the proinflammatory TNF- α and IL-12 by mouse peritoneal macrophages [14, 16]. The biologically active form of IL-12 is a heterodimeric molecule (p70) composed of two subunits of 40 kDa (p40) and 35 kDa (p35) [20]. Here, we showed that IL-12p35 and IL-12p40 mRNA were highly and faintly expressed, respectively, and that only IL-12p40 mRNA was induced by leptin treatment. It is, thus, likely that induction of IL-12p40 facilitates a formation and production of heterodimeric IL-12p35 and IL-12p40 complex, although we did not measure IL-12 production due to limitation of bovine

IL-12 assay system.

Unlike TNF- α and IL-12 transcriptional induction, IL-1 β and IL-18 mRNA were highly expressed in bovine monocyte/macrophages and were not induced by leptin treatment. It is well described that IL-1 β is initially synthesized as an inactive 31- to 33-kDa precursor protein (pro-IL-1 β). Similarly, IL-18 is synthesized as a 24 kDa precursor (pro-IL-18) lacking a signal peptide. Both are structurally related, and require an intracellular cysteine protease caspase-1, formerly termed IL-1 β -converting enzyme to generate the mature secretory proteins [11]. Importantly, this study showed that the mRNA of bovine caspase-1 that we cloned (Genbank accession number AB285205) was induced by leptin treatment, and consequently increased IL-1 β in the culture medium. It is intriguing to note that leptin induces IL-1 β immunoreactivity in the hypothalamus that is suggested to be required for leptin-induced suppression of food intake [17]. However, in THP-1 human monocytic cells, leptin even at high doses fails to stimulate the production of IL-1 β [10], but this discrepancy might result from the cell conditions used; that is, if THP-1 cells were not differentiated into macrophages, even LPS failed to induce IL-1 β [10]. However, if the cells were differentiated into macrophages by dihydroxy-vitamin D3 for three days, LPS could induce IL-1 β production [23]. In the present study, monocyte/macrophages were isolated from PBMC by adherence on FBS-coated dishes, a process that could have primed the cells and enable the responsiveness to leptin induction of caspase-1 and production of IL-1 β .

The results described above also strongly suggest that IL-18 is released from bovine monocyte/macrophages by the aid of caspase-1 activity, although IL-18 production were not measured, because of unavailability of bovine IL-18 assay. It is noteworthy that leptin-deficient *ob/ob* mice are unresponsive to Con A, resulting from defect in IL-18 and TNF- α production [6]. In addition, IL-18 acts synergistically with IL-12 to enhance IL-12-driven T helper 1 (Th1) cell

differentiation resulting in stimulation of IFN- γ production [21, 25]. Isolated T lymphocytes treated with leptin are shown to shift a profile of cytokine production toward Th1 phenotype by stimulating the synthesis of IFN- γ [15, 18, 19]. Taken together with these findings, it is suggested that leptin modifies the T lymphocyte functions directly and indirectly through the production of IL-12 and IL-18 from monocyte/macrophages. Moreover, we have recently demonstrated that TNF- α , but not leptin, enhances superoxide production from bovine neutrophils [2]. Therefore, it is also likely that leptin modulates neutrophil functions indirectly through the production of TNF- α from monocyte/macrophages, as observed previously [28].

In summary, we clearly demonstrated that the stimulatory effect of leptin on mitogenic response of bovine PBMC. We also demonstrated that leptin increased expression of genes including IL-12 and caspase-1, the latter converts pro-IL-1 β and pro-IL-18 into active forms.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid of the investigation on the etiology and prevention of bovine mastitis (No. 2210) by the Ministry of Agriculture, Forestry and Fisheries of Japan, and by grants from the Ministry of Education, Science and Culture of Japan and the Japan Society for the Promotion of Science (JSPS). The work was also supported in part by JSPS postdoctoral fellowships for foreign researchers to M.S. and K.M. and for young scientist to D.Y. and Y. O.-O.

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Table 1. PCR primers

Ob-Rb (U62385, 288bp) [58 °C, 1 min, 35 cycles]

Forward primer: 5'-GCCAGCAACT-ACAGATGCTCTAC-3'

Reverse primer: 5'- TCCCATGATCTCTTAGAGGAAG -3'

IL-1 β (M35589, 726bp) [60 °C, 1 min, 30 cycles]

Forward primer: 5'-ATGGCAACCGTACCTGAACCCA -3'

Reverse primer: 5'-GCTCGAAAATGTCCCAGGAA -3'

IL-12p40 (U11815, 415bp) [55 °C, 1 min, 30 cycles]

Forward primer: 5'-GCAGTACACCTGTCACAAAG-3'

Reverse primer: 5'-CTACCACGACCTCAATAAGC-3'

IL-12p35 (U14416, 444bp) [59°C, 1 min, 30 cycles]

Forward primer: 5'-CGACTACTCCCAAACCTGCT -3'

Reverse primer: 5'-AGTTCTTTCAGGGAGGGTTTCT -3'

IL-18 (NM-174091, 216bp) [56 °C, 1 min, 30 cycles]

Forward primer: 5'-AGATAATGCACCCCAGACCA-3'

Reverse primer: 5'-ATCATCATGTCCTGGAACAC-3'

TNF- α (AF348421, 467bp) [60 °C, 1 min, 30 cycles]

Forward primer: 5'-ACTCAGGTCCTCTTCTCAAGCC-3'

Reverse primer: 5'-ATGATCCCAAAGTAGACCTGCC-3'

Caspase-1 (AB285205, 327bp) [60 °C, 1 min, 30 cycles]

Forward primer: 5'-AATAAATGGCTTGCTGGATGAG -3'

Reverse primer: 5'- CCTCCTGGTCCTGAAGATGC-3'

G3PDH (U85042, 452bp) [59 °C, 30 sec, 25 cycles]

Forward primer: 5'-ACCACTGTCCACGCCATCAC-3'

Reverse primer: 5'-TCCACCACCTGTTTGCTGTA-3'

In the parenthesis, GenBank accession number of bovine genes and size of PCR product are shown. In the square brackets, annealing temperature and time, and number of PCR cycle of respective genes are shown, while temperature and time of denaturation and elongation steps of each PCR cycle are 94 °C, 30 sec and 72 °C, 60 sec, respectively.

FIGURE LEGENDS

Fig. 1. Expression of Ob-Rb in bovine monocyte/macrophages.

Total RNA was prepared from monocyte/macrophages of 5 different cows (lane 1-5) and mRNA expression of Ob-Rb was analyzed by RT-PCR. The expected size of PCR product was 288bp. Lane 6, negative control (PCR was performed using total RNA from bovine brain without RT reaction) ; lane 7, RNA from bovine brain.

Fig. 2. Effect of leptin on Con A-induced PBMC proliferation.

PBMC were cultured in triplicates or quadruplicates in 96-well plates at 10^4 cells per well with increasing concentrations of Con A either in the absence (A) or presence (B) of leptin for 72 h. Cell proliferation was assessed as described in Materials and Methods section and expressed as means \pm SE of 12 independent experiments using 6 different cows. * $p < 0.05$ vs. no Con A treatment (A) and no leptin treatment (B).

Fig. 3. Effect of leptin on mRNA expression of cytokines in bovine monocyte/macrophages.

Monocyte/macrophages were cultured in the absence or presence of either leptin (0.1-10 nM) or LPS (10 ng/ml) for 24 h. Total RNA was isolated and the mRNA expression of TNF- α , IL-1 β , IL-12p35, IL-12p40, IL-18 and G3PDH were analyzed by RT-PCR. Shown are representative results of 5 independent experiments.

Fig. 4. Effect of leptin on cytokine production in bovine monocyte/macrophages.

Monocyte/macrophages were cultured in the absence or presence of leptin (10 nM) for 24 h. The concentration of TNF- α and IL-1 β were determined by respective ELISA systems. Results of 5 samples are expressed as relative to control. * $p < 0.05$ vs. control (no treatment).

Fig. 5. Effect of leptin on mRNA expression of caspase-1 in bovine monocyte/macrophages.

Monocyte/macrophages were cultured in the absence or presence of either leptin (0.1-10 nM) or LPS

(10 ng/ml) for 24 h. C is control without any stimulation. Total RNA was isolated and the mRNA expressions of caspase-1 and G3PDH were analyzed by RT-PCR. Shown in A are representative results of 5 independent experiments.

Fig. 1

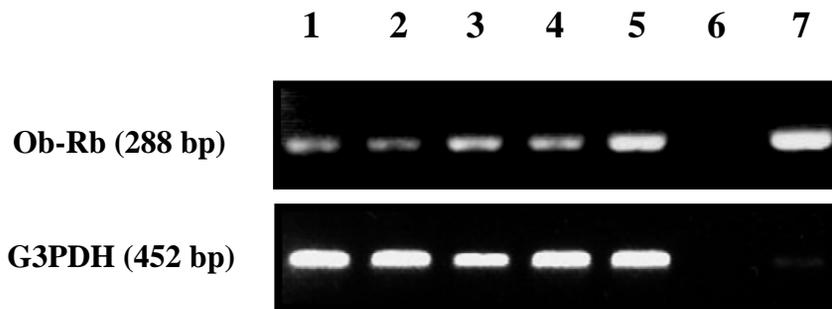


Fig 2

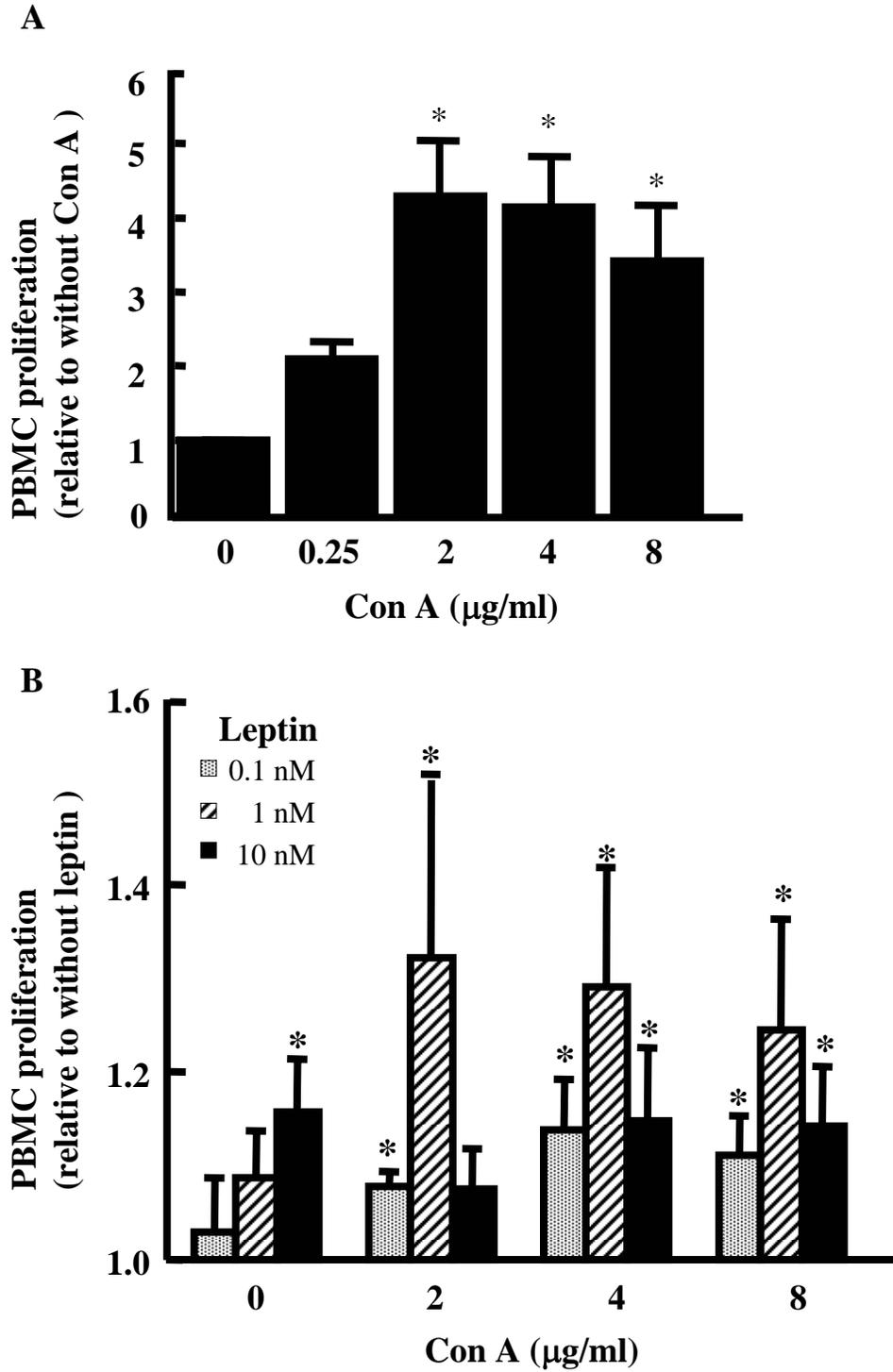


Fig. 3

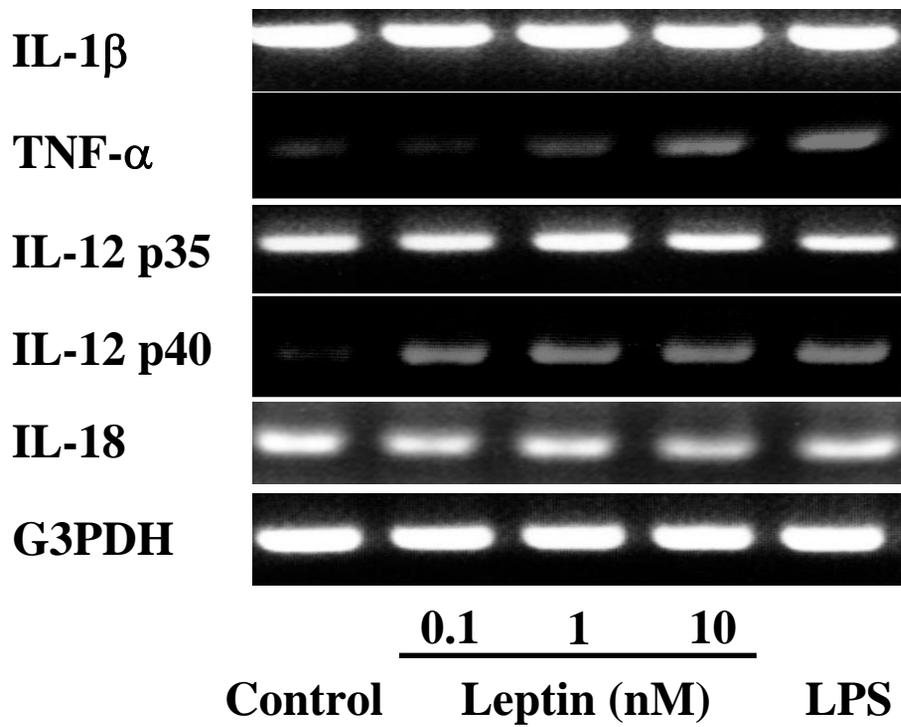


Fig. 4

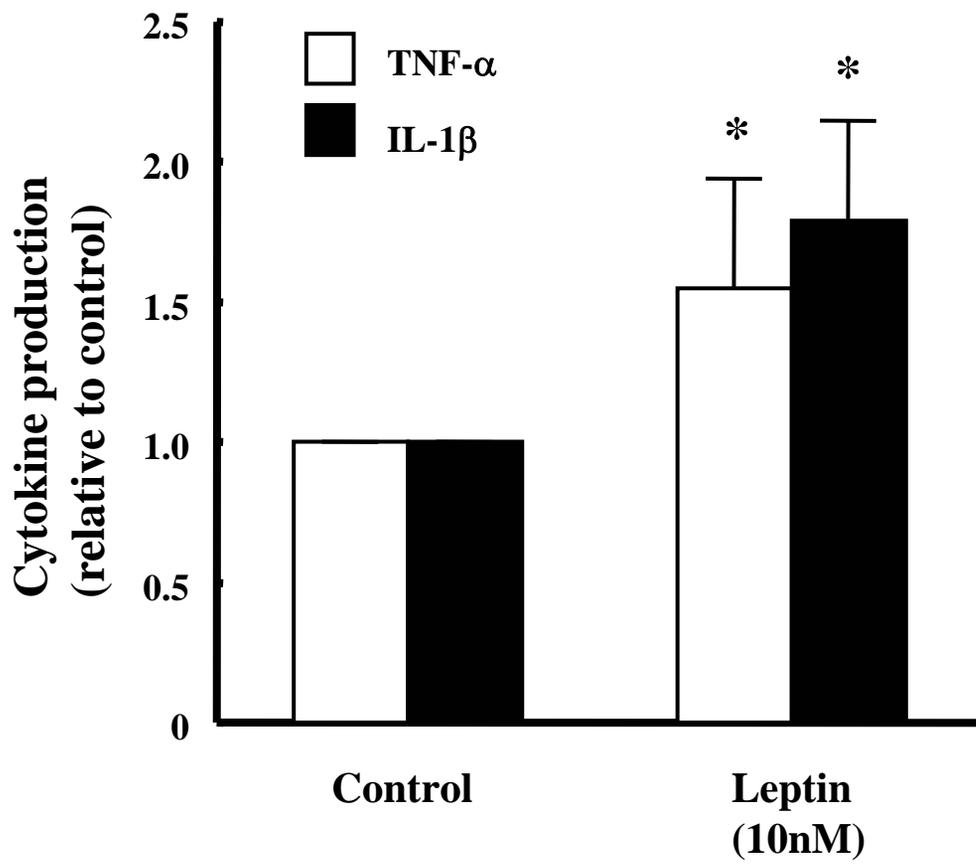


Fig. 5

