J. Microbiol. Biotechnol. (2004), 14(4), 722–729

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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# Effect of Leptin on the Expression of Lipopolysaccharide-Induced Chemokine KC mRNA in the Mouse Peritoneal Macrophages

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Received: September 8, 2003
Accepted: February 24, 2004

**Abstract** Leptin is an adipocyte-secreted hormone and its plasma levels correlate with total body fat mass, however, it also plays a regulatory role in immunity, inflammation, and hematopoiesis. Chemokine is known as a chemoattractant cytokine in inflammatory reaction, but its role in leptin reaction has not been well studied. In this study, the direct effect of leptin on the expression of chemokine mRNAs and lipopolysaccharide (LPS)-induced chemokine KC mRNA in mouse peritoneal macrophages was investigated. Leptin did not induce the expression of lymphotactin, RANTES, eotaxin, MIP-1β, MIP-1α, MIP-2, MCP-1, IP-10, TCA-3, and KC mRNA in mouse peritoneal macrophages, and had no direct effect on the expression of these LPS-induced chemokine mRNAs except KC mRNA. The synergistic effect of leptin on the expression of LPS-induced KC mRNA occurred late in the time course of response to LPS. The increased expressions of Ob-Rb mRNA and leptin receptor protein were detected during the LPS treatment. Leptin produced a substantial increase in the stability of the LPS-induced KC mRNA, and the synergistic effect of leptin on LPS-induced KC mRNA expression was further augmented by cycloheximide (CHX). Pyrrolidine dithiocarbamate (PDTC) did not block the synergistic effect of leptin on LPS-induced KC mRNA expression in mouse peritoneal macrophages. These data suggest that although leptin has no direct effect on the expression of lymphotactin, RANTES, eotaxin, MIP-1β, MIP-1α, MIP-2, MCP-1, IP-10, TCA-3, and KC mRNA in mouse peritoneal macrophages, the synergistic effect of leptin on the expression of LPS-induced KC mRNA has the possibility that LPS might induce the expression of the Ob-Rb receptor or an unknown gene(s) that sensitizes macrophages to the synergistic function of leptin. Therefore, further studies are necessary to examine leptin as a regulatory factor of chemokine production.

**Key words:** Leptin, lipopolysaccharide, chemokine, KC

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Leptin, a 16 kDa protein, is an adipocyte-secreted hormone encoded by the *ob* gene [31] and its plasma levels correlate with total body fat mass. Leptin has multiple biological actions on food intake, energy homeostasis, reproduction, and neuroendocrine axis, and also plays a regulatory role in immunity, inflammation, and hematopoiesis [5].

Leptin signals through the leptin receptor (Ob-R), which is a member of the class 1 cytokine receptor family [26, 29]. The Ob-R is widely distributed in many tissues and expresses on human circulating leukocytes, predominantly on monocytes [6]. Ob-Rb (long isoform) is known to be involved in the activation of JAK-STAT tyrosine kinases in the signaling pathways of several proinflammatory cytokines and the direct effects of leptin to Ob-R have been demonstrated in a number of studies [9, 19, 27]. Leptin has been shown to increase proinflammatory immune response by having an effect on peripheral blood mononuclear cells (PBMC) and macrophages [5, 10, 18]. Leptin with high dose induces inflammatory cytokines production from human PBMCs [8] and it modulates helper T lymphocyte activation toward Th1 phenotype by stimulating the synthesis of IL-2 and IFN-γ [17]. Furthermore, leptin induces the production of GM-CSF and G-CSF in murine peritoneal macrophages, and monocyte chemoattractant protein-1 (MCP-1) in human umbilical vein endothelial cells [2]. These studies provide evidence that leptin has a direct effect on the production of many cytokines and has a potential role as a mediator in the cytokine network [16].

Although leptin has been shown to induce the production of various proinflammatory cytokines, the direct effect of leptin on the expression of chemokine genes has not been well elucidated. In this study, the direct effect of leptin on the expression of chemokine mRNAs in mouse peritoneal macrophages was investigated. The results demonstrated that leptin does not induce expression of lymphotactin, RANTES, eotaxin, MIP-1β, MIP-1α, MIP-2, MCP-1, TCA-3, IP-10, and KC mRNA, and has no direct effect on the expression of these LPS-induced chemokine mRNAs

except KC mRNA in mouse peritoneal macrophages. However, leptin has a synergistic effect on the expression of LPS-induced KC mRNA.

## MATERIALS AND METHODS

### Reagents

Mouse recombinant leptin was purchased from R&D systems (Minneapolis, MN, U.S.A.). RiboQuant, Multi-probe Rnase protection assay system and mCK-5b as template set were purchased from Pharmingen (San Diego, CA, U.S.A.). RNAzol reagent for total RNA isolation was from AMS Biotechology (Abingdon, Oxon, U.K.), DMEM (Dulbecco's Modified Eagle Medium, Life Technologies Gibco BRL), RPMI 1640, Dulbecco's phosphate-buffered saline (PBS), Hank's balanced salt solution (HBSS), L-glutamin, trypsin, agarose, fetal bovine serum (FBS), phenol, and formamide were obtained from Gibco/BRL (Life Technologies, Gaithersburg, MD, U.S.A.). The Magna nylon transfer membrane was purchased from Micron Separation Inc. (Westboro, KS, U.S.A.). The high prime kit was purchased from Boehringer Mannheim (Indianapolis, ID, U.S.A.), [$\alpha$-$^{32}$P]dCTP and [$\alpha$-$^{32}$P]UTP were obtained from Dupont-New England Nuclear (Boston, MA, U.S.A.). Trihydroxymethyl aminomethane (Tris), sodium dodecyl sulfate (SDS), and *E. coli* lipopolysaccharide (LPS, O111:B4) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Mouse leptin receptor antibody for immunocytochemistry was purchased from R&D system (Abingdon, Oxon, U.K.), and secondary biotinylated antigoat antibody was purchased from Vector Biolabs Inc. (Burlingame, CA, U.S.A.). The plasmids encoding KC, IP-10, and the GAPDH genes were kindly provided by Dr. Hamilton at the Department of Immunology, Lehner Research Institute, Cleveland Clinic Foundation, U.S.A. All other reagents were commercial preparations of pure grade.

### Mice

Specific pathogen free, inbred C57BL/6 mice, 8 to 12 weeks of age, were purchased from Hyeuchang Science (Daegu, Korea). The utmost precautions were taken to ensure that the mice remained free from infection by environmental pathogens, thereby ensuring that the degree of spontaneous activation of tissue macrophages would be minimal.

### Preparation of Cell Lines and Mouse Peritoneal Macrophages

Raw264.7 cells and 3T3-L1 adipocytes obtained from Korean Cell Line Bank (KCLB, Korea) were seeded ($10^6$/well) on plastic dishes in complete medium (DMEM supplemented with penicillin, streptomycin, and 10% FBS). After seeding overnight, the medium was replaced by serum-starved DMEM supplemented with 2.5% FBS, and then cultured in the presence or absence of leptin (1 μg/ml) for 4 h or indicated times.

Thioglycollate (TG)-elicited peritoneal macrophages were obtained by the previously described method [14]. Briefly, macrophages in a complete medium (RPMI 1640 supplemented with penicillin, streptomycin, and 10% FBS) were plated in 60-mm tissue culture dishes, incubated for 2 h at 37°C in an atmosphere of 5% $CO_2$, and then washed three times with HBSS to remove any non-adhering cells. The macrophages were cultured overnight in a complete medium at 37°C in 5% $CO_2$. After overnight, the medium was replaced by serum-starved RPMI 1640 supplemented with 2.5% FBS and then cultured in the presence or absence of stimuli for the indicated times.

### Preparation of RNA and Northern Hybridization Analysis

Total RNA was extracted using RNAzol solution according to the manufacturer's instructions. This is a single-step method to remove protein and DNA from the RNA based on the guanidiniumthiocyanate-phenol-chloroform extract procedure. The quantity of the RNA obtained was checked by measuring optical density (OD) at 260 and 280 nm.

For Northern blot analysis, an equal amount of RNA (7 μg/sample) was loaded in each lane of the gel. The RNA was denatured, separated by electrophoresis in a 1% agarose/2.2 M formaldehyde gel, and transferred to a nylon membrane as previously described [10]. The blots were prehybridized for 6 h at 42°C in 50% formamide, 1% SDS, 5× saline sodium citrate (SSC), 1× Denhardt's solution (0.02% bovine serum albumin and 0.02% polyvinylpyrrolidone), 0.25 mg/ml denatured herring testis DNA, and 50 mM of sodium phosphate buffer, pH 6.5. Hybridization was carried out at 42°C for 18 h with $1\times10^7$ cpm of denatured plasmid DNA containing KC, IP-10, and GAPDH cDNA inserts. The blots were rinsed with a solution of 0.1% SDS-0.2×SSC, washed at 42°C for 30 min and at 65°C for 15 min. The blots were then dried and exposed using XAR-5 X-ray film (Eastman Kodak Co. Rochester, NY, U.S.A.) at -70°C.

To prepare the Ob-Rb probe, cDNA was obtained from RNA isolated as described above by the reverse transcriptase-PCR method using the following primer pairs; Ob-Rb sense: 5'-gtgtgagcatctctcctggag-3', antisense: 5'-accacaccagaccctgaaag-3'.

### Ribonuclease Protection Assay (RPA)

RPA for 8 chemokines (lymphotactin, RANTES, eotaxin, MIP-1β, MIP-1α, MIP-2, MCP-1, and TCA-3) were performed according to the instruction of Multi-probe RNase protection assay system using RiboQuant. Briefly, the mCK-5b was used to obtain radiolabeled antisense

RNA probes. *In vitro* transcription was carried out by incubation in a buffer containing 10 mM ATP, 10 mM CTP, 10 mM GTP, 250 μCi α-[$^{32}$P]UTP, and T7 RNA polymerase in transcription buffer. The mixture was incubated at 37°C for 60 min and then treated with DNase I at 37°C for 30 min. After that, the mixture was extracted with a mixture of phenol and chloroform. Extracted RNA was precipitated with ethanol and collected by centrifugation at 4°C, and was then resuspended in 50 μl hybridization buffer and diluted to $3\times10^5$ cpm/μl. Two μl of the resalting solution was used for the reaction. The PBMC RNA samples (10 μg RNA/sample) were dried in a vacuum evaporator and resuspended in 8 μl hybridization buffer. The RNA was annealed to the probe by incubating successively at 95°C for 3 min and at 56°C overnight in a total volume of 10 μl. RNase was added to each sample for removal of single-stranded RNA at 30°C for 45 min, and then the protected RNA duplexes were purified by phenol/chloroform extraction and ethanol precipitation. After the pelleted RNA was resuspended in 5–6 μl of gel loading buffer, and incubated at 95°C for 3 min, RNA was then quickly quenched on ice and analyzed by electrophoresis on 5% polyacrylamide/8 M urea gels. The gel was adsorbed to filter paper, dried under vacuum, and exposed on film (XAR; Kodak, Rochester, NY, U.S.A.) with intensifying screens at -70°C.

### Immunocytochemistry

Macrophages over coverglass were fixed in 4% paraformaldehyde for 5 min at room temperature. Covers were placed in 0.3% hydrogen peroxide in methanol for 20 min and nonspecific activity was blocked by horse serum for 20 min. Primary mouse leptin receptor antibody was incubated at room temperature for 30 min at 10 μg/ml concentration. Secondary biotinylated antigoat antibody was applied and incubated for 30 min at 5 μg/ml concentration. They were then further incubated for 30 min with avidin-biotin complex. The subsequent chromogen 3,3-diaminobenzidine was applied for 10 min. The cells were counterstained with hematoxylin after preincubation in 50 units/mg RNase for 20 min and then dehydrated and mounted in balsam.

## RESULTS

### Effect of Leptin on the Expression of Chemokine mRNAs in Mouse Peritoneal Macrophages, RAW264.7, and 3T3-L1 Adipocytes

After the thioglycollated (TG)-elicited peritoneal macrophages, RAW264.7 cells, and 3T3-L1 adipocytes were stimulated with leptin (1 μg/ml) for 4 h, RPA was performed. As shown in Fig. 1A, leptin has no effect on the expression

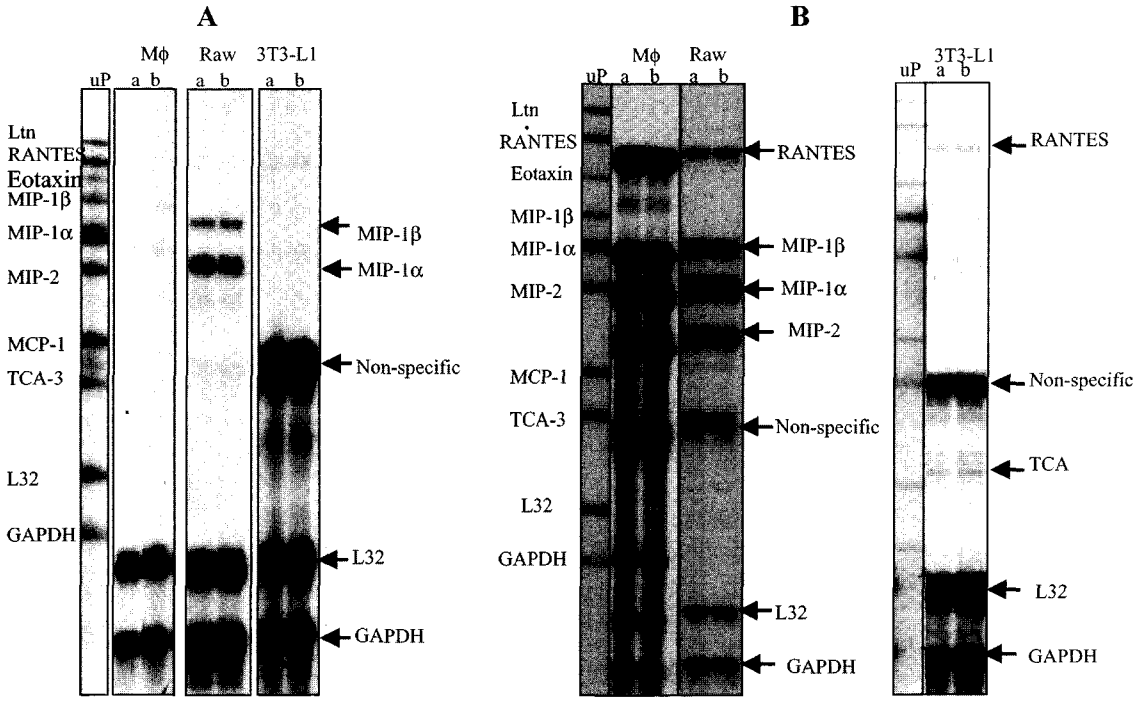


**Fig. 1.** Leptin does not induce the mRNA expression of 8 chemokines and does not affect the LPS-induced mRNA levels of chemokines in mouse peritoneal macrophages and RAW 264.7 and 3T3-L1 cells.
A: Thioglycollate (TG)-elicited C57BL/6 mouse peritoneal macrophages (Mϕ), RAW264.7 cells (RAW), or 3T3-L1 adipocytes were untreated (a) or treated (b) with leptin (1 μg/ml) for 4 h. B: TG-elicited Mϕ, RAW, or 3T3-L1 were treated with LPS (100 ng/ml, a) or LPS plus leptin (1 μg/ml, b) for 4 h. Total RNA was extracted by TRIzol and 10 μg of isolated RNA per sample were subjected to analyses by multiple-probe RPA (mCK-5b). uP: unprotected probe.

of lymphotactin, RANTES, eotaxin, MIP-1β, MIP-1α, MIP-2, MCP-1, and TCA-3 mRNAs in all cell types. To determine the leptin effect on the expression of these LPS-induced chemokine mRNAs, three kinds of cells were stimulated with LPS (100 ng/ml) and leptin (1 μg/ml) simultaneously (leptin/LPS) for 4 h, and RPA was performed. The relative capacity of LPS to induce chemokine mRNAs expression was dependent on cell types. 3T3-L1 cells were less responsive to LPS than other cell types. However, the expression patterns of leptin/LPS-induced chemokine genes were the same as those of LPS-induced chemokine mRNAs (Fig. 1B).

## Leptin Synergized with LPS to Induce KC mRNA Expression

To gain a further insight into LPS-induced KC and IP-10 mRNA expression in three cell types by leptin, the macrophages were stimulated for 4 h with leptin/LPS, and the total RNA was analyzed by a Northern blot analysis. Leptin showed a synergistic effect on the expression of LPS-induced chemokine KC mRNA in all three cell types, but leptin did not affect the expression of LPS-induced IP-10 mRNA in all three cell types (Fig. 2).

The synergistic induction of KC mRNA in the peritoneal macrophages stimulated with leptin/LPS was not dependent upon the dose of leptin (data not shown) but dependent upon the dose of LPS (Fig. 3A). One-hundred ng/ml and 1 μg/ml of LPS used in the macrophages showed almost the same effect on induction of KC mRNA expression, but KC mRNA was not detected in the macrophages stimulated with 1 ng/ml of LPS. In order to determine whether the synergistic action of LPS-induced KC mRNA expression by leptin is dependent on the time of macrophage exposure to leptin, peritoneal macrophages were treated with leptin either 1 h before or after or at the same time as LPS. Then, KC mRNA levels were measured

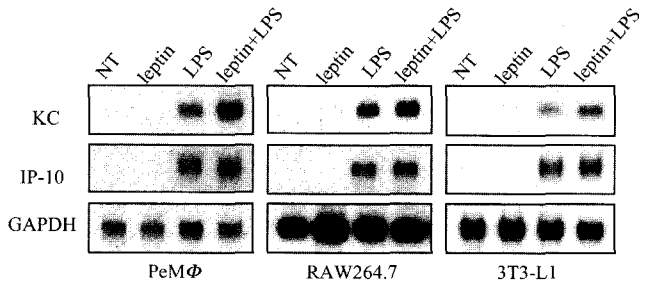


**Fig. 2.** Synergistic effect of leptin on LPS-induced KC mRNA expression in mouse peritoneal macrophage, RAW 264.7, and 3T3-L1 adipocytes.
TG-elicited peritoneal macrophages (PeMϕ), RAW264.7 cells, or 3T3-L1 adipocytes were untreated (NT) or treated with leptin (1 μg/ml) and/or LPS (100 ng/ml) for 4 h. The total mRNA was isolated, and a Northern blot analysis was performed. Similar results were obtained in three separate experiments.

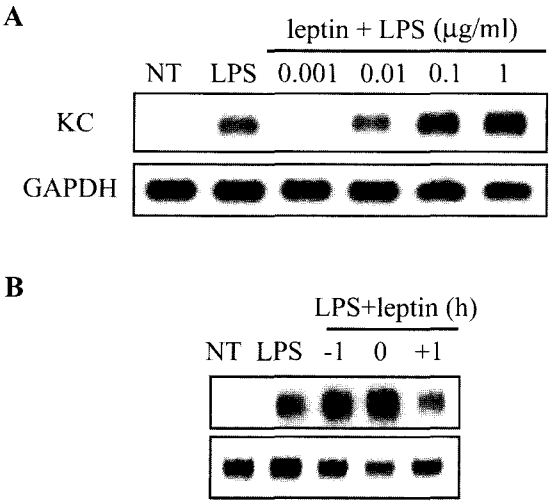


**Fig. 3.** Dose effect of LPS and time effect of leptin in the synergistic action of leptin on the expression of LPS-induced KC mRNA in mouse peritoneal macrophages.
A: TG-elicited PeMϕ was untreated (NT) or treated with LPS (100 ng/ml) alone or treated with leptin (1 μg/ml) and varying doses of LPS simultaneously for 4 h. B: TG-elicited PeMϕ was untreated (NT) or treated with LPS (100 ng/ml) for a period of 4 h. Samples were exposed to leptin (1 μg/ml) 1 h before (-1), simultaneously (0), or 1 h after (+1) the addition of LPS. Total RNA was prepared and analyzed for KC and GAPDH mRNA levels as described in Materials and Methods. These data are representative of three experiments.

4 h after the addition of LPS. The most synergistic action of leptin was detected in cells treated with leptin and LPS simultaneously (Fig. 3B).

## Delayed Action of Leptin on the Expression of LPS-induced KC mRNA Expression in Mouse Peritoneal Macrophages

The time dependence for the leptin-mediated synergistic effect on LPS-induced KC mRNA expression was determined in mouse peritoneal macrophages treated for various time periods (Fig. 4). The results demonstrated that the kinetics of the KC mRNA expression were comparable in the LPS-treated macrophages with and without added leptin. The LPS alone-induced KC mRNA expression reached a maximum as early as 2 h after treatment and then

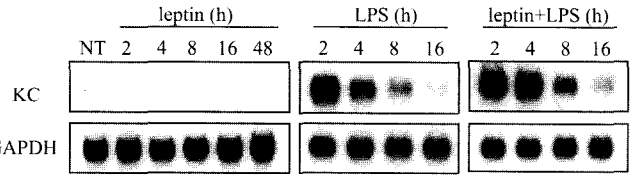


**Fig. 4.** Time course of leptin/LPS-induced KC mRNA expression in mouse peritoneal macrophages.
TG-elicited PeMϕ were untreated (NT) or treated with leptin (1 μg/ml) and/or LPS (100 ng/ml) for the indicated times. The mRNA levels were determined by Northern hybridization.

gradually declined up to 16 h. The expression of KC mRNA at 2 h after treatment with leptin/LPS was almost the same as the cells treated with LPS alone. However, maximum expression of KC mRNA sustained for 4 h and declined to basal levels by 16 h. This result suggests that the step that is sensitive to leptin occurs relatively late in the response of macrophages to LPS. Alternatively, at least 4 h of exposure to leptin may be required to induce an unknown necessary component(s) of the synergistic mechanism.

Moreover, LPS might modulate the amount or functional chacteristics of leptin receptor. To confirm the effect of LPS on Ob-Rb mRNA expression in mouse peritoneal macrophages, the macrophages were stimulated with LPS for 2 and 4 h and the total RNA was analyzed by a Northern blot analysis. Time dependence of the expression of Ob-Rb mRNA by LPS was detected in mouse peritoneal macrophages (Fig. 5A). Ob-Rb mRNA showed stronger expression at 4 h treatment of LPS than 2 h. In order to confirm the expression of Ob-Rb induced by LPS, immunocytochemical studies were performed on mouse peritoneal macrophages *in vitro*. As shown in Fig. 5B, the expression of leptin receptor protein was dependent on the treatment time of LPS. Cells treated with LPS were polygonal shape, and the cytoplasm became foamy in appearance, and the reaction intensity was also stronger than in LPS-untreated cells.

## Mechanisms of Synergistic Effect of Leptin on LPS-Induced KC mRNA Expression

Alteration of KC mRNA levels induced by leptin may be caused by modulating the rate of mRNA degradation. To elucidate this mechanism that is involved in the synergy of LPS-induced KC mRNA by leptin, mRNA stability was analyzed. Macrophages were stimulated with LPS in the presence or absence of leptin for 2 or 4 h before treatment with actinomycin D (Act.D) to prevent further transcription. After additional incubation up to 90 min, KC mRNA levels were assessed by Northern hybridization and quantified by Statview software (SAS Institute, Cary, NC, U.S.A.) analysis (Fig. 6). KC mRNA in cells treated with

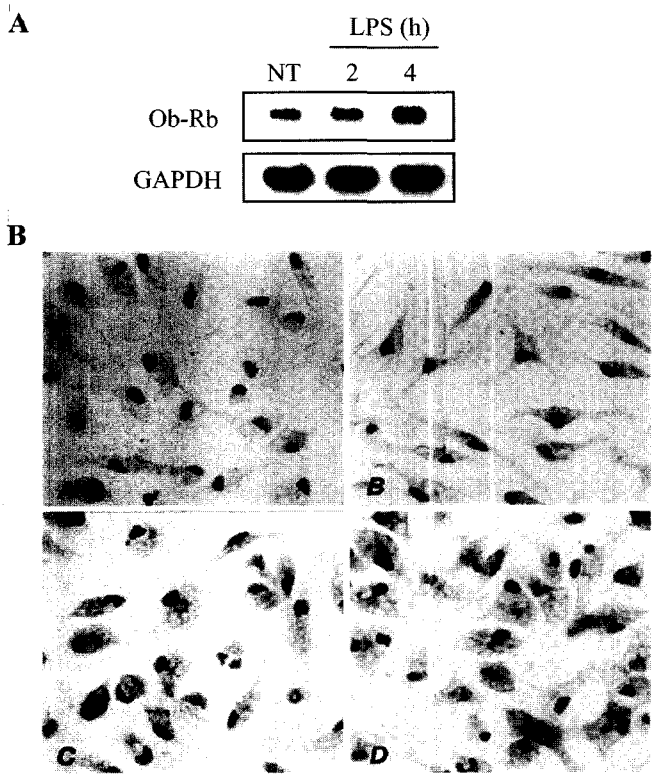


**Fig. 5.** Time response of LPS-induced leptin receptor expression in mouse peritoneal macrophages.
A: TG-elicited PeMϕ were untreated (NT) or treated with LPS (100 ng/ml) for the indicated times. The mRNA levels were determined by Northern hybridization. Ob-Rb; long isoform of leptin receptor. B: Immunocytochemical detection of leptin receptor protein in mouse peritoneal macrophages. Primary antibody was replaced by PBS in negative control (A); TG-elicited PeMϕ were untreated (B) or treated with LPS (100 ng/ml) for 4 h (C) and overnight (D). Nuclei were counterstained with hematoxylin after RNase treatment. Magnification 400×.

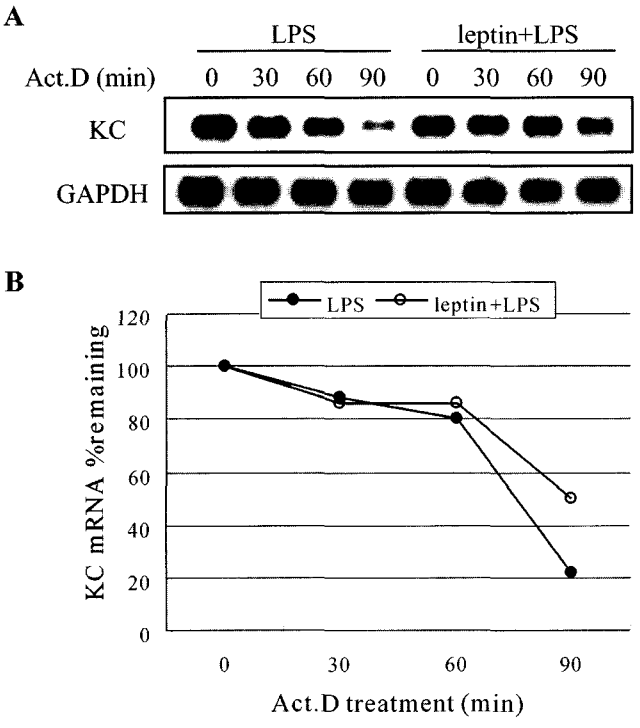


**Fig. 6.** Effect of leptin on stability of LPS-induced KC mRNA.
A: TG-elicited PeMϕ were untreated (NT) or treated with LPS (100 ng/ml) in the absence or presence of leptin (1 μg/ml) for 4 h. Actinomycin D (Act.D, 5 μg/ml) was added to all cultures and incubated continuously for the indicated times before an analysis of the KC and GAPDH mRNA levels by Northern hybridization. B: The blots were quantified by Statview software (SAS Institute, Cary, NC) analysis, KC mRNA levels normalized for GAPDH content of each sample, and plotted as percent mRNA remaining versus time. Similar results were obtained in three experiments.

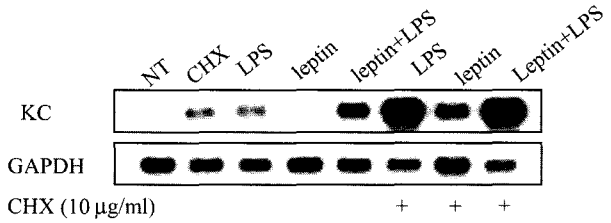


**Fig. 7.** Leptin-mediated synergy of LPS-induced KC mRNA expression is not prevented by an inhibitor of protein synthesis.
TG-elicited PeMϕ were untreated (NT) or treated with LPS (100 ng/ml) with leptin (1 μg/ml), and/or with cycloheximide (CHX, 10 μg/ml) for 4 h. The mRNA was isolated, and a Northern analysis was performed. These data are representative of three similar experiments.

leptin/LPS decayed more slowly and declined with a half-life of approximately 90 min. In the absence of leptin, KC mRNA decayed more rapidly and the level of the mRNA was already decreased by 22% at 90 min of exposure to Act.D.

The synergistic action of leptin could reflect the need for an inducible intermediate gene product. To examine the possibility of a role for one or more leptin/LPS-inducible proteins, peritoneal macrophages were stimulated with leptin and/or LPS in the presence or absence of cycloheximide (CHX), an inhibitor of protein synthesis. Synergistic effect of leptin on LPS-induced KC mRNA expression was further augmented by CHX (Fig. 7). This result confirmed that leptin increased accumulation of KC mRNA without *de novo* protein synthesis.

Next, the role of NF-κB activation in the synergistic effect of leptin on LPS-induced KC mRNA expression was investigated. An antioxidant, PDTC, selectively blocks the dissociation of Iκ-B from the cytoplasmic NF-κB, thus preventing the activation and nuclear translocation of NF-κB. Mouse peritoneal macrophages were treated with leptin and/or LPS in the presence or absence of PDTC (10 μM/ml) for 4 h. PDTC did not block the synergistic effect of leptin on LPS-induced KC mRNA expression

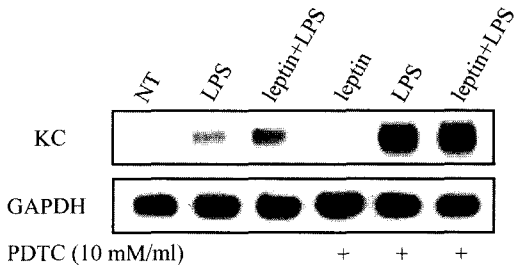


**Fig. 8.** Inhibiton of NF-κB activation does not suppress the synergistic effect of leptin on LPS-induced KC mRNA expression in mouse peritoneal macrophage.
TG-elicited PeMϕ were treated with leptin (1 μg/ml) and/or LPS (100 ng/ml) in the absence or presence of PDTC (10 mM/ml) for 4 h. Total mRNA was isolated, and Northern blot analysis was performed. Similar results were obtained in two separate experiments.

(Fig. 8). This finding suggests that the synergistic action of leptin/LPS on KC mRNA expression is not mediated via activation of NF-κB.

## DISCUSSION

The objective of this study was to evaluate the possibility that leptin can induce proinflammatory chemokines in mouse peritoneal macrophages and to explore the mechanism by which this could occur. However, leptin had no effect on the expression of lymphotactin, RANTES, eotaxin, MIP-1β, MIP-1α, MIP-2, MCP-1, TCA-3, IP-10, and KC mRNA in mouse peritoneal macrophages, RAW 264.7 cells, and 3T3-L1 adipocytes. Also, leptin had no effect on these LPS-stimuated chemokine mRNAs expression except chemokine KC. But, in this study, it was found for the first time that leptin synergizes LPS-induced KC gene expression by enhancing KC mRNA stability at a late stage in the response to LPS.

Although the experimental design and the kinds of cytokine studied were different, Hamid *et al.* [8] reported that high-dose leptin can activate human leukocytes. They used 250 ng/ml and 1 μg/ml of leptin. When cells were treated with endotoxin and leptin simultaneously, the productions of TNF-α, IL-6, and IFN-γ were enhanced. In particular, the production of cytokine was further enhanced when leptin was used at a concentration of 1 μg/ml rather than 250 ng/ml. In addition, according to Sanros-Alvarez *et al.* [23], the production of TNF-α and IL-6 by leptin in monocytes was dependent on the leptin dose (0.01 to 100 nM). However, inducing the mRNA expression of KC and IP-10 using various doses of leptin ranging from 50 ng/ml to 5 μg/ml (data not shown) failed.

One of the studies for regulation of chemokine by leptin can be exemplified by the report of Yamagishi *et al.* [30] that the MCP-1, which is produced when aortic endothelial cells were stimulated by genistein, was produced more if leptin treatment was additionally associated. But, there has been no study for the chemokine gene expression by leptin alone. Based on previous reports [4, 23] that leptin enhances the stimulatory effect on LPS or PMA, mouse peritoneal macrophages, RAW 264.7 cells, and 3T3-L1 adipocytes were stimulated with LPS or leptin/LPS. As compared with the mRNA expression of 10 kinds of chemokines, 9 kinds of chemokines except KC remained unchanged, but the general expression pattern of these chemokines showed difference according to reactive cells. While the expression of LPS-induced RANTES, MIP-1α, MIP-1β, MIP-2, IP-10, and KC mRNA was clearly distinguishable in RAW 264.7 cells and peritoneal macrophages, the expression appeared weak or insignificant in 3T3-L1 adipocytes. The reason may be that the adipocytes react to LPS relatively less than macrophages.

The relatively late appearance of the synergistic action of leptin on the expression of LPS-induced KC mRNA suggests the possibility that LPS might induce expression of a gene that sensitizes the macrophages to synergistic function of leptin. For example, LPS might modulate the amount or functional characteristics of the leptin receptor. Leptin receptors (Ob-R) are found in several alternatively spliced forms such as Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, and Ob-Re [6, 26]. Ob-Rb (long isoform) has a long cytoplasmic domain which activates the JAK-STAT intracellular signal transduction pathway. Other isoforms have no or short cytoplasmic domains and could not activate STAT protein [7, 27]. Constitutive levels of mRNA for leptin receptors (predominantly the short isoform, Ob-Ra) are highly expressed in several peripheral tissues and cells, including kidney, lung, liver, spleen, and macrophages. This study observed that resting mouse peritoneal macrophages expressed Ob-Rb mRNA, and the expression of Ob-Rb mRNA was enhanced in LPS-treated macrophages, and this enhancement of Ob-Rb was dependent upon the time of LPS treatment. This result suggests that the degree of Ob-Rb expression might affect the delayed synergistic effect of leptin on KC expression.

Chemokine KC is a rapidly upregulated gene and a potent neutrophil chemoattractant *in vitro* and *in vivo* [1, 3, 24]. KC gene expression in macrophages appears to be regulated by both transcriptional and posttranscriptional mechanisms [13, 21, 22]. However, little is known about the mechanism of KC gene expression induced by leptin/LPS. Leptin produced a substantial increase in the stability of the LPS-induced KC mRNA. Most of the studies on regulation of cytokine production by leptin concentrated on measuring the production of proinflammatory cytokines such as TNF-α, IL-6, IL-12, IL-2, and IL-1 [15, 17, 20, 25], and no study observed the functional mechanism of leptin for those cytokines on transcription level or mRNA stability level. There are a number of studies for expression mechanism of KC mRNA [11, 12, 21, 22, 28], and a few studies dealt with NF-κB related to the expression of LPS-induced KC mRNA [21]. Therefore, although leptin activates STAT protein through the Ob-Rb receptor [27], this study examined the effect of PDTC, the selective inhibitor of NF-κB, to define whether the synergistic effect of leptin /LPS includes the activation of NF-κB. According to the NF-κB-related study by Ohmori *et al.* [21], LPS-induced KC gene transcription needs two κB sequence motifs on the KC gene in RAW 264.7 cells and NIH 3T3 fibroblast. However, when we used PDTC in mouse peritoneal macrophages, the expression of leptin/LPS-induced KC mRNA was not repressed but enhanced, and so was LPS-induced KC mRNA. This may be because not NF-κB but other transcription factors or mechanisms are involved to induce the expression of leptin/LPS-induced KC mRNA in mouse peritoneal macrophages, and also because the functional mechanisms of extracellular stimulating factors to control the expression of the KC gene are variant according to the kinds of reactive cells. Further studies with electrophoretic mobility shift assay (EMSA) or transfection of a specific gene for the evaluation of other molecular mechanisms are needed to investigate the transcription factors related to mechanisms underlying the synergistic effect of leptin on LPS-induced KC mRNA expression.

This study was focused on the effect of leptin as an immunoregulatory factor in the expression of chemokines. Because there are few studies on leptin-related chemokines and no collective analysis on the changes in chemokine genes expression, we tried to analyze the function of leptin on chemokine gene expression in mouse peritoneal macrophages. The results showed that leptin did not induce the expression of the 10 kinds of chemokine mRNAs studied, and also had little affect on LPS-induced chemokines except KC. However, the synergistic effect of leptin on the expression of LPS-induced chemokine KC mRNA was detected. The synergistic effect of leptin occurred late in the time course of response to LPS, and was dependent on KC mRNA stability. But it did not seem to be directly involved in the activation of NF-κB.

These results suggest that although leptin has been known to regulate directly the production of some proinflammatory cytokines, leptin does not affect the expression of lymphotactin, RANTES, eotaxin, MIP-1β, MIP-1α, MIP-2, MCP-1, TCA-3, IP-10, and KC mRNA in mouse peritoneal macrophages. Therefore, further studies are necessary to examine leptin as a regulatory factor of chemokine production.

## Acknowledgment

This work was supported by a research grant from the Advanced Research Center in Yeungnam University (Grant No. 105098).

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