Leptin Increases Lipoprotein Lipase Secretion by Macrophages: Involvement of Oxidative Stress and Protein Kinase C

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Recent data suggest that plasma leptin may represent a cardiovascular risk factor in diabetic patients. To gain further insight into the role of leptin in atherogenesis associated with diabetes, we investigated in the present study the role of this hormone in the regulation of macrophage lipoprotein lipase (LPL), a proatherogenic cytokine overexpressed in patients with type 2 diabetes. Treatment of human macrophages with leptin (1–10 nmol/l) increased LPL expression, at both the mRNA and protein levels. Pretreatment of these cells with anti–leptin receptor (Ob-R) antibody, protein kinase C (PKC) inhibitors, calphostin C, and GF109203X, or the antioxidant *N*-acetylcysteine (NAC) blocked the effects of leptin. Similar results were observed in leptin-treated J774 macrophages. In these cells, leptin increased the membrane expression of conventional PKC isoforms and downregulation of endogenous PKC expression abolished the effects of leptin on macrophage LPL expression. In leptin-treated J774 cells, enhanced LPL synthetic rate and increased binding of nuclear proteins to the activated protein-1 (AP-1) consensus sequence of the LPL gene promoter were also observed. This latter effect was abrogated by GF109203X. Overall, these data demonstrate that binding of leptin at the macrophage cell surface increases, through oxidative stress- and PKC-dependent pathways, LPL expression. This effect appears to be exerted at the transcriptional level and to involve AP-1 activation. *Diabetes* 52: 2121–2128, 2003

Leptin is a 16-kDa peptide hormone that is produced mainly by adipocytes and plays a central role in the regulation of body weight. Plasma leptin levels correlate with BMI (1) and are three to four times higher in obese and diabetic patients (2,3). The broad distribution of leptin receptor expression in a variety of tissues accounts for the pleiotropic functions of this hormone in lipid metabolism, hematopoiesis, pancreatic cell function, thermogenesis, and response to lipopolysaccharide (4). Recently, a role for leptin in the control of inflammation has been proposed. Indeed, it has been demonstrated that leptin stimulates the proliferation, differentiation, and functional activation of hemopoietic cells, including monocytes/macrophages, and increases the production of proinflammatory cytokines (5–7). These results and the findings that leptin modulates key processes involved in atherogenesis, including angiogenesis, oxidative stress, vascular calcification, and thrombosis (8–12), suggest a role for leptin as a potential cardiovascular risk factor in obese and diabetic patients. This possibility is further supported by recent observations showing that leptin receptors are expressed in human atherosclerotic lesions and that plasma leptin is independently associated with the intima-media thickness of the carotid artery and represents an independent predictor of first-ever acute myocardial infarction and hemorrhagic stroke (13–15).

Lipoprotein lipase (LPL) is a key enzyme in lipid metabolism. Contrary to the antiatherogenic effect of plasma LPL, LPL secreted by macrophages in the arterial wall is a proatherogenic molecule (16,17). Indeed, it has been shown that LPL promotes the retention of lipoproteins in the subendothelial space (18) and favors monocyte adhesion to the endothelium (19,20). It also stimulates the transformation of macrophages into foam cells, the production of proinflammatory cytokines, and the proliferation of smooth muscle cells (21–24).

We have previously demonstrated that macrophages of patients with type 2 diabetes overexpress LPL (25) and have characterized the role of metabolic factors in this alteration (26–28). In the present study, we investigated the role of leptin in the induction of macrophage LPL in diabetes. Our data, which demonstrate that leptin increases macrophage LPL secretion in vitro, support the possibility that leptin may represent a macrophage LPL-stimulatory factor in diabetes. Leptin-induced macrophage LPL may contribute to the increased incidence of atherosclerotic events seen in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

Reagents. Fetal calf serum (FCS) was purchased from Wisent (St-Bruno, PQ, Canada). Dulbecco's modified Eagle medium (DMEM), RPMI-1640 medium, penicillin-streptomycin, phorbol myristate acetate (PMA), and Trizol reagent were purchased from Gibco-BRL (Burlington, ON, Canada). Human recombinant leptin, rabbit monoclonal antibody against the leptin receptor recogniz-

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AP-1, activated protein-1; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; LPL, lipoprotein lipase; MAPK, mitogen-activated protein kinase; NAC, *N*-acetylcysteine; NP-40, Nonidet-P-40; Ob-R, leptin receptor; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; PMA, phorbol myristate acetate; PMSF, phenylmethylsulfonyl fluoride; PPAR, peroxisome proliferator–activated receptor.

ing both the Ob-Ra and Ob-Rb isoforms of the leptin receptor, and IgG_1 neutralizing antibody were obtained from R&D Systems (Minneapolis, MN) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Endotoxin content of the leptin preparation (1 nmol/l) was determined by the Limulus amoebocyte lysate assay (Sigma, St. Louis, MO) and was consistently found to be <6 pg/ml. 4α-PMA and heparin were purchased from Sigma. Calphostin C, *N*-acetylcysteine (NAC), GF109203X, wortmannin, and PD98059 were obtained from Calbiochem (La Jolla, CA). ^{35}S was provided by ICN Biochemicals (Costa Mesa, CA).

Macrophages. Human monocytes were isolated as previously described (29). Peripheral blood mononuclear cells were isolated from venous blood of healthy subjects by density-gradient sedimentation using Ficoll, allowed to aggregate in the presence of FCS, then further purified by the rosetting technique. After density centrifugation, highly purified monocytes (85–90%) were recovered as assessed by flow cytometry (FACScan; Becton Dickinson, Rutherford, NJ) using phycoerythrin-conjugated anti-CD14 monoclonal antibody (Becton Dickinson). Differentiation of monocytes into macrophages was achieved by culturing the cells in RPMI-1640 medium supplemented with 1% (vol/vol) penicillin/streptomycin and 20% (vol/vol) autologous serum. The cells were incubated for 8 days at 37°C in a humidified 5% CO_2/95% air atmosphere. The culture medium was changed at days 4 and 8. Levels of LPL mass and activity were measured 24 h after the last medium change.

The J774 murine macrophage cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD). J774 cells were cultured in DMEM containing 10% FCS and 100 μg/ml penicillin-streptomycin.

Analysis of LPL mRNA expression. Expression of the LPL gene in human macrophages was performed by semiquantitative PCR. Total RNA for use in the PCR was extracted from human macrophages by an improvement of the acid-phenol technique of Chomczynski and Sacchi (30). Briefly, cells were lysed with Trizol reagent, and chloroform was added to the solution. After centrifugation, the RNA present in the aqueous phase was precipitated and resuspended in diethyl pyrocarbonate water. cDNA was synthesized from RNA by incubating 1 μg of total cellular RNA with 0.1 μg oligodT (Pharmacia, Piscataway, NJ) for 5 min at 98°C, then by incubating the mixture with reverse transcription buffer for 60 min at 37°C. The cDNA obtained was amplified by using 0.8 μmol/l of two synthetic primers specific for human LPL (5′-GAGATTTCTCTGTATGGCACC-3′; 5′-CTGCAAATGAGACACTTTCTC-3′), and human GAPDH (5′-CCCTTCATTGACCTCAACTACATGG-3′; 5′-AGTCTTCTG GGTGGCAGTGATGG-3′) used as the internal standard in the PCR mixture. A 277-bp human LPL cDNA fragment and a 456-bp human GAPDH cDNA fragment were amplified enzymatically by 21 repeated cycles at 95°C for 60 s, 60°C for 40 s, and 72°C for 90 s. An aliquot of each reaction mixture was then subjected to electrophoresis on a 1% agarose gel containing ethidium bromide. The intensity of the bands was measured by an image analysis scanning system (Alpha Imager 2000; Packard Instruments, Meriden, CT). Titrating the cDNA samples ensured that the signal lies on the exponential part of the standard curve.

Determination of LPL gene expression in murine macrophages was performed by Northern blot analysis. Six million J774 cells were plated in plastic Petri dishes (100×200 mm) (Falcon, Lincoln Park, NJ). After treatment, cells were lysed with Trizol reagent. Total RNA was isolated and separated in a 1.2% agarose gel containing 2.2 mol/l formaldehyde. The blots were prehybridized for 6 h. The mRNA expression was analyzed by hybridization with [^{32}P]dCTP-labeled LPL and S28 cDNA probes. Hybridization was detected by autoradiography with Kodak X-Omat-AR films (Rochester, NY). mRNA expression was quantified by high-resolution optical densitometry (Alpha Imager 2000, Packard Instruments).

DNA probes. The cDNA probe for detection of murine LPL was prepared by the PCR technique. cDNA was obtained from total RNA using a reverse transcription reaction. Two synthetic primers spanning bases 255–287 and 1,117–1,149 of the LPL cDNA were used to enzymatically amplify an 894-bp region of the LPL probe. The cDNA probe for detection of murine S28 was purchased from ATCC. A 20-mer double-stranded oligonucleotide (5′-GGGC ACCTGACTAAGGCCAG-3′; 5′-TGTGCTGGCCTTAGTCAGGT-3′) containing the consensus sequence for the activated protein-1 (AP-1) responsive element of the murine LPL gene promoter was synthesized with the aid of an automated DNA synthesizer. After annealing, the double-stranded oligonucleotide was labeled with [γ-^{32}P]ATP by using the Boehringer Mannheim 5′ end-labeling kit (Indianapolis, IN).

Electrophoretic mobility shift assay. The isolation of the nuclei was performed as follows. Briefly, 5×10^7 J774 cells were collected, washed with cold PBS, and lysed in 1 ml ice-cold buffer A (15 mmol/l KCl, 2 mmol/l $MgCl_2$, 10 mmol/l HEPES, 0.1% phenylmethylsulfonyl fluoride [PMSF], and 0.5% Nonidet-P-40 [NP-40]). After a 10-min incubation on ice, lysed cells were centrifuged, and the nuclei were washed with buffer A without NP-40. The nuclei were then lysed in a buffer containing 2 mol/l KCl, 25 mmol/l HEPES, 0.1 mmol/l EDTA, and 1 mmol/l dithiothreitol (DTT). After a 15-min incubation period, a dialysis buffer (25 mmol/l HEPES, 1 mmol/l DTT, 0.1% PMSF, 2 μg/ml aprotinin, 0.1 mmol/l EDTA, and 11% glycerol) was added to the nuclei preparation. Nuclei were collected by centrifugation for 20 min at 13,000 rpm. Aliquots (50 μl) of the supernatants were frozen at −80°C, and the protein concentration was determined. DNA retardation (mobility shift) electrophoresis assays were performed as previously described by Fried and Crothers (31). Briefly, 5 μg nuclear extracts were incubated for 15 min in the presence of 5× binding buffer (125 mmol/l HEPES [pH 7.5], 50% glycerol, 250 mmol/l NaCl, 0.25% NP-40, and 5 mmol/l DTT). End-labeled double-stranded consensus sequences of the LPL promoter AP-1–enhancing element (20,000 cpm per sample) were then added to the samples for 30 min. Samples were analyzed on a 4% nondenaturating PAGE containing 0.01% NP-40. The specificity of the nuclear protein binding was assessed by incubating the nuclear proteins isolated from murine macrophages with labeled DNA probe in the presence of a 1,000-fold molar excess of unlabeled DNA probe.

Determination of extracellular LPL immunoreactive mass and activity. One hour before the end of the incubation period, 50 units/ml heparin was added to the medium. The amount of LPL immunoreactive mass in the supernatants was measured by enzyme-linked immunosorbent assay using the Markit-F LPL kit (Dainippon Pharmaceutical, Osaka, Japan) (32). Extracellular LPL activity was determined using the Confluolip kit (Progen, Heidelberg, Germany) (33). Levels of macrophage LPL mass and activity were normalized to levels of total cell proteins.

Western blot analysis for PKC isoforms. After appropriate treatment, adherent J774 macrophages were recovered and homogenized (Dounce: 15 strokes) in 500 μl of ice-cold buffer A (20 mmol/l Tris [pH 7.5], 0.5 mmol/l EDTA, 0.5 mmol/l ethylene glycol-bis tetraacetic acid [EGTA], 25 μg/ml aprotinin, and 25 μg/ml leupeptin). The membrane and cytosolic fractions were separated by ultracentrifugation (100,000*g* for 30 min at 4°C). After recovery of high-speed supernatants containing cytosolic PKC, the corresponding membrane pellets were homogenized in 500 μl of buffer A containing 0.5% Triton X-100. Protein extracts (10 μg) were applied to 10% SDS-PAGE and transferred to a nitrocellulose membrane using a Bio-Rad transfer blotting system at 100 V for 1 h. Nonspecific binding was blocked with 3% BSA for 1 h at room temperature. After washing with PBS-Tween 0.1%, blots were incubated overnight with PKC-α, -β_I, -β_{II}, -γ, -ζ, and -ε or anti–β-actin antibodies (Santa Cruz Biotechnology). After further wash, membranes were incubated for 1 h at room temperature with a horseradish peroxidase–conjugated donkey anti-mouse or anti-rabbit IgG (1/5,000). Antigen detection was performed with an enhanced chemiluminescence detection system (Amersham).

Immunoprecipitation assay. Following treatment with leptin, 2×10^6 cells were washed twice with PBS and incubated for 1 h with methionine-free DMEM. Cells were then metabolically labeled with 100 μCi [^{35}S]methionine for 30 min and chased for 1 h with complete DMEM. Radiolabeling was ended by the addition of lysis buffer (50 mmol/l Tris-HCl [pH 7.5], 150 mmol/l NaCl, 100 μg/ml PMSF, and 1% Triton X-100). Immunoprecipitation of LPL in cell lysates was performed after centrifugation of homogenates at 17,000*g* for 20 min at 4°C; the supernatant was collected and used for immunoprecipitation. The samples (200 μg total cytoplasmic proteins) were incubated at 4°C for 4 h with 10 μg of monoclonal anti-LPL antibody 5D2 (received from J.D. Brunzell, University of Washington, Seattle, WA) followed by incubation with 10 μl goat anti-mouse IgG antiserum (BioRad, Hercules, CA). The immunocomplexes were collected on protein A/G PLUS-agarose beads (Santa Cruz, CA), washed twice with Tris-buffered saline (TBS) containing 0.2% NP-40, 2 mmol/l EDTA, and 500 mmol/l NaCl, then with TBS only. The pellet was resuspended in 1× SDS-loading buffer (50 mmol/l Tris-HCl [pH 6.8], 100 mmol/l DTT, 2% SDS, 0.1% bromophenol blue, and 10% glycerol) and boiled for 10 min, and the samples were subjected to 8% SDS-PAGE. Immunoprecipitation of cytoplasmic proteins isolated from heparin-stimulated macrophages was used as a positive control. Immunoprecipitation with the irrelevant anti-mouse IgG (10 μg) (Santa Cruz Biotechnology) was used as negative control. After autoradiography, the intensity of the bands was quantified by densitometry using an image analysis scanning system.

Determination of cell viability. Cell viability after treatment with leptin was assessed by trypan blue exclusion and was consistently found to be >90%.

Determination of total protein concentration. Total protein content was estimated according to the Bradford method (34) using a colorimetric assay (BioRad, Mississauga, ON, Canada).

Statistical analysis. Statistical analysis of the results was done by one-way ANOVA followed by the Student-Newman-Keuls test for multiple comparisons or by the unpaired Student's *t* test. All values were expressed as means ± SE. $P < 0.05$ was considered significant.

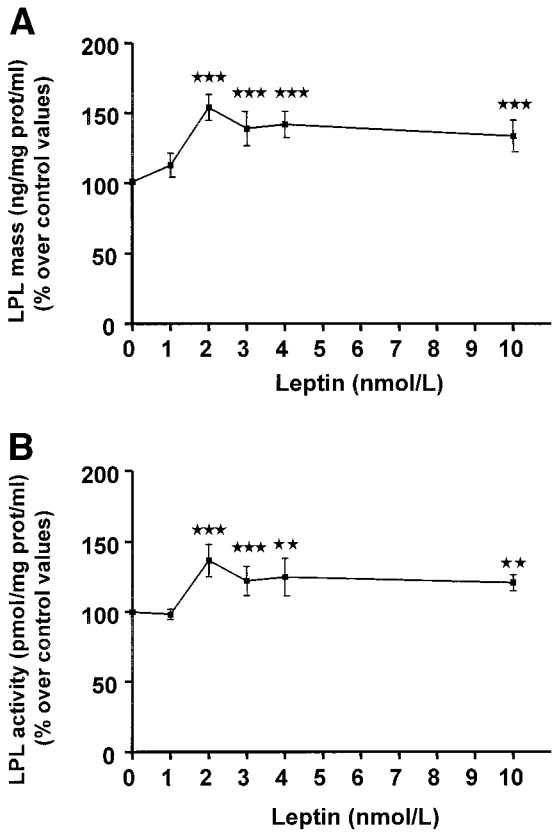

FIG. 1. Dose-dependent effect of leptin on human macrophage extracellular LPL immunoreactive mass (*A*) and activity (*B*). Human macrophages were incubated for 24 h with increasing doses of leptin (1–10 nmol/l). LPL immunoreactive mass (*A*) and activity (*B*) were measured in the culture medium and normalized to total cell protein levels. Data are means ± SE of four different experiments, $^{**}P < 0.01$ vs. medium (Med) and $^{***}P < 0.001$ vs. medium.

RESULTS

Effect of leptin on human macrophage LPL secretion. Human monocyte-derived macrophages were incubated for 24 h with increasing doses of leptin (1–10 nmol/l). At concentrations ranging from 2 to 10 nmol/l, leptin significantly increased extracellular LPL mass (Fig. 1*A*) and activity (Fig. 1*B*). Maximal effect was observed with 2 nmol/l leptin (LPL mass [% increase over control values]: leptin [2 nmol/l] 154 ± 9, $P < 0.001$; LPL activity [% increase over control values]: leptin [2 nmol/l] 137 ± 12, $P < 0.001$). This stimulatory effect of leptin on human macrophage LPL mass and activity was still observed at 48 h (LPL mass: leptin [2 nmol/l] 126 ± 7, $P < 0.05$; LPL activity: leptin [2 nmol/l] 124 ± 9, $P < 0.05$).

Effect of leptin on human macrophage LPL mRNA expression. To investigate whether leptin modulates macrophage LPL at the gene level, human monocyte-derived macrophages were incubated with leptin for 1–6 h, and LPL mRNA levels were determined by PCR analysis. Incubation of these cells for 1 and 3 h led to a significant increase in LPL mRNA levels. This effect was maximal at 1 h, declining toward baseline at 6 h (Fig. 2). Under these experimental conditions, no modulation of the mRNA expression of GAPDH was observed. LPL mRNA levels normalized to the levels of GAPDH mRNA are presented in Fig. 2 (LPL mRNA levels [% increase over control values] at 1 h 152 ± 15, $P < 0.01$; at 3 h 141 ± 17, $P < 0.05$).

Leptin effect on human macrophage LPL secretion is mediated by the leptin receptor (Ob-R). To determine whether the Ob-R is responsible for mediating the stimulatory effect of leptin on macrophage LPL secretion, human monocyte-derived macrophages were pretreated for 1 h with saturating amounts (10 μg/ml) of a monoclonal antibody against the Ob-R before the addition of leptin (2 nmol/l). Pretreatment of these cells with the anti–Ob-R antibody resulted in a total inhibition of the stimulatory effect of leptin on macrophage extracellular LPL mass (Fig. 3*A*) and activity (Fig. 3*B*), whereas addition of the irrelevant murine anti-IgG_1 antibody did not affect these parameters.

Role of PKC and oxidative stress on leptin-stimulated human macrophage LPL secretion. To evaluate the role of PKC in mediating leptin-induced macrophage LPL secretion, human monocyte-derived macrophages were preincubated for 1 h with the pan-specific PKC inhibitor, calphostin C (0.1 μg/ml). Pretreatment of the cells with this compound totally prevented the stimulatory effect of leptin on macrophage extracellular LPL mass (Fig. 4*A*) and activity (Fig. 4*B*). A similar effect was observed when human macrophages were pretreated with the conventional PKC selective inhibitor, GF109203X (20

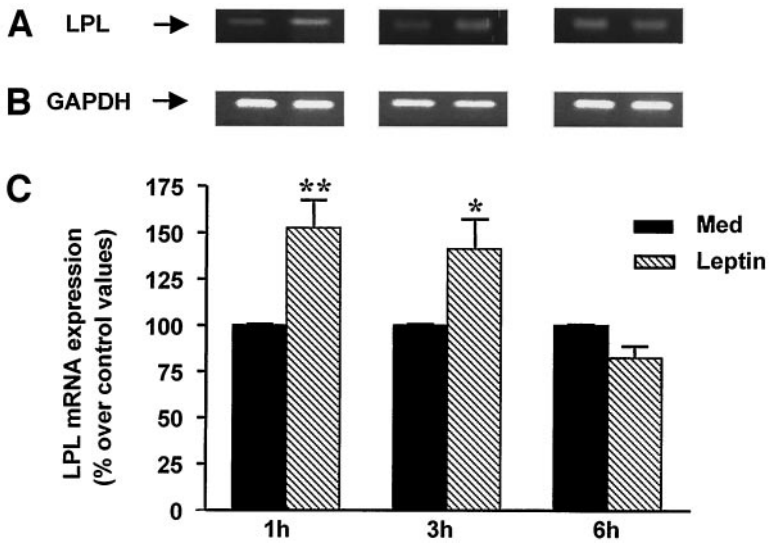

FIG. 2. Effect of leptin on human macrophage LPL mRNA expression. Human macrophages were incubated for 1, 3, and 6 h in the presence of 10 nmol/l leptin. At the end of these incubation periods, cells were lysed and total RNA was extracted and analyzed by PCR analysis for LPL (*A*) and GAPDH (*B*) mRNA expression. Levels of LPL mRNA were normalized to the levels of GAPDH mRNA (*C*). Results are means ± SE of four different experiments, $^{*}P < 0.05$ vs. medium (Med) and $^{**}P < 0.01$ vs. medium.

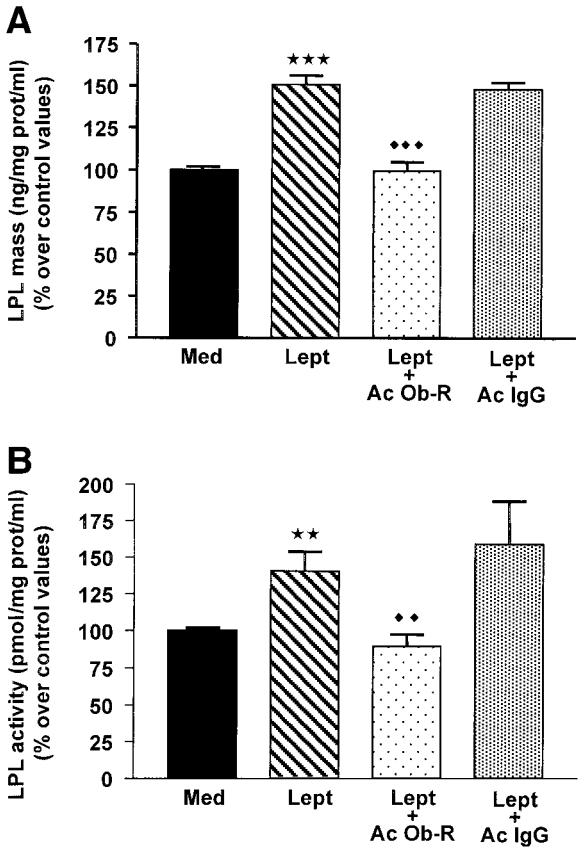

FIG. 3. Effect of Ob-R antibody on human macrophage extracellular LPL immunoreactive mass and activity. Human macrophages were preincubated for 1 h with saturating amounts of an anti–Ob-R antibody (10 μg/ml) or anti-IgG_1 neutralizing antibody before incubation with 2 nmol/l leptin for 24 h. At the end of this incubation period, LPL immunoreactive mass (*A*) and activity (*B*) were measured in the culture medium and normalized to total cell protein levels. Data are means ± SE of four different experiments, $^{**}P < 0.01$ vs. medium (Med), $^{***}P < 0.001$ vs. medium, $^{♦♦}P < 0.01$ vs. leptin, and $^{♦♦♦}P < 0.001$ vs. leptin.

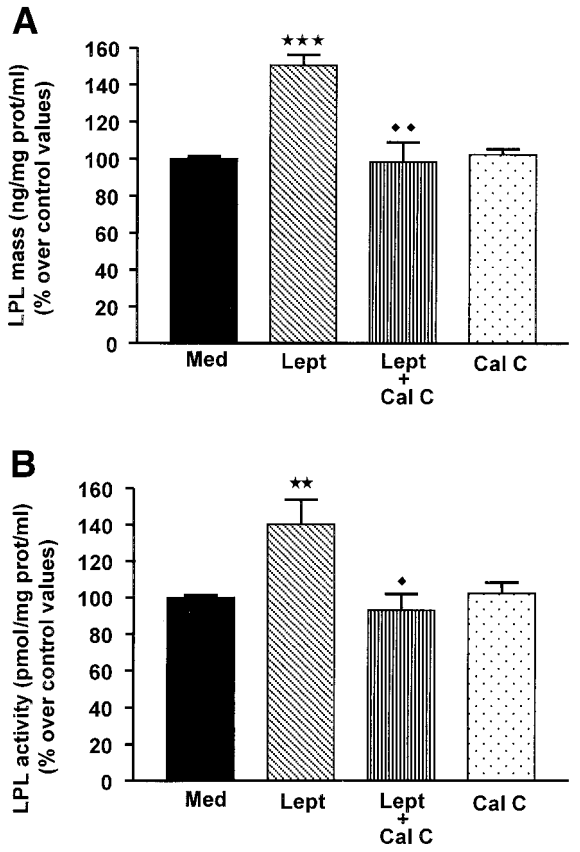

FIG. 4. Effect of PKC inhibitor on human macrophage extracellular LPL immunoreactive mass and activity. Human macrophages were preincubated for 1 h with calphostin C (Cal C) (0.1 μg/ml) before incubation with 2 nmol/l leptin for 24 h. At the end of this incubation period, LPL immunoreactive mass (*A*) and activity (*B*) were measured in the culture medium and normalized to total cell protein levels. Data are means ± SE of four different experiments, $^{**}P < 0.01$ vs. medium (Med), $^{***}P < 0.001$ vs. medium, $^{♦}P < 0.05$ vs. leptin, and $^{♦♦}P < 0.01$ vs. leptin.

nmol/l) (LPL mass [% increase over control values]: leptin 156 ± 4; GF109203X 93 ± 3; leptin + GF109203X 100 ± 3, $P < 0.01$ vs. leptin; LPL activity [% increase over control values]: leptin 133 ± 2; GF109203X 105 ± 8; leptin + GF109203X 88 ± 5, $P < 0.01$ vs. leptin). The stimulatory effect of leptin on macrophage extracellular LPL mass and activity was also completely abolished in the presence of the antioxidant NAC (10 mmol/l) (LPL mass [% increase over control values]: leptin 153 ± 5; NAC 101 ± 3; leptin + NAC 100 ± 2, $P < 0.01$ vs. leptin; LPL activity [% increase over control values]: leptin 132 ± 3; NAC 97 ± 5; leptin + NAC 90 ± 3, $P < 0.01$ vs. leptin).

Effect of leptin on murine macrophage LPL secretion and gene expression: role of oxidative stress and PKC. Given the limited amount of biological material that can be obtained from human macrophages, the molecular mechanisms involved in leptin-induced LPL expression were studied in the J774 murine macrophage cell line. To ascertain the validity of this model, LPL secretion and gene expression were first measured in leptin-treated J774 macrophages. Incubation of murine macrophages with leptin led to a significant increase in LPL secretion by these cells. Maximal stimulatory effect of leptin on extracellular LPL mass was observed after a 24-h incubation period with 4 nmol/l of leptin. This effect was abolished by the Ob-R antibody (10 μg/ml) (LPL mass [% increase over control values]: leptin [4 nmol/l] 175 ± 12, $P < 0.001$; leptin + Ob-R antibody 104 ± 8, $P < 0.01$ vs. leptin; leptin + IgG_1 antibody 184 ± 23). Leptin (4 nmol/l) also significantly increased murine macrophage LPL mRNA levels. This effect was maximal at 1 h, declining toward baseline at 6 h (LPL mRNA levels [% increase over control values] at 1 h 176 ± 20, $P < 0.05$; at 3 h 128 ± 3, $P < 0.001$). Pretreatment of J774 macrophages with NAC (10 mmol/l) or GF109203X (20 nmol/l) abolished the stimulatory effect of leptin on macrophage LPL gene expression (Fig. 5).

While incubation of J774 cells for 1 h with PMA (500 nmol/l), but not with the nonactive analogue of PMA, 4α-PMA, increased LPL mRNA levels (LPL mRNA levels [% increase over control values] PMA 292 ± 52, $P < 0.01$; 4α-PMA 106 ± 19), endogenous PKC depletion by overnight PMA treatment totally abolished the stimulatory effect of leptin on macrophage LPL expression (LPL mass [% increase over control values]: leptin 103 ± 5; LPL activity [% increase over control values]: leptin 98 ± 6) and PKC-β expression (data not shown).

As illustrated in Fig. 6, treatment of J774 cells for 20 min with 4 nmol/l leptin or PMA, used in these experiments as positive control, increased the expression of PKC-α, -$β_I$,

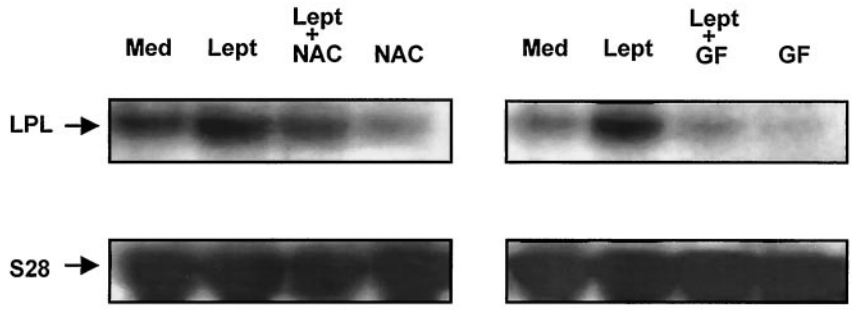

FIG. 5. Effect of antioxidant and PKC inhibitor on leptin-stimulated murine macrophage LPL gene expression. J774 cells were preincubated for 1 h in the presence of NAC (10 mmol/l) or GF109203X (20 nmol/l) and then treated for 1 h with 4 nmol/l leptin. At the end of these incubation periods, cells were lysed and total RNA was extracted and analyzed by Northern blot analysis for LPL (upper panel) and S28 (lower panel) mRNA expression. Representative blots from three independent experiments are shown.

-β_{II}, and -γ by 2.2-, 5.8-, 4.2-, and 5.5-fold, respectively. Conversely, the expression of PKC-ζ and PKC-ϵ was not enhanced by leptin (data not shown). Under these experimental conditions, no modulation of β-actin, used as internal control, was observed.

To evaluate whether other signaling pathways that are known to be activated by leptin might be involved in the stimulatory effect of leptin of macrophage LPL expression, J774 cells were incubated with leptin in the presence or absence of the phosphatidylinositol 3-kinase (PI3K) wortmannin (100 μmol/l) or the mitogen-activated protein kinase (MAPK) inhibitor PD98059 (50 μmol/l). Exposure of macrophages to these inhibitors did not reduce the stimulatory effect of leptin on macrophage LPL secretion (LPL mass [% increase over control values]: leptin 175 ± 12, $P < 0.001$; leptin + wortmannin 185 ± 67; leptin + PD98059 201 ± 43).

Effect of leptin on murine macrophage LPL synthetic rate. To determine the effect of leptin on macrophage LPL synthesis, J774 cells were treated for 24 h with leptin (4 nmol/l) and were then pulse-labeled. Cell extracts were immunoprecipitated to obtain the radiolabeled LPL protein. Heparin-stimulated cells and immunoprecipitation with an irrelevant antibody were used as positive and negative controls, respectively. As shown in Fig. 7, leptin led to a significant increase in macrophage LPL synthetic rate (LPL synthetic rate [% increase over control values]: leptin [4 nmol/l] 166 ± 18, $P < 0.01$).

Effect of leptin on the binding of nuclear proteins to the AP-1 sequence of the murine LPL gene promoter. To test whether incubation of J774 cells in the presence of leptin results in changes at the level of LPL gene binding proteins, we measured the binding activity of nuclear proteins extracted from leptin-treated J774 cells to the AP-1 consensus sequence of the LPL gene promoter. Electrophoretic mobility shift experiments showed that a 45-min exposure of J774 cells to leptin led to a considerable increase in the binding of nuclear proteins to the AP-1 consensus sequence of the LPL gene promoter. This binding complex was specifically competed in the presence of a 1,000-fold molar excess of the unlabeled AP-1 oligonucleotide. Pretreatment of the cells with GF109203X abolished the stimulatory effect of leptin on the binding of nuclear proteins to the AP-1 sequence of the LPL gene promoter. In contrast, no effect of PI3K and MAPK inhibitors on leptin-induced AP-1 activation was observed (Fig. 8).

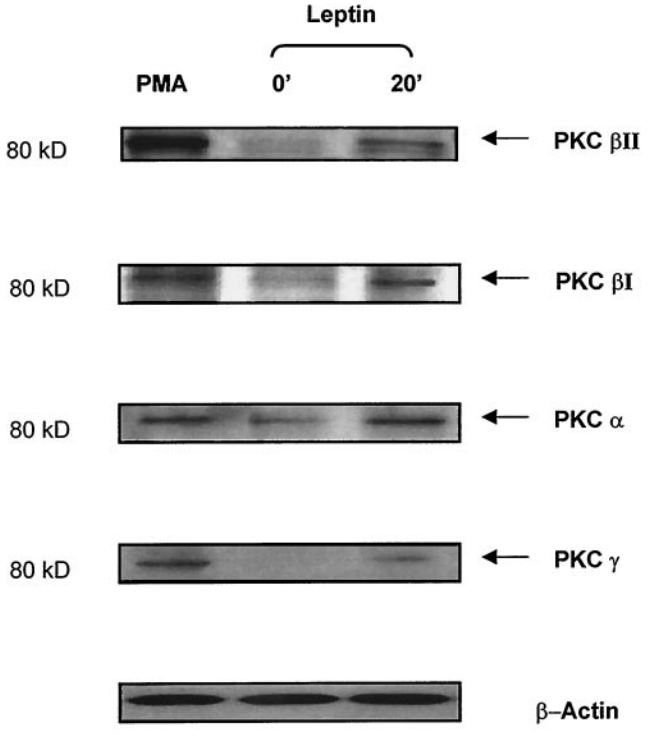

FIG. 6. Effect of leptin on conventional PKC isoform expression. J774 cells were incubated with 4 nmol/l leptin for 20 min. Membrane fractions were assayed for conventional PKC isoforms by Western blot analysis as described in RESEARCH DESIGN AND METHODS. Cells stimulated with PMA (500 nmol/l) for 30 min were used as positive control. The figure illustrates the results of one representative experiment of three.

DISCUSSION

Immune cells, and especially macrophages, play a crucial role in the development and progression of atherosclerotic lesions (35). Among the multiple molecules secreted by macrophages in the arterial wall, LPL has unique features in that it influences atherogenesis by a dual effect, namely a peripheral effect whereby it affects the composition of plasma lipoproteins and a local vascular effect whereby it favors foam cell formation and atherosclerosis in vivo (16,17,21,36,37).

Atherosclerotic cardiovascular disease is the leading cause of death in and the major complication of diabetes (38,39). Several cardiovascular risk factors, including hyperglycemia, glycoxidative modifications of lipoproteins, and advanced glycation end products, have been identified in patients with diabetes. Evidence that leptin is enhanced in the plasma of patients with diabetes (3) and promotes inflammation (5–7) supports the possibility that leptin may represent one of such factors. Consistent with this hypothesis, the present study demonstrates that leptin stimulates the secretion of macrophage LPL in vitro. In accordance with one previous study using preadipocytes (40), we

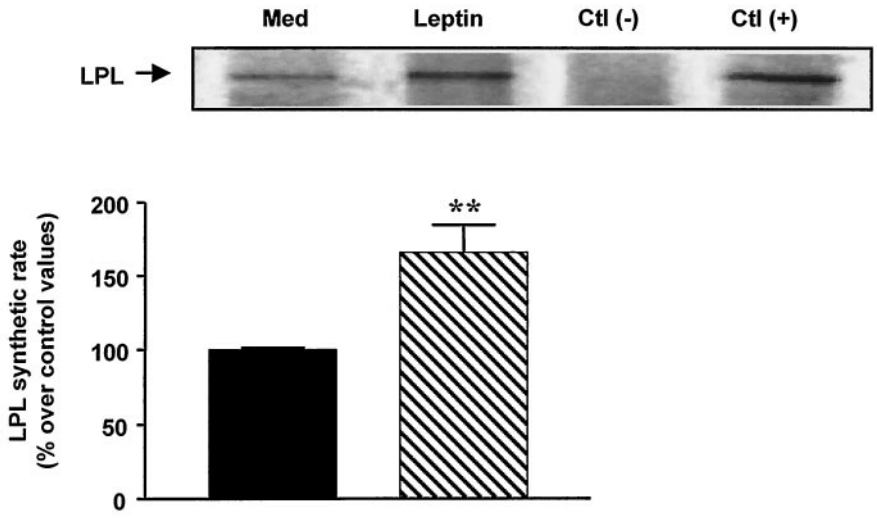

FIG. 7. Effect of leptin on murine macrophage LPL synthetic rate. J774 cells were incubated for 24 h with 4 nmol/l leptin and then incubated for 1 h with methionine-free medium. Cells were next labeled with [^{35}S]methionine for 30 min and chased for 1 h with complete DMEM. LPL was immunoprecipitated by incubating total cytoplasmic proteins with 10 μg of the monoclonal anti-LPL antibody 5D2. Heparin-stimulated cells and immunoprecipitation with an irrelevant antibody (IgG) were used as positive and negative controls, respectively. Data are means ± SE of four different experiments, $^{**}P < 0.01$ vs. medium (Med).

found that leptin enhances LPL gene expression in macrophages. Although we did not perform run-on assays, our data showing that induction of LPL mRNA levels by leptin parallels the increase in LPL synthesis and secretion suggest that macrophage LPL regulation in response to leptin takes place at the transcriptional level. This possibility is further supported by our results demonstrating a stimulatory effect of leptin on nuclear protein binding to the AP-1 sequence of the LPL gene. Macrophages possess the molecular machinery required to respond to leptin stimulation. Indeed, these cells express high levels of the long form of the leptin receptor and their stimulation with physiological concentrations of leptin results in the activation of intracellular signaling pathways linked to this receptor (7,41). Evidence that the Ob-R antibody inhibits the induction of macrophage LPL by leptin demonstrates that this hormone regulates macrophage LPL through its functional receptors. Our findings that NAC prevented the stimulatory effect of leptin on macrophage LPL further indicate that leptin stimulates macrophage LPL through an oxidative stress–dependent pathway. This observation is consistent with previous results showing that reactive oxygen species are effective enhancers of macrophage LPL expression (42) and that leptin induces oxidative stress in human endothelial cells (9,10).

PKC is implicated in the pathogenesis of vasculopathies associated with diabetes, and preferential activation of the conventional PKC isoforms is documented in diabetic vascular tissues (43,44). In accordance with the results of Takekoshi et al. (45) in chromaffin cells, we found that leptin enhances the expression of PKC -α, -β_I, -β_{II}, and -γ in macrophages. To the best of our knowledge, this is the

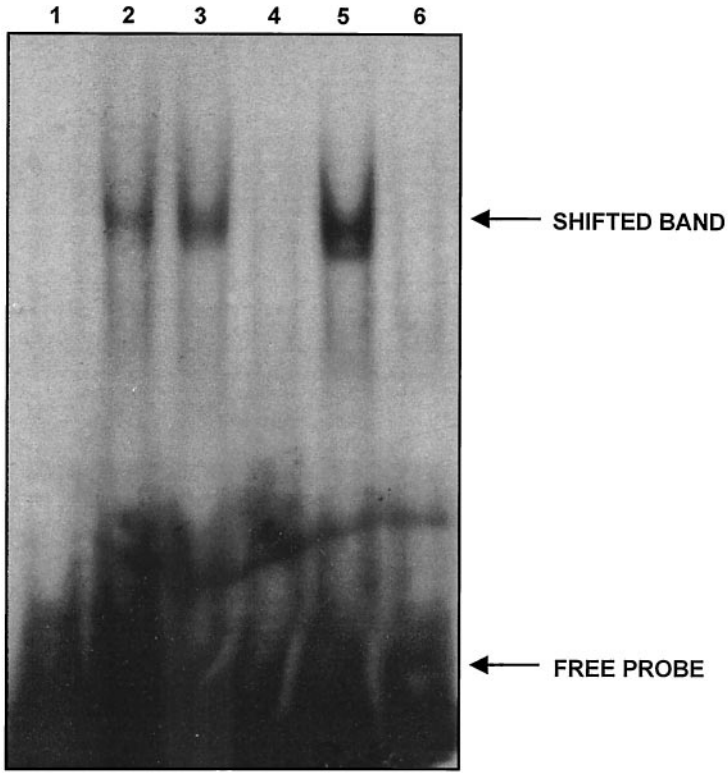

FIG. 8. Effect of leptin on the binding of nuclear proteins extracted from murine macrophages to the regulatory AP-1 sequence of the LPL gene promoter. J774 cells were incubated with 4 nmol/l leptin for 45 min in the presence or absence of PD98059 (50 μmol/l), GF109203X (20 nmol/l), or wortmannin (100 μmol/l). Nuclear proteins isolated from these cells were incubated with double-stranded AP-1 regulatory element of the LPL gene promoter. Retardation was assessed by gel electrophoresis in 4% PAGE. The figure shows one representative experiment of three. *Lane 1*: untreated cells; *lane 2*: leptin-treated cells; *lane 3*: leptin + PD98059; *lane 4*: leptin + GF109203X; *lane 5*: leptin + wortmannin; and *lane 6*: competition of the leptin-induced binding complex in the presence of 1,000-fold molar excess of the unlabeled AP-1 oligonucleotide.

first report that leptin activates PKC in vascular cells. Evidence that the conventional PKC selective inhibitor, GF109203X, abrogates the effect of leptin on macrophage LPL expression further stresses the key role of the conventional PKC isoenzymes in the regulation of macrophage LPL by leptin. Previous studies have demonstrated that PKC is involved in AP-1 activation (46) and that leptin stimulates c-*fos* expression (40). On the basis of these results, it is tempting to postulate that the activation of PKC by leptin that we documented in the present study could result in the binding of its downstream target, c-*fos*, to the AP-1 sequence of the LPL gene promoter, thereby leading to an increased transcription rate of this gene. This possibility is supported by our results showing that GF109203X inhibits the stimulatory effect of leptin on the binding of nuclear proteins to the AP-1 regulatory sequence of the LPL gene. Besides PKC and AP-1, leptin activates in mononuclear cells other signaling pathways, including PI3K and MAPK (47,48), and increases the expression of peroxisome proliferator–activated receptors (PPARs) (40). Evidence that insulin, a well-known activator of PI3K and MAPK, does not enhance human macrophage LPL (F.M., G.R., unpublished observations) and that PI3K and MAPK inhibitors do not inhibit the stimulatory effect of leptin on macrophage LPL secretion do not argue for a role of these kinases in leptin-induced macrophage LPL expression. Furthermore, our results showing that leptin does not affect PPAR activation in human macrophages do not support the involvement of PPARs in the regulation of the macrophage LPL gene by leptin.

Evidence has been provided that LPL secreted in the vessel wall exerts proatherogenic effects through both its structural and catalytic properties. Our results showing that leptin, at concentrations similar to those found in the plasma of diabetic patients, stimulates the release of increased amounts of active macrophage LPL suggest that the effect of leptin on macrophage LPL may promote atherogenesis in diabetes. In support for this possibility, O'Rourke et al. (49) recently reported that under hyperglycemic conditions, leptin significantly reduced the level of hormone-sensitive lipase in J774 macrophages. Establishing whether the in vivo effect of leptin on macrophage function favors atherogenesis in humans is of clinical interest especially in patients with diabetes who have increased plasma levels of leptin and demonstrate enhanced secretion of proatherogenic cytokines by macrophages, including tumor necrosis factor (TNF)-α and LPL (25).

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