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Leptin Induces Mitochondrial Superoxide Production and Monocyte Chemoattractant Protein-1 Expression in Aortic Endothelial Cells by Increasing Fatty Acid Oxidation via Protein Kinase A*

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Leptin, a circulating hormone secreted mainly from adipose tissues, is involved in the control of body weight. The plasma concentrations are correlated with body mass index, and are reported to be high in patients with insulin resistance, which is one of the major risk factors for cardiovascular disease. However, the direct effect of leptin on vascular wall cells is not fully understood. In this study, we investigated the effects of leptin on reactive oxygen species (ROS) generation and expression of monocyte chemoattractant protein-1 (MCP-1) in bovine aortic endothelial cells (BAEC). We found that leptin increases ROS generation in BAEC in a dose-dependent manner and that its effects are additive with those of glucose. Rotenone, thenoyltrifluoroacetone (TTFA), carbonyl cyanide m-chlorophenylhydrazone (CCCP), Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), uncoupling protein-1 (UCP1) HVJ-liposomes, or manganese superoxide dismutase (MnSOD) HVJ-liposomes completely prevented the effect of leptin, suggesting that ROS arise from mitochondrial electron transport. Leptin increased fatty acid oxidation by stimulating the activity of carnitine palmitoyltransferase-1 (CPT-1) and inhibiting that of acetyl-CoA carboxylase (ACC), pace-setting enzymes for fatty acid oxidation and synthesis, respectively. Leptin-induced ROS generation, CPT-1 activation, ACC inhibition, and MCP-1 overproduction were found to be completely prevented by either genistein, a tyrosine kinase inhibitor, H-89, a protein kinase A (PKA) inhibitor, or tetradecylglycidate, a CPT-1 inhibitor. Leptin activated PKA, and the effects of leptin were inhibited by the cAMP antagonist Rp-cAMPS. These results suggest that leptin induces ROS generation by increasing fatty acid oxidation via PKA activation, which may play an important role in the progression of atherosclerosis in insulin-resistant obese diabetic patients.

Leptin, a circulating hormone secreted mainly by adipose tissues, is involved in the control of body weight through the effects on food intake and energy expenditure (1, 2). In humans, the plasma concentrations of leptin are markedly correlated with body mass index and are also reported to be higher in insulin-resistant first-degree relatives of patients with non-insulin-dependent diabetes mellitus (NIDDM)[1] and type I diabetes compared with normal individuals (3–5). Disorders associated with hyperleptinemia such as obesity and insulin resistance are major risk factors for cardiovascular diseases (6, 7). Recently, the leptin receptor has been identified on endothelial cells, and leptin has been shown to promote both angiogenesis and inflammation (8, 9). However, the mechanism by which leptin induces inflammation remains to be elucidated.

Adipocytes have recently been shown to secrete pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-6 (10, 11), and plasminogen activator inhibitor-1, a serine protease inhibitor of fibrinolysis, which together may play an active role in the pathogenesis of accelerated atherosclerosis in diabetes (12–14). We hypothesized that leptin is also one of the mediators promoting atherosclerosis. We have very recently shown that hyperglycemia-induced mitochondrial overproduction of reactive oxygen species (ROS) serves as a causal link between elevated glucose and hyperglycemic vascular damage (15). Therefore, we investigated the direct effects of leptin on ROS generation, and the mechanism by which this induced expression of the redox-sensitive chemokine, monocyte chemoattractant protein-1 (MCP-1) (16) in bovine aortic endothelial cells (BAEC).

EXPERIMENTAL PROCEDURES

Cell Culture Conditions—Confluent BAEC cells (passage 4–10) were maintained in Eagle's minimal essential medium (Life Technologies, Inc., Grand Island, NY) containing 0.4% fetal bovine serum, essential and nonessential amino acids and antibiotics. Cells were incubated with various concentrations of human recombinant leptin plus either 5 mM glucose, 10 mM glucose, 30 mM glucose, 5 mM glucose plus 5 μM rotenone, 10 μM thenoyltrifluoroacetone (TTFA), 0.5 μM carbonyl cyanide m-chlorophenylhydrazone (CCCP), 100 μM Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), uncoupling protein-1 (UCP1) HVJ-liposomes, manganese superoxide dismutase (MnSOD) HVJ-liposomes, 50 μM genistein, 10 μM H-89 dihydrochloride, 25 μM Rp-cAMPS, or 20 μM tetradecylglycidate (TDGA),[2] as indicated. Leptin, rotenone, TTFA, CCCP, digitonin, palmitoyl-CoA, and butyryl-CoA were purchased from

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[1] The abbreviations used are: NIDDM, non-insulin-dependent diabetes mellitus; ROS, reactive oxygen species; MCP-1, monocyte chemoattractant protein-1; BAEC, bovine aortic endothelial cells; TTFA, thenoyltrifluoroacetone; CCCP, carbonyl cyanide m-chlorophenylhydrazone; MnTBAP, 100 μM Mn(III)tetrakis (4-benzoic acid) porphyrin; UCP-1, uncoupling protein-1; MnSOD, manganese superoxide dismutase; Rp-cAMPS, the Rp diastereoisomer of adenosine 3′, 5′-cyclic monophosphothionate, TDGA, tetradecylglycidate; 2-DG, 2-deoxy-D-glucose; PBS, phosphate-buffered saline; TCA, tricarboxylic acid; CPT-1, carnitine palmitoyltransferase-1; ACC, acetyl-CoA carboxylase; PKA, protein kinase A.

[2] TDGA was kindly given by Dr. J. M. Lowenstein (Brandeis University, Waltham, MA).

Sigma Chemical Co. MnTBAP, genistein, and H-89 dihydrochloride were purchased from Calbiochem (La Jolla, CA). 2-deoxy-D-[1-^{3}H]glucose, [1-^{14}C]palmitic acid, L-[*methyl*-^{3}H]carnitine, and [1-^{14}C]acetyl-CoA were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). 5 μl of each HVJ-liposome was added for experiments in 96-well plates (80,000 cells). Cells were washed after 2-h incubation. Transfection efficiency was >90% as assessed by FACS analysis of eGFP expression. Reagents alone had no effect on the variables measured in Figs. 1, 4, 5, 6, and 7 (data not shown).

Intracellular ROS—The intracellular formation of ROS was detected by using the fluorescent probe CM-H_2DCFDA (Molecular Probes Inc., Eugene, OR). Cells (1×10^5/ml) were loaded with 10 μM CM-H_2DCFDA, incubated for 60 min at 37 °C, and analyzed in an HTS 7000 Bio Assay Fluorescent Plate Reader (PerkinElmer Life Sciences) using the HTSoft program. ROS production was determined from an H_2O_2 standard curve (10–200 μM).

2-Deoxy-D-Glucose (2-DG) Uptake—Cells were incubated with 10 ng/ml leptin for 45 min in medium containing 5 mM glucose. Then cells were washed five times with phosphate-buffered saline (PBS) and incubated with 1 ml of PBS containing 1 μCi 2-deoxy-D-[1-^{3}H]glucose for 5 min. Cells were solubilized in 1 ml of 1 N NaOH for 60 min at 37 °C, and the radioactivity was counted as described previously (17).

Glucose Flux through Glycolysis—Cells were incubated with 10 ng/ml leptin for 45 min in medium containing 5 mM glucose. 10 μCi/ml of 5-^{3}H was added, and the conversion to [^{3}H]H_2O was quantitated as described previously (15).

Glucose Flux through the Tricarboxylic Acid (TCA) Cycle—Cells were incubated with 10 ng/ml leptin for 45 min in medium containing 5 mM glucose. 1 μCi/ml of U-^{14}C glucose was added, and the conversion to [^{14}C]CO_2 was quantitated as described previously (15).

Fatty Acid Oxidation—Cells were prelabeled overnight in medium containing 50 μM [1-^{14}C]palmitic acid. After trypsinization, cells were incubated with 10 ng/ml leptin for 2 h in medium containing 5 mM or 30 mM glucose. The conversion to [^{14}C]CO_2 was quantitated as described previously (18).

Epstein-Barr Virus Replicon Vectors—Rat UCP1 sense and antisense cDNAs were generously provided by Dr. Daniel Riquier, Center National de la Recherche Scientifique-Unite Propre 1511, Meudon, France. Human MnSOD cDNA was generously provided by Dr. Larry Oberley, University of Iowa College of Medicine, Iowa City, IA. These cDNAs were cloned into the Epstein-Barr virus replicon-based plasmid pEB (19) and used to prepare HVJ-liposomes.

Preparation of HVJ-Liposomes—Cationic HVJ-liposomes were prepared as described previously (19), using 9.75 mg of the dried lipids and 200 μg of plasmid DNA.

Assay for CPT-1 Activity—Cells were preincubated with or without TDGA, a specific irreversible inhibitor of CPT-1, for 45 min (20). Then the medium was aspirated, and cells were washed twice with PBS. Cells were then treated with either genistein or H-89. After 45 min, cells were incubated with or without 10 ng/ml leptin and were subsequently permeabilized with 700 μl of a medium containing 50 mM imidazole (pH 7.1), 70 mM KCl, 80 mM sucrose, 1 mM EGTA, 2 mM $MgCl_2$, 1 mM dithioerythritol, 1 mM KCN, 1 mM ATP, and 0.1% (w/vol) defatted and dialyzed bovine serum albumin (medium A), supplemented with 100 μl of 0.2 mg/ml digitonin. The medium was aspirated after 3 min, and reactions were started by addition of 700 μl of medium A supplemented with 100 μl of 0.4 mM palmitoyl-CoA plus 1.2 mM L-[*methyl*-^{3}H]carnitine. After 4-min incubation at 37 °C, the reactions were stopped with 0.8 ml of 2 N HCl, and [^{3}H]palmitoylcarnitine product was extracted with *n*-butanol (21).

Assay for Acetyl-CoA Carboxylase (ACC) Activity—ACC activity was determined in digitonin-permeabilized BAEC as the incorporation of radiolabeled acetyl-CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction. Cells were pretreated with or without either genistein or H-89 for 45 min and then treated with or without 10 ng/ml leptin. Cells were washed twice with PBS and then trypsinized. Reactions were subsequently started with 100 μl of cell suspension plus 100 μl of assay mixture containing 126 mM Hepes (pH 7.9), 21 mM NaCl, 4.2 mM $MgCl_2$, 1 mM citric acid, 20 mM $KHCO_3$, 4 mM ATP, 1 mM NADPH, 0.5 mM EGTA, 0.5 mM dithioerythritol, 1 mM KCN, 1 mM ATP, and 0.85% (w/v) defatted and dialyzed bovine serum albumin, 125 μM butyryl-CoA, 125 μM [1-^{14}C]acetyl-CoA, 3 munits of purified rat fatty acid synthase and 0.39 mg/ml digitonin. After 5-min incubation at 37 °C, the reactions were stopped with 100 μl of 10 N NaOH, and fatty acids were extracted by petroleum ether (22).

MCP-1 Expression—Cells were cultured with or without 10 ng/ml leptin in the presence or absence of genistein, H-89, Rp-cAMPS, or TDGA for 24 h. MCP-1 content in the medium was analyzed using an enzyme-linked immunosorbent assay kit derived from R&D systems (Minneapolis, MN), according to the manufacturer's instruction.

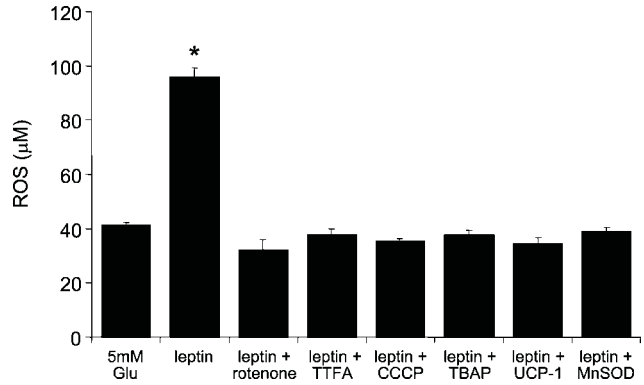

FIG. 1. **Effects of agents that alter mitochondrial metabolism on leptin-induced ROS generation in BAEC.** Cells were preincubated with or without rotenone, TTFA, CCCP, or MnTBAP for 45 min, then treated with or without 10 ng/ml leptin for 45 min, and then ROS was quantitated. Two days after transfection with HVJ-UCP-1 or HVJ-MnSOD liposomes, cells were treated with or without 10 ng/ml leptin for 45 min and then ROS was quantitated. *, $p < 0.01$ compared with cells incubated with 5 mM glucose alone.

PKA Assay—Cells were incubated with or without 10 ng/ml leptin for 45 min, lysed, and PKA activity was measured using a commerical protein kinase A assay kit (Calbiochem) according to the manufacturer's instructions.

cAMP Assay—Cells were incubated with or without 10 ng/ml leptin, lysed, and cAMP concentrations in whole-cell lysates were determined using the cyclic Amp (^{3}H) assay system (Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's instructions.

Statistical Analysis—Data were analyzed using the one-factor analysis of variance (ANOVA) procedure to compare the means of all the groups. The Tukey-Kramer multiple comparisons procedure was used to determine which pairs of means were different. Data are shown as mean ± S.E.

RESULTS

Effects of Leptin on Intracellular ROS Production in BAEC—To determine the site of leptin-induced intracellular ROS production, BAEC were incubated with either rotenone, an inhibitor of complex I, TTFA, an inhibitor of complex II, or CCCP, an uncoupler of oxidative phosphorylation that abolishes the mitochondrial membrane proton gradient, MnTBAP, a stable cell-permeable SOD mimetic, UCP-1 HVJ-liposomes, or MnSOD HVJ-liposomes. Compared with basal conditions (5 mM glucose), 10 ng/ml leptin increased ROS production to about 2.3-fold (Fig. 1). Furthermore, all the reagents were found to completely prevent the effects of leptin on ROS production in BAEC, demonstrating that ROS generation arises from mitochondrial electron transport chain.

We have very recently shown that hyperglycemia-induced ROS generation also arises exclusively from the mitochondrial electron transport chain (15). Therefore, we next investigated the additive effects of high glucose and leptin on ROS production in BAEC. As shown in Fig. 2, leptin increased ROS production in a dose-dependent manner at every concentrations of glucose tested, and the effect was synergistic with that of glucose at the highest leptin concentration.

Effects of Leptin on Glucose Transport, Glycolysis, and TCA Cycle—The effects of leptin on glucose transport activity of BAEC was assayed with a glucose analogue, 2-DG, in confluent cultures preexposed to 10 ng/ml leptin for 45 min. Leptin did not affect the uptake of 2-DG into BAEC (leptin-treated *versus* control cells; 7.29 ± 0.2 *versus* 7.18 ± 0.34 μmol/mg/min). Intracellular glucose oxidation begins with glycolysis in the cytoplasm, leading to the generation of pyruvate, which can also be transported into the mitochondria where it is oxidized by the TCA cycle. Therefore, we next investigated the effects of

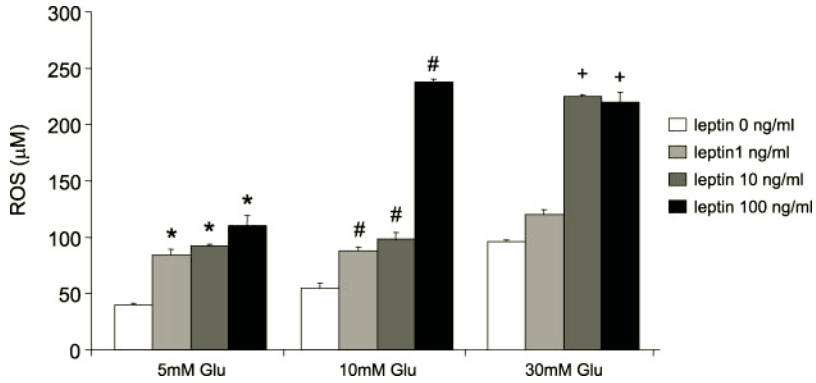

FIG. 2. **Effects of various concentrations of glucose and leptin on ROS generation in BAEC.** Cells were incubated with the indicated concentrations of glucose for 24 h and for the last 45 min with the indicated concentrations of leptin and then ROS was quantitated. *, $p < 0.01$, #, $p < 0.01$, or +, $p < 0.01$ compared with cells incubated with 5 mM glucose, 10 mM glucose, or 30 mM glucose alone, respectively.

leptin on glycolysis and the TCA cycle. Ten ng/ml leptin affected neither glycolysis nor the TCA cycle in BAEC (glycolysis and TCA cycle of leptin-treated *versus* control cells; 1.39 ± 0.36 *versus* 1.73 ± 0.16 nmol/mg/min and 0.131 ± 0.003 *versus* 0.123 ± 0.009 nmol/mg/min, respectively). These results suggest that neither glycolysis nor the TCA cycle is the source of increased ROS generation induced by leptin.

Effects of Leptin on Fatty Acid Oxidation in BAEC—We next investigated the effects of leptin on fatty acid oxidation in BAEC. For this, BAEC were incubated with [^{14}C]palmitate in the presence or absence of leptin and then $^{14}CO_2$ production was determined. Compared with basal conditions (5 mM glucose), 10 ng/ml leptin increased fatty acid oxidation about 1.5-fold (Fig. 3). Furthermore, high glucose was found not to inhibit the leptin-induced increase in fatty acid oxidation in BAEC. These results suggest that leptin produces ROS generation through an increase of fatty acid oxidation that is independent of glucose concentrations.

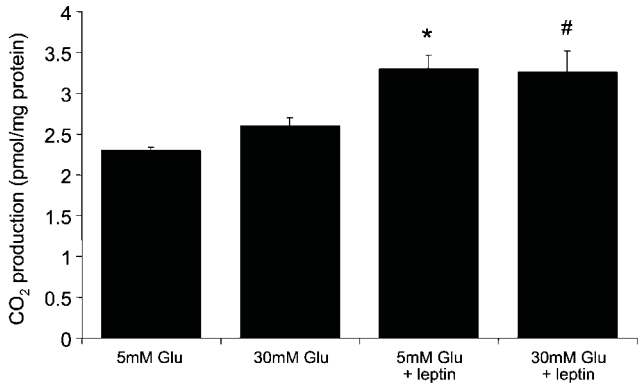

FIG. 3. **Effects of leptin on fatty acid oxidation in BAEC.** Cells were prelabeled overnight with 50 μM [1-^{14}C]palmitic acid, and the conversion to [^{14}C]CO_2 was quantitated under the indicated conditions. *, $p < 0.05$ compared with cells incubated with 5 mM glucose alone. #, $p < 0.05$ compared with cells incubated with 30 mM glucose alone.

Effects of Leptin on CPT-1 and ACC Activity in BAEC—CPT-1, located in the mitochondrial outer membrane, catalyzes the pace-setting step of long chain fatty acid translocation into the mitochondrial matrix, and is a key regulatory site of fatty acid oxidation. We investigated the effects of leptin on CPT-1 activity in BAEC. As shown in Fig. 4, leptin increased CPT-1 activity about 1.7-fold. Genistein, an inhibitor of tyrosine kinases and H-89, an inhibitor of protein kinase A (PKA), completely prevented the leptin-induced increase in CPT-1 activity in BAEC. Because CPT-1 is subject to inhibition by malonyl-CoA, the product of the reaction catalyzed by ACC, a key regulatory enzyme of fatty acid synthesis, we next studied the effects of leptin on ACC activity in BAEC. In contrast to the case of CPT-1, leptin was found to decrease ACC activity to about 50% of that of control cells (Fig. 5). Genistein or H-89 completely prevented the leptin-induced decrease in ACC activity in BAEC. Rp-cAMPS treatment similarly inhibited the leptin-induced increase in CPT-1 and the decrease in ACC activity (data not shown). These results suggest that activation of the leptin receptor tyrosine kinase increases fatty acid oxidation by increasing CPT-1 activity and decreasing ACC activity via PKA activation.

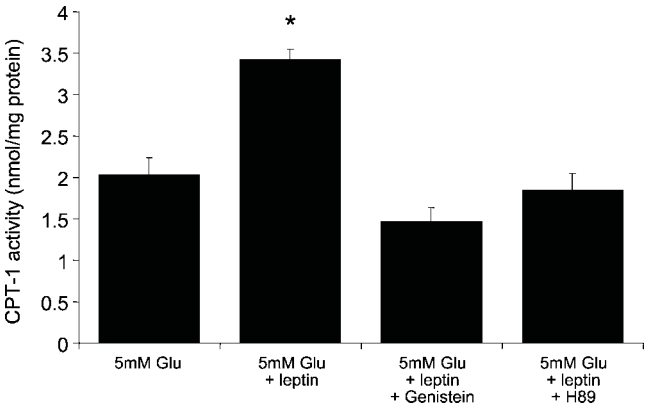

FIG. 4. **Effects of leptin on CPT-1 activity.** Cells were pretreated with either genistein or H-89 for 45 min and then incubated with 10 ng/ml leptin for 45 min. CPT-1 activity was measured as described under "Experimental Procedures." Enzyme activities are expressed as nmol of product/min/mg cell protein. *, $p < 0.01$ compared with cells incubated with 5 mM glucose alone.

Effects of Genistein, H-89, Rp-cAMPS, or TDGA on Leptin-induced ROS Production—We next investigated whether the inhibition of fatty acid oxidation induced by leptin could block leptin-induced ROS generation in BAEC. As shown in Fig. 6, genistein, H-89, Rp-cAMPS, or TDGA completely inhibited the production of ROS induced by leptin. The results indicate that leptin-induced ROS production is derived from PKA-induced fatty acid oxidation.

Effects of Genistein, H-89, Rp-cAMPS, or TDGA on Leptin-induced MCP-1 Production in BAEC—Because MCP-1 production is known to be induced by ROS (14), we studied whether leptin can stimulate the production of MCP-1 in BAEC. As shown in Fig. 7, compared with basal conditions (5 mM glucose), 10 ng/ml leptin increases the MCP-1 production about 1.4-fold. Genistein, H-89, Rp-cAMPS, or TDGA was found to completely inhibit the leptin-induced MCP-1 production in BAEC. The results suggest that the leptin-induced ROS production enhances the production of MCP-1 in BAEC by increasing fatty acid oxidation via PKA activation.

Effect of Leptin on PKA Activity—Because both H89 and Rp-cAMPS blocked the effects of leptin on ROS and MCP-1 production, PKA activity was directly measured in the presence and absence of 10 ng/ml leptin. Leptin induced a 2-fold increase in PKA activity compared with controls (56.0 ± 7.8 *versus* 27.2 ± 1.6 pmol/min/mg protein).

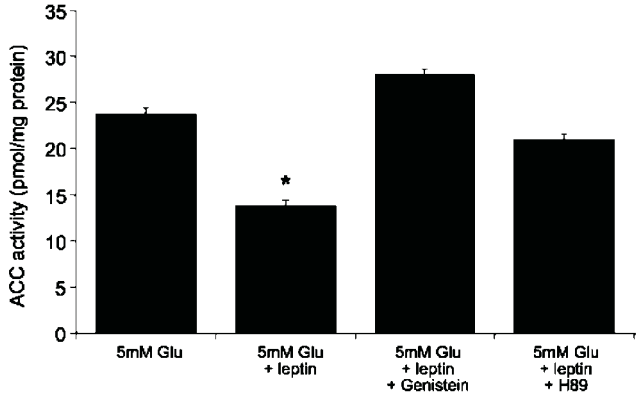

FIG. 5. **Effects of leptin on ACC activity.** Cells were pretreated with either genistein or H-89 for 45 min and then incubated with 10 ng/ml leptin for 45 min. ACC activity was measured as described under "Experimental Procedures." Enzyme activities are expressed as pmol of product/min/mg cell protein. *, $p < 0.01$ compared with cells incubated with 5 mM glucose alone.

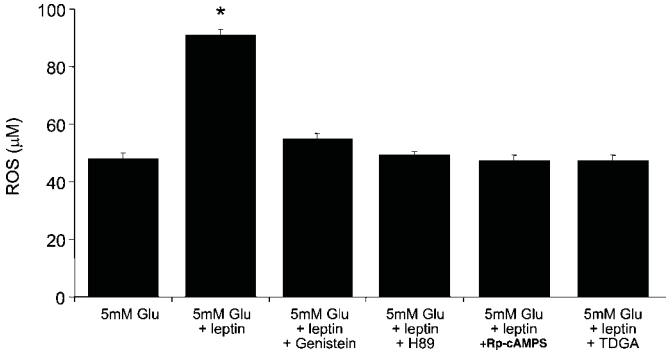

FIG. 6. **Effects of genistein, H-89, Rp-cAMPS, or TDGA on the leptin-induced ROS generation in BAEC.** Cells were preincubated with or without either genistein, H-89, Rp-cAMPS, or TDGA for 45 min and then treated with or without 10 ng/ml leptin. After 45 min, ROS were quantitated. *, $p < 0.01$ compared with cells incubated with 5 mM glucose alone.

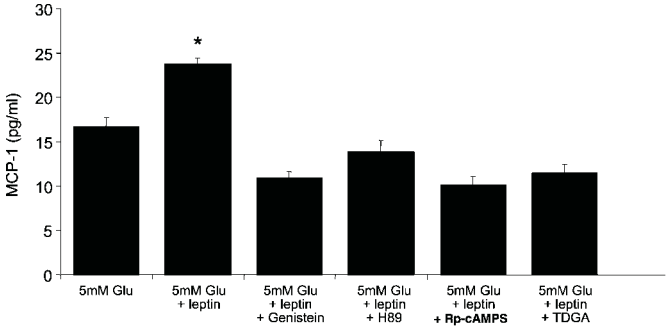

FIG. 7. **Effects of leptin on MCP-1 expression in BAEC.** Cells were incubated with or without 10 ng/ml leptin in the presence or absence of either genistein, H-89, Rp-cAMPS, or TDGA for 24 h. Medium was collected and MCP-1 content in the medium was analyzed with an enzyme-linked immunosorbent assay kit. *, $p < 0.01$ compared with cells incubated with 5 mM glucose alone.

Effect of Leptin on cAMP Concentration—To confirm that leptin induced cAMP, levels were determined after leptin stimulation. Leptin induced a 1.3-fold increase in cAMP in total cell lysate (13.28 ± 1.08 *versus* 10.33 ± 1.25 pmol/mg protein, $p < 0.03$).

DISCUSSION

In this study, we found for the first time that leptin induces mitochondrial superoxide production and MCP-1 expression in BAEC by increasing fatty acid oxidation via PKA. Like the AMP-activated protein kinase, PKA may stimulate CPT-1, a key enzyme of fatty acid oxidation, by two mechanisms: one is by decreasing malonyl-CoA levels through phosphorylation and inactivation of ACC, and the other is by modulating the interactions between CPT-1 and the cytoskeleton through phosphorylation of intermediate filaments in a malonyl-CoA-independent manner (23, 24). Our results suggest that PKA activation is a key step for the leptin-induced ROS generation in BAEC. According to the glucose fatty acid cycle hypothesis of Randle (25), glucose oxidation inhibits fatty acid oxidation. However, we did not observe this effect in our system. This likely reflects the fact that PKA and AMP-activated kinase may inhibit ACC activation by citrate derived from glucose oxidation and thus prevent the malonyl-CoA inhibition of CPT-1, which is the basis for Randle cycle (25, 26). Thus, the additive effects of leptin-induced fatty acid oxidation and glucose most likely reflect the fact that hyperglycemia enhances ROS formation through increased pyruvate oxidation in the TCA cycle (15), whereas leptin enhances ROS formation through increased beta oxidation and oxidation of the resultant acetyl-CoA through the TCA cycle.

Plasma levels of leptin in healthy subjects are less than 10 ng/ml. In obese subjects and insulin resistant first-degree relatives of patients with NIDDM, they are increased to 10–100 ng/ml, and similar values have been reported in type I diabetes (3–5). In this study, 10 ng/ml leptin was sufficient to nearly triple fatty acid-induced mitochondrial overproduction of ROS. The effect of 10 and 100 ng/ml leptin was further accentuated by diabetic levels of hyperglycemia. These data suggest that hyperleptinemia accelerates atherosclerosis in obese diabetic patients through ROS overproduction.

In insulin resistant states, the activity of hormone-sensitive lipase in adipose tissues is known to be increased, contributing to the elevation of free fatty acids (27). Therefore, the coexistence of hyperleptinemia and insulin resistance-induced elevation of free fatty acids may further enhance the ROS generation in vascular endothelial cells, promoting atherosclerosis.

ROS is recently reported to up-regulate many genes that are involved in various steps of atherosclerosis by inducing redox-sensitive transcriptional factors such as nuclear factor-κB and activator protein-1 (28–30). Among these mediators, MCP-1, a CC chemokine, plays an important role in the early phase of atherosclerosis by initiating monocyte/macrophage recruitment to the vessel wall (31), and its expression is actually known to be up-regulated in human atherosclerotic plaques (32). Furthermore, the selective targeting of CCR2, the receptor for MCP-1, was recently shown to markedly decrease atheromataous lesion formation in apoE knockout mice (33). Therefore, MCP-1 overproduction induced by leptin would promote fatty streak formation, the earliest histopathological hallmark of atherosclerosis. The elimination of ROS generation induced by leptin via fatty acid oxidation may provide a new therapeutic strategy for the treatment of accelerated atherosclerosis in diabetic patients.

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