Leptin modulates ACAT1 expression and cholesterol efflux from human macrophages

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Hongo S, Watanabe T, Arita S, Kanome T, Kageyama H, Shioda S, Miyazaki A. Leptin modulates ACAT1 expression and cholesterol efflux from human macrophages. Am J Physiol Endocrinol Metab 297: E474–E482, 2009; doi:10.1152/ajpendo.90369.2008.—Leptin is an adipose tissue-derived hormone implicated in atherosclerosis and macrophage foam cell formation. The current study was conducted to examine the effect of leptin on cholesterol ester accumulation in human monocytes/macrophages. Exogenously added leptin at 5 nM during differentiation of monocytes into macrophages for 7 days accelerated acetylated LDL (acetyl-LDL)-induced cholesterol ester accumulation by 30–50%. Leptin did not affect endocytic uptake of acetyl-LDL; however, it increased ACAT1 activity 1.8-fold and ACAT1 protein expression 1.9-fold. Among the four ACAT1 mRNA transcripts, two shorter transcripts (2.8 and 3.6 kb) were upregulated ~1.7-fold upon leptin treatment. The enhanced expression of ACAT-1 protein by leptin was suppressed by inhibitors of Janus-activated kinase2 (JAK2) and phosphatidylinositol 3-kinase (PI3K). HDL-mediated cholesterol efflux was suppressed by leptin, which was canceled by K-604, an ACAT-1 inhibitor. Expression of the long form of leptin receptor was upregulated during monocyte differentiation into macrophages and sustained after differentiation. Thus, the results suggest that leptin accelerates cholesterol ester accumulation in human monocyte-derived macrophages by increasing ACAT1 expression via JAK2 and PI3K, thereby suppressing cholesterol efflux.

 leptin receptor; acyl-coenzyme A:cholesterol acyltransferase-1; acyl-coenzyme A:cholesterol acyltransferase inhibitor; atherosclerosis

LEPTIN is a 16-kDa peptide hormone secreted by adipose tissues that targets the hypothalamus to regulate appetite and energy expenditure (56). When recombinant leptin is administered to ob/ob mice, which are characterized by expression of a truncated inactive leptin protein and marked obesity due to uncontrolled food intake, these animals show reductions in both food intake and weight (38). However, the concentration of circulating leptin is elevated in obese human subjects, suggesting leptin resistance in obesity (26). Obesity is regarded as a major risk factor for hypertension, diabetes, and other cardiovascular diseases. Elevation of plasma leptin has been suggested to promote cardiovascular diseases, including atherosclerosis (1, 49). In support of this notion, leptin-deficient ob/ob mice and leptin receptor (Ob-R)-deficient db/db mice, both of which are defective in leptin signaling, develop only small lesions after arterial injury despite severe obesity and deranged glucose and lipid metabolism on an atherogenic diet (41). Furthermore, a recent study indicates that when apolipoprotein (apo)E-deficient mice were injected daily with leptin for 4 wk, these mice had increased atherosclerotic lesions (4). Nevertheless, there remains argument about the role of leptin in atherogenesis in vivo.

The accumulation of macrophage-derived foam cells in the subendothelial space is a hallmark of early atherosclerotic lesions (14). Macrophages incorporate modified LDL, such as oxidized LDL and acetylated LDL (acyl-LDL), through scavenger receptors (16) and hydrolyze cholesteryl ester to free cholesterol, which is reesterified to cholesteryl ester or is exported to extracellular cholesterol acceptors. Intracellular cholesterol esterification is catalyzed by acyl-coenzyme A:cholesterol acyltransferase (ACAT), which is located in the rough endoplasmic reticulum (7). ACAT1 is expressed at high levels by macrophage-derived foam cells in atherosclerotic lesions (31) and upregulated during differentiation of human monocytes into macrophages in vitro (31, 50). Without showing changes in protein and mRNA levels, O’Rourke et al. (35) demonstrated the increase in ACAT activity by leptin treatment in the murine macrophage J774.2 cell line. Excess cholesterol is removed from macrophages by cholesterol efflux. ATP-binding cassette transporter A1 (ABC1) plays an essential role in efflux of cellular cholesterol to apolipoprotein A-I (apoA-I), a major HDL apolipoprotein (39). ATP-binding cassette transporter G1 (ABC1) and scavenger receptor class B type I (SR-BI) also mediate cholesterol efflux to particle HDL from macrophages (21).

Leptin elicits its biological function by binding to its specific receptor, Ob-R. Recently, Ob-R has been shown to be widely expressed in peripheral cell types (5, 33, 34, 55), including macrophages, outside the hypothalamus (43). Leptin can stimulate the proliferation of monocytes and production of cytokines from these cells (40) and increase phagocytotic activity of macrophages (12). Western blotting analyses of the murine macrophage cell line J774.2 indicated the presence of the long form of the leptin receptor (ObRb; 210 kDa) as well as multiple shorter forms (36). However, time-dependent changes in the expression of Ob-R protein during differentiation of human monocytes into macrophages have not yet been reported.

To examine whether leptin directly affects human monocytes/macrophages to promote atherosclerosis, we studied the effects of leptin on cholesteryl ester accumulation induced by acetyl-LDL in primary culture systems in the presence of high glucose. We also studied the effects of leptin on endocytic uptake of acetyl-LDL, ACAT1 expression, and cholesterol efflux. Furthermore, we first analyzed the time-dependent changes in the expression of Ob-R protein in primary cultured human monocytes/macrophages during differentiation.
MATERIALS AND METHODS

The study was approved by the Ethics Committee of Showa University. Cell culture. Human monocytes were isolated from the blood of healthy volunteers by Ficoll density gradient centrifugation (11) and subsequent cold aggregation (28). Monocytes (4 × 10^6 cells/6-cm dish) were allowed to adhere for 1 h with standard RPMI 1640 (R8758; Sigma, St. Louis, MO) that contains 2.0 g/l glucose and were further incubated for 7 days with RPMI 1640 containing 10% pooled human serum to induce differentiation into macrophages in the presence of recombiant human leptin (L4146; Sigma). The medium was changed every 3 days.

Western blotting analyses. Cells were extracted with 100 µl of 10% SDS and subjected to Western blotting (44) using specific antibodies raised against human ACAT1 (DM10) (6), human scavenger receptor class A (SR-A) (MAB2708; R & D Systems, Minneapolis, MN), human ABCA1 (NB400-105; Novus Biologicals, Littleton, CO), human ABCG1 (NB400-132; Novus Biologicals), mouse SR-BI (NB400-104; Novus Biologicals), or human leptin receptor (MAB867; R & D Systems). For analysis of SR-A protein, samples were incubated with 5% 2-mercaptoethanol at 95°C for 5 min prior to loading.

Evaluation of signal transduction pathway of ACAT1 expression. Monocytes (1.2 × 10^6 cells/3.5-cm dishes) were incubated for 7 days with the Janus-activated kinase 2 (JAK2) inhibitor tyrphostin AG490 (30 µM), the MEK1 inhibitor PD-98059 (1 µM), the phosphatidylinositol 3-kinase (PI3K) inhibitor LY-294002 (1 µM), or the protein kinase C (PKC) inhibitor rottlerin (1 µM) in the presence of leptin (5 nM). Inhibitor concentrations were based on our previous study (52) and IC50 for each inhibitor. We confirmed that the inhibitors alone were not cytotoxic.

Labeling of cellular cholesterol and subsequent efflux. apoE-free HDL (density = 1.063 to 1.21) was prepared as described (17, 32). In a standard experiment, monocytes (8 × 10^5 cells/12-well plates) were incubated for 7 days with leptin (0–10 nM) and loaded for 24 h in the presence of leptin with 10 µg/ml acetyl-LDL that had been preincubated for 8 h at 37°C with 74 kBq/ml [1,2-3H(N)]cholesterol (PerkinElmer, Boston, MA) in RPMI 1640 containing 10% human serum. After being washed, cellular lipids were extracted twice for 30 min with 0.6 ml of hexane/isopropanol (3:2) (vol/vol) and dried up under nitrogen. Samples were dissolved in 60 µl of isopropanol, applied on a thin-layer chromatography plate, and developed with hexane-diethyl ether-acetic acid-methanol (85:20:1:1) (vol/vol/vol/vol). Free and esterified [3H]cholesterol were cut out for liquid scintillation counting.

For cholesterol efflux assay (48), macrophages loaded for 24 h with acetyl-LDL prelabelled with [3H]cholesterol were incubated for 16 h with HDL or apoA-I (15 µg/ml; Academy Bio-Medical, Houston, TX) in RPMI 1640 containing 0.1% (wt/vol) bovine serum albumin. Cells were washed and lysed with 0.3 ml of 0.2 N NaOH. Cholesterol efflux was expressed as the percentage of the medium radioactivity of the sum of the medium and lysate radioactivities. During efflux period, 1 µM K-604, an ACAT1 inhibitor (Kowa Tokyo, Tokyo, Japan) (20), was added to test its effect on cholesterol efflux.

Quantitative real-time reverse transcription-polymerase chain reaction. Levels of mRNA expression were determined by quantitative real-time reverse transcription-polymerase chain reaction. Total RNA was isolated from the cultures on 3.5-cm dishes with the QiAshredder and RNaseasy Protect Mini Kit (Qiagen, Tokyo, Japan). Total RNA (1 µg) per 20-µl reaction was reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA), and 2 µl of each cDNA was amplified in a total volume of 50 µl using the SYBR Premix Ex Taq II kit (Takara Bio, Otsu, Japan). PCR assays were carried out according to the manufacturer’s instructions in the ABI Prism 7000 sequence detection system (Applied Biosystems). Each sample was run in triplicate. Messenger RNA levels were determined after normalization of RNA concentration with human GAPDH, and values were expressed as fold changes against control. The primers used in this study were as follows: leptin receptor, transcript variant 1, mRNA (accession no. NM_002303), 5′-TGTTCCTGGGCAACAGGACTA-3′ (forward) and 5′-CACAGTTGTCGATCATCTCATC-3′ (reverse); leptin receptor, transcript variant 2, mRNA (accession no. NM_001003679), 5′-ACTGAAACCTCAAGACCTCTG-3′ (forward) and 5′-AAAGTTTCTGTCCTCAAGTA-3′ (reverse); leptin receptor, transcript variant 3, mRNA (accession no. NM_001003680), 5′-TGTTTCTGGGCAACAGGACCTA-3′ (forward) and 5′-TGTTGTCCTGGGTACTTGAAG-3′ (reverse); GAPDH (accession no. NM_002046), 5′-GACACCTCAGGCTGAGAAC-3′ (forward) and 5′-TGTTGAAGACCGCAGTGG-3′ (reverse).

Fig. 1. Effect of leptin on acetylated LDL (acetyl-LDL)-induced cholesteryl ester accumulation in human monocytes/macrophages. A: human monocytes were incubated for 7 days to induce differentiation into macrophages in the absence or presence of leptin (5 nM), followed by incubation for 15 h with the indicated concentrations of acetyl-LDL in the presence of 0.1 nM [3H]oleate. Cellular cholesteryl ester accumulation was determined by measuring the radioactivity of cholesteryl [3H]oleate. Data are expressed as means ± SE from duplicate determinations of 4 independent experiments with monocytes from 4 different donors. Control values (0 µg/ml acetyl-LDL, 0 nM leptin) in individual experiments are expressed as 1. Average control value was 0.19 nmol·mg cell protein^-1·15 h^-1. *P < 0.05, †P < 0.001 vs. 0 nM leptin. B: dose-dependent effect of leptin on cholesteryl ester accumulation. Cells were incubated with the indicated concentrations of leptin as described above and loaded with 10 µg/ml acetyl-LDL and 0.1 mM [3H]oleate for 15 h. Data are expressed as means ± SE from duplicate determinations of 3 independent experiments with monocytes from 4 different donors. Control values (10 µg/ml acetyl-LDL, 0 nM leptin) in individual experiments are expressed as 1. Average control value was 1.6 nmol·mg cell protein^-1·15 h^-1. ‡P < 0.001, ††P < 0.0001 vs. 0 nM leptin.
**Immunocytochemical analysis.** The presence of leptin receptors on human monocytes/macrophages was studied by immunocytochemistry with anti-leptin receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA) that can recognize both long (Ob-RL) and short forms (Ob-RS) of human leptin receptors. Peripheral monocytes were cultured for 3 days and fixed in 4% paraformaldehyde in PBS for 30 min. These cells were incubated in 10% normal horse serum in PBS for 1 h on ice and then incubated with goat anti-leptin receptor antibody (1:100) at 4°C overnight followed by incubation with Alexa fluor 546 donkey anti-goat IgG (1:400; Invitrogen Carlsbad, CA) for 1.5 h at room temperature. Immunolabeling was detected with a fluorescence microscope (Nikon Eclipse TE300; Nikon, Tokyo, Japan). Cells to be used as controls were incubated without primary antibody.

**Other assays.** ACAT enzyme activity was determined by the reconstitution assay (8, 11). Expression of ACAT1 mRNA was analyzed by Northern blotting as described (44). LDL (density = 1.019–1.063) and acetyl-LDL were prepared as described (30, 42). Incorporation of exogenous [3H]oleate into cellular cholesteryl [3H]oleate (cholesterol esterification) was determined as described (15, 29). Acetyl-LDL was iodinated with Na125I (PerkinElmer), using ICl (27) to a specific radioactivity of 1.03 × 106 counts·min⁻¹·μg⁻¹ protein followed by determination of endocytic degradation and cell association of [125I]acetyl-LDL (30).

**Statistical analysis.** Values expressed as means ± SE were analyzed statistically using one-way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons or Student’s t-test for unpaired data. Differences were considered statistically significant at P < 0.05.

**RESULTS**

**Leptin increases acetyl-LDL-induced cholesteryl ester accumulation in human monocyte-derived macrophages.** We examined cholesteryl ester accumulation by incorporation of exogenous [3H]oleate into cellular cholesteryl [3H]oleate. Acetyl-LDL induced accumulation of cholesteryl [3H]oleate in a dose-dependent manner, which was further increased by leptin ~1.5-fold (Fig. 1A). Even in the absence of acetyl-LDL, cholesteryl [3H]oleate formation was significantly increased by leptin, implying that ACAT-accessible cholesterol pool was increased by leptin in nonloaded cells. Figure 1B shows that significant effects of leptin were detected at 0.5 nM and maximized at 2 nM.

**Leptin does not influence endocytic uptake of acetyl-LDL by human monocyte-derived macrophages.** We first focused on endocytic uptake of acetyl-LDL by macrophages. Figure 2A shows that leptin (5 nM) had no significant effect on cell association or endocytic degradation of [125I]acetyl-LDL. In addition, leptin did not change the level of SR-A protein that is involved in uptake of acetyl-LDL (Fig. 2B), indicating that enhancement of cholesteryl ester accumulation by leptin (Fig. 1) is due to a postreceptor event.

**Leptin increases ACAT1 expression.** We next focused on ACAT1. Incubation of monocytes for 7 days with leptin led to a dose-dependent increase in ACAT1 protein, with a near-maximal response (1.9-fold) at 5 nM (Fig. 3A). The enhanced effect of leptin (5 nM) on ACAT1 protein expression was recognizable from day 3 to day 5 and became prominent on day 7 (data not shown). Radioimmunoassays (leptin RIA kit; SRL, Tokyo, Japan) showed that concentrations of endogenous leptin in the medium were 0.023 ± 0.0021 nM on day 0, 0.013 ± 0.0036 nM on day 3, and 0.017 ± 0.0021 nM on day 7, which were negligible compared with exogenous leptin (2–20 nM). Consistent with the increase in ACAT1 protein (Fig. 3A), leptin (5 nM) significantly increased ACAT activity 1.8-fold (Fig.
Northern blotting analyses showed that leptin selectively increased two shorter ACAT1 mRNA species of 2.8 and 3.6 kb (Fig. 3C, left) 1.6- and 1.7-fold, respectively (Fig. 3B, right), without significant changes in the 4.3- and 7.0-kb species. To determine how leptin upregulates ACAT1 expression, we tested specific inhibitors of JAK2 (tyrphostin AG490), MEK1 (PD-98059), PI3K (LY-294002), and PKC (rottlerin) on leptin-induced ACAT1 expression. The increase in ACAT1 protein homogenates were solubilized and determined for ACAT activity using the reconstituted assay. Data are expressed as means ± SE from duplicate determinations of 6 independent experiments using monocytes from 6 different donors. Control value = 36 pmol min⁻¹ mg⁻¹ protein. This value is expressed as 1. *P < 0.0005 vs. 0 nM leptin control. C, left: total RNA was extracted, and ACAT1 mRNA was detected by Northern blotting analysis. C, right: bar graphs show the results of densitometric analysis, in which the intensity of 18S rRNA band was used as an internal control to normalize the intensity of ACAT1 mRNA subspecies. Intensities of 4.3-kb bands were measured after prolonged exposure to X-ray film. Data are expressed as means ± SE from 3 independent experiments using monocytes from 3 different donors. †P < 0.005, ††P < 0.01 vs. 0 nM leptin control. D: human monocytes were incubated for 7 days without [negative control (cont)] or with leptin (5 nM; positive cont) in the presence or absence of JAK2 inhibitor tyrphostin AG490 (AG490; 30 μM), MEK1 inhibitor PD-98059 (1 μM), phosphatidylinositol 3-kinase inhibitor LY-294002 (1 μM), or PKC inhibitor rottlerin (1 μM). Cells were subjected to Western blotting analyses for ACAT1. The bar graph shows densitometry of ACAT1 normalized to β-actin. Data are expressed as means ± SE from 3 independent experiments using monocytes from 3 different donors. *P < 0.05 vs. positive control.
Human monocytes/macrophages were loaded with \[^{3}H\]cholesterol as above, and efflux was initiated by exposing cells for 16 h to HDL (15 g/ml). apoA-I-mediated efflux in the absence of leptin or K-604 was normalized to 100%. Data are expressed as means ± SE from 3 independent experiments using monocytes from 3 different donors. Control values (0 nM leptin) in individual experiments are expressed as 1. Average control values for free \[^{3}H\]cholesterol and esterified \[^{3}H\]cholesterol were 4,700 and 840 disintegrations·min⁻¹·μg⁻¹·cell protein, respectively. #p < 0.0005 vs. 0 nM leptin control. B: following \[^{3}H\]cholesterol loading for 24 h as above, efflux was initiated by adding HDL (15 μg/ml). After 16 h, the radioactivity in the medium and cells was measured. Cholesterol efflux is expressed as the percentage of the medium radioactivity of the total radioactivity (cells + medium). Data are indicated as means ± SE from duplicate determinations of 3 independent experiments using monocytes from 3 different donors. *p < 0.05, †p < 0.01, ‡p < 0.005 vs. 0 nM leptin control. C: effect of K-604, an ACAT1 inhibitor, on leptin-dependent suppression of cholesterol efflux to HDL. Human monocytes/macrophages were loaded with \[^{3}H\]cholesterol as above, and efflux was initiated by exposing cells for 16 h to HDL (15 μg/ml) in the absence or presence of K-604 (1 μM). HDL-mediated efflux in the absence of leptin or K-604 was normalized to 100%. Data are indicated as means ± SE from duplicate determinations of 3 independent experiments using monocytes from 3 different donors. †p < 0.001, *p < 0.01. D: effect of K-604 on cholesterol efflux to apolipoprotein A-I (apoA-I) in the presence of leptin. Human monocytes/macrophages were loaded with \[^{3}H\]cholesterol as above, and efflux was initiated by exposing cells to apoA-I for 16 h (15 μg/ml) in the absence or presence of K-604 (1 μM). apoA-I-mediated efflux in the absence of leptin or K-604 was normalized to 100%. Data are indicated as means ± SE from duplicate determinations of 3 independent experiments using monocytes from 3 different donors. *p < 0.01. NS, no significant difference.

We examined the proteins involved in cholesterol efflux. Western blotting analyses for ABCA1, ABCG1, and SR-BI were performed using human monocytes/macrophages that were incubated for 7 days with various concentrations of leptin. However, repeated analyses did not show significant changes in the expression of these proteins by leptin (Fig. 5).

**Upregulation of long form of Ob-R during differentiation of human monocytes into macrophages.** The biological function of leptin is mediated through its receptor (Ob-R) on the cell surface (10). We further examined whether the level of Ob-R could be altered during differentiation of monocytes into macrophages. Western blotting analysis of Ob-R revealed 150- and 80-kDa proteins in human monocytes/macrophages (Fig. 6A). The 150-kDa protein was verified as the long form of Ob-R (Ob-RL) because a band with an equivalent migration rate was detected in COLO320DM cell lysate used as a positive control of Ob-RL (46). The expression of 150-kDa Ob-R was very weak on day 1, was increased by day 3, and reached an almost maximal level from day 7 to day 10 during monocytic differentiation into macrophage (Fig. 6, A and B). The elevated level of Ob-RL was sustained until day 14. The level of the short form of Ob-R (Ob-RS; 80 kDa) decreased during the monocytic differentiation from day 1 to day 7 and elevated slightly from day 7 to day 14.

We then studied the change in the expression of Ob-R mRNA isoforms, transcript variant 1 (long form) and variants 2 and 3 (short forms). Quantitative real-time RT-PCR indicated that expression of variants 1 and 3 increased, whereas variant 2 decreased, during differentiation from monocytes (day 1) into macrophages (day 7) (Fig. 6C). The pattern of change in variant 2 mRNA expression resembled that of 80-kDa Ob-R protein. Variant 3 mRNA may not play a major role in production of Ob-R protein in macrophages. Thus, increase in the expression of Ob-RL during differentiation from monocytes into macrophages was shown at protein and mRNA levels.

Immunocytochemical analyses confirmed the expression of Ob-R in human monocytes cultured for 3 days (Fig. 6D).
Leptin receptor-like immunoreactivity was not detected in control cells incubated in the absence of primary antibody (data not shown).

DISCUSSION

The accumulation of macrophage-derived foam cells is a hallmark of atherosclerosis. Leptin has been implicated in the development of atherosclerosis and cardiovascular diseases (1, 4, 41) with little information about its effect on cholesterol status in human macrophages. The results of the present study indicated that leptin increases cellular accumulation of cholesteryl ester induced by acetyl-LDL in human monocytes/macrophages (Fig. 1) without influencing endocytic uptake of acetyl-LDL (Fig. 2). This was explained by upregulation of ACAT1 and suppression of cholesterol efflux by leptin. (Figs. 3 and 4). These effects of leptin were observed at physiological concentrations (2–5 nM). Plasma leptin level is elevated with obesity: 43 ± 9.4 ng/ml in obese subjects compared with the

Fig. 6. Expression of leptin receptor (Ob-R) in human monocytes/macrophages. A: human monocytes were incubated for the indicated times in the medium containing 10% human serum to induce differentiation into macrophages. Ob-R protein was analyzed by Western blotting using an antibody directed against recombinant human Ob-R extracellular domain. Ob-R proteins were revealed as 150- and 80-kDa bands. Lysate of COLO320DM (human colon cancer cell) was used as positive control. B: densitometric analysis of the change in Ob-R expression. Data are expressed as means ± SE from 3 independent experiments using monocytes from 3 different donors. Expression of 150-kDa Ob-R on day 14 and expression of 80-kDa Ob-R on day 7 during differentiation of monocytes into macrophages. Ob-R variant 1 mRNA (long form) and variants 2 and 3 mRNA (short forms) were quantified by real-time RT-PCR. Amounts of mRNA were normalized with those of GAPDH. Data are expressed as means ± SE from 4 independent experiments with monocytes from 4 different donors. *P < 0.001 vs. day 1 control. C: expression of Ob-R mRNA on day 1 and day 7 during differentiation of monocytes into macrophages. Ob-R variant 1 mRNA (long form) and variants 2 and 3 mRNA (short forms) were quantified by real-time RT-PCR. Amounts of mRNA were normalized with those of GAPDH. Data are expressed as means ± SE from 4 independent experiments with monocytes from 4 different donors. *P < 0.001 vs. day 1 control. D: immunocytochemical detection of Ob-R in primary human monocytes cultured for 3 days. Scale bar = 40 μm.
value of 5.6 ± 1.3 ng/ml in normal-weight subjects (19). Other groups reported that the plasma leptin concentration reached values as high as 97.9 (6.1 nM) (37) to 200 ng/ml (12.5 nM) (26) in obese subjects. Our results indicated that physiological concentrations of leptin (2–5 nM) can enhance cellular accumulation of cholesteryl ester. Thus, it seems reasonable to speculate that elevated leptin concentrations accelerate foam cell formation and atherosclerosis in human.

O’Rourke et al. (35) reported that leptin at 10 nM significantly increased deposition of cholesteryl ester and ACAT activity in the murine macrophage cell line J774.2 at a high concentration (20 mM, 3.6 g/l) of glucose. To clarify physiological implications of this phenomenon, we used primary cultured human monocytes/macrophages as a model system instead of a mouse cell line. For cell culture, we used a standard RPMI 1640 medium containing 2.0 g/l d-glucose, which is higher than normal plasma concentration around 1.0 g/l but reachable in the diabetic blood. We showed increases of ACAT1 mRNA and protein levels in addition to its enzyme activity. Furthermore, we first demonstrated that leptin suppressed HDL-mediated cholesteryl efflux from human macrophages (Fig. 4). This is an essential difference between the current study and the previous one using J774.2 macrophages (35).

Although HDL-mediated cholesterol efflux was suppressed by leptin (Fig. 4, B and C), there was no significant change in the expression of the key molecules involved in cholesteryl efflux, such as ABCG1, ABCA1, and SR-BI (Fig. 5) (21). However, in contrast to our result with human macrophages, leptin injection into ob/ob mice increased hepatic SR-BI expression (25), indicating tissue-specific regulation of SR-BI by leptin in hepatocytes.

Reduced HDL-mediated cholesterol efflux by leptin is due in part to upregulation of ACAT1 by leptin that increases ACAT-accessible cholesterol pool (Fig. 1) in endoplasmic reticulum, thereby reducing HDL-accessible cholesterol pool in the cell surface. This notion was supported by the results that an ACAT1 inhibitor, K-604, reversed the inhibition of cholesterol efflux by leptin (Fig. 4C). Other scenarios are also possible. Cholesterol removal from cells involves several biophysical or biological processes, such as by intracellular cholesterol trafficking, apolipoprotein-mediated membrane microsolubilization (13), aqueous diffusion of cholesterol from plasma membrane (22), and cholesterol secretion in a complex with macrophage-derived apoE (57). These processes might be modulated by leptin independently of increased ACAT activity.

The biological functions of leptin are mediated through its receptors (Ob-R), which belong to the class I cytokine receptor family (47). In rodents, at least six differentially spliced mRNAs have been described with different 3’-terminal exons (23, 51). The long-form Ob-R (Ob-Rb) is widely expressed in the hypothalamus and many other peripheral tissues (18), whereas the short forms of Ob-R are present mainly in peripheral tissues (24). Recent studies indicated that different isoforms of Ob-R are coexpressed in various tissues (24, 55). In humans, the presence of the long form of Ob-R (Ob-RL) and three short forms of Ob-R (Ob-RS; Hub219.1–Hub219.3) have been reported (2, 9). Leptin receptors were shown to be expressed on peripheral platelets (33) and vascular cells (5, 34). The expression of Ob-R protein in macrophages was demonstrated by Western blotting analysis only in the murine J774.2 cell line (36).

In the present study, we demonstrated time-dependent changes in the expression of Ob-R protein detected as 150- and 80-kDa bands (Fig. 6). A molecular mass of human Ob-RL is calculated as 132 kDa, and those of three Ob-RS isoforms are calculated as ~105 kDa from amino acid sequences. Therefore, the 150-kDa band detected in the present study can be regarded as that of Ob-RL. Molecular masses of 150 and 160 kDa have been reported for mouse brain long form of Ob-R (53) and mouse recombinant long form of Ob-R (54), respectively, as estimated from mobilities in Western blotting analyses. The value of 150 kDa indicated in our study is consistent with these data. The expression of 150-kDa Ob-RL was up-regulated following differentiation of monocytes into macrophages from day 3 to day 7 and sustained until day 14 (Fig. 6, A and B). However, despite high expression of Ob-R in differentiated macrophages (days 10–14), ACAT1 protein as well as acetyl-LDL-induced cholesteryl ester accumulation was not increased by leptin when added after differentiation (data not shown), indicating that regulation of ACAT1 by leptin is specific for the stage of monocytic differentiation. Because the apparent molecular mass (80 kDa) coincides with the published values of the short form of Ob-R (53) and the time-dependent changes are similar between the 80-kDa band and variant 2 mRNA, we regarded the 80-kDa Ob-RL as 80-kDa Ob-RL (53) and the published values of the short form of Ob-R (53) and the molecular masses of 150 and 160 kDa have been reported for mouse brain long form of Ob-R (53) and mouse recombinant long form of Ob-R (54), respectively. Thus, the role of macrophage Ob-R in atherogenesis is still controversial.

In summary, leptin accelerates the accumulation of cholesteryl ester by upregulating ACAT1 expression and suppressing HDL-mediated cholesteryl efflux in human macrophages. Although this may explain one of the molecular mechanisms for accelerated atherosclerosis by leptin, its in vivo implication should be addressed in the future studies.

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