Effects of leptin on acyl-CoA: cholesterol O-acyltransferase-1 expression and activity during foam cell differentiation in human mononuclear THP-1 cells

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Abstract: The mechanism of atherosclerosis is not yet entirely clear. The transformation of mononuclear cells into macrophages has been shown to be one of early events leading to atherosclerosis. This study was to detect the effects of leptin on the acyl coenzyme A: cholesterol acyltransferase-1 (ACAT-1) expression and activity during foam cell differentiation in human acute monocyctic leukemia cell line (THP-1). Human mononuclear THP-1 cells were induced to differentiate into macrophages and foam cells following thawing. Leptin concentration gradients and leptin/anti-leptin antibodies were added to culture medium, and ACAT-1 mRNA and protein expression was analyzed to determine the effects of leptin on ACAT-1 expression. During mononuclear cell differentiation into macrophages, as well as from macrophages into foam cells, leptin upregulated ACAT-1 mRNA and protein expression in a dose- and time-dependent manner, with highest expression (10 mmol/L) on day 3. The upregulatory effects of leptin were completely blocked by anti-leptin antibody. The effects of leptin on the ACAT-1 expression and activity during foam cell differentiation were in a time- and dose-dependent manner.

Keywords: Macrophages, foam cells, cholesterol acyltransferase-1, leptin, anti-leptin antibody

Introduction

Atherosclerosis is a condition where the arteries become narrowed and hardened due to an excessive build up of plaque around the artery wall. The prevalence of atherosclerosis is common in the modern society. Under the role of various risk factors, transformation of mononuclear cells into macrophages has been shown to be one of early events leading to atherosclerosis [1-4]. The primary cause of lipid streak formation in early atherosclerosis is the transformation of macrophages into foam cells following lipid phagocytosis, in particular oxidized low-density lipoprotein (ox-LDL). Studies have shown that acyl-CoA: cholesterol O-acyltransferase-1 (ACAT-1) expression plays an important role during transformation of mononuclear cells into macrophages and foam cells. Recently, the relationship between leptin and atherogenesis has caused widespread concern.

Leptin is a non-glycosylated protein hormone encoded by the obese gene and is primarily secreted by adipocytes. The leptin receptor is widely distributed on the karyocyte surface. Clinical epidemiology has shown that hyperleptinemia/leptin resistance, hyperglycemia, hyperlipidemia, hypertension, hyperinsuline-mia/insulin resistance, and obesity are the main risk factors of atherosclerosis [5]. The purpose of the present study was to determine the effects of leptin on ACAT-1 expression and activity during differentiation of the human mononuclear cell line THP-1 towards foam cells, and to understand the relationship between leptin and atherogenesis.

Materials and methods

Reagent

RPMI-1640 medium and fetal bovine sera were purchased from Gibco, USA. Phorbol-12-
Leptin and ACAT-1 expression and activity

myristate 13-acetate (PMA) was purchased from Calbiochem, CA. The mononuclear cell line THP-1 was provided by Preservation Center for Typical Culture, Wuhan University, China. Transwell culture plates, culture flasks, and one-off 0.21-µmol/L filters were purchased from Costar, USA. Copper sulfate was purchased from Shanghai Shenneng Bocai Biological Science and Technology, China. Trizol reagent was sourced from Invitrogen, USA. Reverse transcription kit, Taq DNA enzyme, deoxyribonucleoside 5’-triphosphate (dNTP) and DNA Marker were purchased from Fermentas, USA. For reverse transcription-polymerase chain reaction, recombinant human leptin protein was purchased from EC, USA, and anti-leptin antibody (mouse anti-human) was purchased from ProSpec-Tany TechnoGene, Israel. ACAT-1: up-stream primer: 5’-CGCCCTACATTATTTACCG-3’, down-stream primer: 5’-AGATTGGCCTTTTCCGAC-3’. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH): up-stream primer: 5’-TCCCTCAAGATTCTCAGCAA-3’, down-stream primer: 5’-AGATCCACAACGGATACATT-3’.

Effects of leptin on ACAT-1 expression

After thawing at 37°C in a water bath, the mononuclear cell line THP-1 was resuspended with RPMI-1640 medium supplemented with 10% fetal bovine sera and cultured at 37.0°C in a 5% CO2 incubator. After three passages, the cell suspension (1 × 109/L) was inoculated into a 100-mL culture flask and incubated as described for groups A and B. Group A was divided into 6 subgroups: group A0, addition of simple PMA, cultured for 72 h; group A1, PMA + leptin 1 mmol/L, 72 h; group A2, PMA + leptin 2.5 mmol/L, 72 h; group A3, PMA + leptin 5 mmol/L, 72 h; group A4, PMA + leptin 10 mmol/L, 72 h; and group A5, PMA + leptin 15 mmol/L, 72 h. Group B was divided into 3 subgroups: group B0, addition of simple PMA, cultured for 24-96 h; group B1, PMA + leptin 10 mmol/L, 24-96 h; and group B2, PMA + leptin 10 mmol/L + anti-leptin antibody 2.5 mmol/L, 24-96 h.

Effects of leptin on ACAT-1 expression and activity

Following culture and induction procedures a described above, macrophages were induced to differentiate into foam cells through the addition of ox-LDL (100 mg/L). For induced differentiation, macrophages were subjected to the following interventions for groups C and D. Group C was divided into 6 subgroups: group C0, addition of simple ox-LDL, cultured for 48 h; group C1, ox-LDL + leptin 1 mmol/L, 48 h; group C2, ox-LDL + leptin 2.5 mmol/L, 48 h; group C3, ox-LDL + leptin 5 mmol/L, 48 h; group C4, ox-LDL + leptin 10 mmol/L, 48 h; group C5, ox-LDL + leptin 15 mmol/L, 48 h. Group D was divided into 3 subgroups: group D0, addition of simple ox-LDL, cultured for 24-96 h; group D1, ox-LDL + leptin 10 mmol/L, 24-96 h; and group D2, ox-LDL + leptin 10 mmol/L + anti-leptin antibody 2.5 mmol/L, 24-96 h.

Change in ACAT-1 mRNA expression

mRNA was routinely extracted from mononuclear cells and induced cells from each group, and mRNA integrity was measured. For cDNA synthesis, a 20-µL reaction volume containing 1.0 µL reverse transcription products, 0.5 µL up-stream primer, 0.5 µL down-stream primer, 1.5 µL dNTP mixture (2 mmol/L), 1.5 µL MgCl2 (25 mmol/L), 2 µL 10 × PCR Buffer, 500 U Taq DNA polymerase, and supplemented polymerase chain reaction water was used. Reaction conditions consisted of 95°C for 5 min, 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, for a total of 35 cycles. For an internal control, 25 amplification cycles of GADPH were used. The amplification products were separated on a 1.5% agarose gel. The gel (10 µL sample/well) was run at constant 120 V, followed by ethidium bromide staining and photographic analysis using a gel imaging system.

Western blot detection of ACAT-1 protein

Following induction of mononuclear THP-1 cells, total protein was extracted by cell lysis. A total of 20 µL total protein from each group was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequent membrane transfer. Specific primary antibody (rabbit anti-human ACAT-1 monoclonal antibody) was added for a 2-h incubation at 37°C, followed by overnight at 4°C. After a 1-h incubation with secondary antibody (horseradish peroxidase-labeled goat anti-rabbit IgG) at room temperature, chromogenic substrate was added for a 2-min coloration in the dark. GAPDH was used as an internal control, and a gel imaging system was applied for semi-quantitative analysis of the ratio of ACAT-1 to GAPDH.
Leptin and ACAT-1 expression and activity

Statistical analysis

All data were statistically analyzed using SPSS 13.0 software and were expressed as mean ± SD. One-way analysis of variance (F test and q test) was employed for comparison between groups. A level of \( P < 0.05 \) was considered statistically significant.

Results

Effects of leptin and leptin/anti-leptin on the ACAT-1 mRNA expression during differentiation of mononuclear THP-1 cells towards macrophages

The effects of leptin and leptin/anti-leptin on the ACAT-1 mRNA expression during differentiation of mononuclear THP-1 cells towards macrophages are shown in Figure 1. Mononuclear THP-1 cells cultured with different concentrations of leptin and PMA could induce macrophage differentiation. The ACAT-1 mRNA expression gradually increased in a dose-dependent manner. The concentration with the highest mRNA expression was 10 mmol/L leptin (group A4), it was twice as high as group A0 (control) after 72 h culture with different concentrations of leptin and PMA (Figure 1A).

Mononuclear cells cultured with 10 mmol/L leptin and PMA for 24-96 h could increase the ACAT-1 mRNA expression in a time-dependent manner. The time point with the highest expression level was at 72 h (Figure 1B).

Mononuclear cells cultured with 10 mmol/L leptin, 2.5 mmol/L anti-leptin antibody, and PMA for 24-96 h could completely block the ACAT-1 mRNA expression (Figure 1C, group B2). There were no significant differences in the expression levels between groups B0 (control, PMA) and B2 (PMA + Leptin 10 mmol/L + Anti-leptin 2.5 mmol/L).

Effects of leptin and leptin/anti-leptin on the ACAT-1 protein expression during macrophage differentiation of mononuclear cells

The Effects of leptin and leptin/anti-leptin on the ACAT-1 protein expression during macrophage differentiation of mononuclear cells are described in Figure 2. The ACAT-1 protein expression gradually increased in a dose-
Leptin and ACAT-1 expression and activity

Figure 3. Effects of leptin and leptin/anti-leptin on the ACAT-1 mRNA expression during foam cells differentiation of macrophages. A: Effects of different leptin concentrations on the ACAT-1 mRNA expression. Lanes C0-C5 were groups C0 (control, ox-LDL), C1 (ox-LDL + Leptin 1 mmol/L), C2 (ox-LDL + Leptin 2.5 mmol/L), C3 (ox-LDL + Leptin 5 mmol/L), C4 (ox-LDL + Leptin 10 mmol/L), and C5 (ox-LDL + Leptin 15 mmol/L), respectively. B: Effects of leptin (10 mmol/L) on the ACAT-1 mRNA expression at different time points, the ACAT-1 mRNA expression increased in a time-dependent manner. C: Effects of leptin/anti-leptin antibody (72 h culture) on the ACAT-1 mRNA expression.

Figure 4. Effects of leptin and leptin/anti-leptin on the ACTA-1 protein expression during foam cell differentiation of macrophages. A: Effects of different leptin concentrations on the ACTA-1 mRNA expression. Lanes C0-C5 represent groups C0 (control, ox-LDL), C1 (ox-LDL + Leptin 1 mmol/L), C2 (ox-LDL + Leptin 2.5 mmol/L), C3 (ox-LDL + Leptin 5 mmol/L), C4 (ox-LDL + Leptin 10 mmol/L), and C5 (ox-LDL + Leptin 15 mmol/L), respectively. B: Leptin upregulates the ACTA-1 protein expression at different time points. C: The effects of leptin on the ACTA-1 protein expression were blocked by anti-leptin antibody (Group D2).

dependent manner after 72 hours culture with different concentrations of leptin. The concentration with the highest expression level was 10 mmol/L leptin (group A4), which was twice as high as group A0 (Figure 2A).

Mononuclear cells cultured with 10 mmol/L leptin and PMA for 24-96 h could upregulate the ACAT-1 protein expression in a time-dependent manner. The time point with the highest protein expression was at 72 h (Figure 2B).

Mononuclear cells cultured with 10 mmol/L leptin and 2.5 mmol/L anti-leptin antibody as well as PMA for 24-96 h could completely block the ACAT-1 protein expression (Figure 2C, group B2), there were no significant differences in the ACAT-1 protein expression between groups B0 (control, PMA) and B2 (PMA + Leptin 10 mmol/L + Anti-leptin 2.5 mmol/L).

Effects of leptin and leptin/anti-leptin on the ACAT-1 mRNA expression during foam cells differentiation of macrophages

The effects of leptin and leptin/anti-leptin on the ACAT-1 mRNA expression during foam cells differentiation of macrophages are represented in Figure 3. Macrophages cultured with different concentrations of leptin and ox-LDL could induce foam cell differentiation. The ACAT-1 mRNA expression gradually increased in a dose-dependent manner. The concentration with the highest mRNA expression was 10 mmol/L leptin (group C4, ox-LDL + Leptin 10 mmol/L), which was 1.5 times as high as group C0 (control, ox-LDL) after 48 h culture with different concentrations of leptin and PMA (Figure 3A).

Macrophages cultured with leptin (10 mmol/L) and ox-LDL for 24-96 h could upregulate the ACAT-1 mRNA expression. The time point with the highest ACAT-1 mRNA expression was at 48 h and was nearly 1.5 times as high as group C0 (Figure 3B).

Macrophages cultured with 10 mmol/L leptin and 2.5 mmol/L anti-leptin antibody, as well as PMA, for 24-96 h could completely block the ACAT-1 mRNA expression. There was no significant difference in the ACAT-1 mRNA expression between groups B0 (control, PMA) and B2 (PMA...
Effects of leptin and leptin/anti-leptin on the ACTA-1 protein expression during foam cell differentiation of macrophages

The effects of leptin and leptin/anti-leptin on the ACTA-1 protein expression during foam cell differentiation of macrophages are revealed in Figure 4. The ACAT-1 protein expression gradually increased in a dose-dependent manner after 48 hours culture with different concentrations of leptin. The concentration with the highest expression level was 10 mmol/L leptin (group C4), which was nearly 1.5 times as high as group C0 (Figure 4A).

Macrophages cultured with 10 mmol/L leptin and ox-LDL for 24-96 h could upregulate the ACAT-1 mRNA expression. The time point with the highest ACAT-1 mRNA expression was at 48 h and was nearly 1.5 times as high as group D0 (Figure 4B).

Macrophages cultured with 10 mmol/L leptin, 2.5 mmol/L anti-leptin antibody, and ox-LDL for 24-96 h could blocked the ACAT-1 mRNA expression, but there were still significant differences in the ACAT-1 mRNA expression between groups D0 and D2 (Figure 4C).

Discussion

In the present study, we employed in vitro models of mononuclear cell-derived foam cells and studied the effects of leptin on ACAT-1 expression and activity to investigate the relationship between leptin and atherosclerosis. The main findings showed that during PMA-induced macrophage differentiation of mononuclear cells, higher than physiological doses of leptin resulted in increased ACAT-1 activity, as well as protein and mRNA expression, in a dose- and time-dependent manner. The highest levels were reached after 3 days of 10 mmol/L leptin treatment, which was nearly double the effect of PMA only-induced cells. In addition, during foam cell differentiation of macrophages, leptin also produced upregulatory effects on ACAT-1 activity, as well as protein and mRNA expression, in a time- and dose-dependent manner. The greatest levels were detected after three days of 10 mmol/L leptin treatment, and were approximately 1.5 times greater than the effect of ox-LDL.

Metabolic syndrome is characterized by hyperleptinemia, hyperglycemia, hyperinsulinemia, and obesity, and has also been shown to promote atherosclerosis. Aggregation of macrophage-derived foam cells leads to the development of atherosclerotic lesions. When vascular endothelial cell function is impaired, blood-derived mononuclear cells subcutaneously differentiate into macrophages, and then which develop into foam cells after phagocytizing much lipids. Foam cell accumulation takes place during early atherosclerosis. Studies have shown that cholesterol is rich in foam cell-derived cholesterol ester, which suggests that foam cell-derived cholesterol is re-synthesized by cholesteryl ester circulation during ACAT catalysis [6]. ACAT is an integral membrane protein and is located in the endoplasmic reticulum. It catalyzes the formation of cholesteryl esters from cholesterol and long-chain fatty acyl coenzyme A. Two ACAT isozymes exist in mammals-ACAT-1 and ACAT-2. ACAT-1 is ubiquitously expressed in various tissues, while ACAT-2 is expressed predominantly in the liver (hepatocytes) and intestine (enterocytes) [7]. In animal models and clinical cases of atherosclerosis, cholesteryl ester synthesis and ACAT-1 activity was increased in foam cells isolated from atherosclerotic plaques, which suggested that ACAT-1 plays a role in foam cell formation [8]. ACAT is the only enzyme that catalyzes the formation of cholesteryl esters from cholesterol and long-chain fatty acyl coenzyme A and plays an important role in cholesterol absorption, transport, and storage [9]. Increased ACAT-1 activity promotes intracellular cholesteryl ester synthesis. During this process, mononuclear macrophages transform into foam cells. Therefore, determining ACAT-1 enzyme activity and the influencing factors during transdifferentiation of mononuclear cells or macrophages would provide a better understanding of the mechanisms involved in foam cell formation and atherosclerosis occurrence. In the human, various substances and cytokines regulate ACAT-1. There is strong evidence demonstrating that transform growth factor-β1, interleukin-1β, 5-hydroxytryptamine, and vasoactive peptide salusin-beta up-regulate ACAT-1 expression [10-13], while taurine decreases ACAT-1 expression and activity [14].

Leptin is a 16-kD, non-glycosylated, protein hormone encoded by the obese gene and is primarily secreted by adipocytes. In addition,
brown adipose tissue, skeletal muscle, gastric mucosa [15], placenta, fetal heart, bone, and cartilage tissue can all secrete leptin. Leptin is highly hydrophilic and is present in plasma in monomeric form. It centrally regulates food intake, energy expenditure, the fixation and storing of fat [16], blood pressure, bone mass, and immunologic function. Leptin also plays an important role in peripheral regulation. For example, it directly regulates immunocytes, B cells, adipocytes, and muscle cells. Recent clinical epidemiological studies have shown that hyperleptinemia/leptin resistance is a key risk factor for atherosclerosis. Reilly et al. [17] demonstrated that serum leptin levels significantly correlate with coronary artery calcification in patients with type-2 diabetes mellitus after controlling for body mass index, high-sensitivity C-reactive protein levels in serum, and waist circumference. Moreover, coronary artery calcification is one of markers for subclinical atherosclerosis. Several in vitro studies have demonstrated that leptin exerts direct actions on vessels and inflammatory cells and activates many signal transduction pathways of monocytes and vascular smooth muscle cells, including phosphoinositide-3 kinase, prekallikrein activator, and mitogen-activated protein kinases, thereby promoting the occurrence and development of atherosclerosis [18-20]. In addition, leptin receptor was shown to be expressed in the above-mentioned cells, as well as atherosclerotic plaques [21]. A large-scale clinical case control study showed that leptin was a predictor of coronary heart disease, independent of conventional risk factors, such as body mass index and C-reactive protein [22]. Leptin also influences lipid metabolism [23].

In summary, hyperleptinemia influenced the entire process of foam cell differentiation of mononuclear cells. When vascular endothelial cell function was damaged, blood-derived mononuclear cells subcutaneously differentiated into macrophages, ACAT-1 transcription and expression was enhanced, and ACAT-1 activity was simultaneously increased. Thus, under the catalysis, cholesteryl esters formed from cholesterol and long-chain fatty acyl coenzyme A, which led to aggregation of cholesteryl esters, the promotion of foam cell formation, and finally increased occurrence and development of atherosclerosis.

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Disclosure of conflict of interest

None.

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Leptin and ACAT-1 expression and activity


