Leptin Induces the Hepatic High Density Lipoprotein Receptor Scavenger Receptor B Type I (SR-BI) but Not Cholesterol 7α-Hydroxylase (Cyp7a1) in Leptin-deficient (ob/ob) Mice*

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Cholesterol elimination from the body involves reverse cholesterol transport from peripheral tissues in which the elimination of high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol by the liver and subsequent biliary excretion as free cholesterol and bile acids are important. In situations of peripheral fat and cholesterol accumulation, such as obesity, these pathways may be overloaded, contributing to increased cholesterol deposition. Leptin has an important role in obesity, suppressing food intake and increasing energy expenditure. This hormone, which is absent in genetically obese ob/ob mice, is also thought to be involved in the coordination of lipid excretion pathways, although available data are somewhat inconsistent. We therefore studied the expression of the hepatic HDL receptor, scavenger receptor class B type I (SR-BI), and the LDL receptor as well as the rate-limiting enzyme in bile acid synthesis, cholesterol 7α-hydroxylase (Cyp7a1), in leptin-deficient ob/ob mice and their wild-type controls. In ob/ob mice, protein levels of both LDL receptor and SR-BI were reduced, whereas LDL receptor mRNA levels were increased and those of SR-BI were reduced, regardless of challenge with a 2% cholesterol diet. In ob/ob mice, the enzymatic activity and mRNA for Cyp7a1 were reduced, and the increase in response to dietary cholesterol was blunted. Upon short-term (2 days) treatment with leptin, a dose-dependent increase was seen in the SR-BI protein and mRNA, whereas the Cyp7a1 protein and mRNA were reduced. Our findings indicate that leptin is an important regulator of hepatic SR-BI expression and, thus, HDL cholesterol levels, whereas it does not stimulate Cyp7a1 and bile acid synthesis.

Leptin is a circulating cytokine-like protein secreted mainly from adipose tissue. It acts to reduce food intake and increase energy expenditure by binding to its receptor in hypothalamic nuclei in the brain (1, 2). A number of additional functions in immune, reproductive, and hormonal systems have been ascribed to this protein (1, 2). Ob/ob mice, with a mutation in the leptin gene (3), display obesity, hypothermia, infertility, hyperglycemia, decreased insulin sensitivity, and hyperlipidemia. The increased level of plasma cholesterol in this mouse model is mainly attributed to higher plasma high density lipoprotein (HDL) cholesterol (4). Detailed metabolic studies have shown that ob/ob mice have defective HDL plasma clearance and disturbances in the intracellular distribution of cholesterol and its processing in the hepatocyte (5, 6). These abnormalities in HDL metabolism are normalized upon treatment with leptin. An increase in plasma apoA-I was found concomitant with a reduced hepatic apoA-I mRNA, further supporting the concept of a reduced hepatic metabolism of HDL in leptin deficiency.

A major pathway for the excretion of body cholesterol is through the hepatic conversion of this sterol into bile acids, which is controlled by the activity of the microsomal enzyme cholesterol 7α-hydroxylase (Cyp7a1). Abnormalities of hepatic cholesterol metabolism in ob/ob mice have been reported (7), but the results are not consistent. Thus, increased Cyp7a1 mRNA levels were reported after 2 days of leptin treatment using transcriptional profiling (8), whereas treatment for 2 weeks did not alter Cyp7a1 enzyme activity or bile acid synthesis (7). The leptin deficiency in ob/ob mice promotes overeating, leading to weight gain which can be counteracted by prolonged leptin substitution (9–11). Prolonged leptin treatment of ob/ob mice may therefore reflect the effects of weight reduction and the consequences thereof, such as increased insulin sensitivity and reduced hepatic fat accumulation.

The present study was undertaken to investigate hepatic cholesterol metabolism in ob/ob mice. We evaluated the effects on cholesterol and bile acid metabolism following challenge with a diet rich in cholesterol, and we assessed the responsiveness to short term leptin treatment at increasing doses. We have found that the hepatic HDL receptor, scavenger receptor class B type I (SR-BI), and the LDL receptor are suppressed in ob/ob mice. The activity and mRNA for Cyp7a1 were reduced and showed an attenuated response to cholesterol feeding. With leptin treatment, the SR-BI protein and mRNA increased dose-dependently, whereas the Cyp7a1 protein and mRNA were reduced.

**EXPERIMENTAL PROCEDURES**

*The abbreviations used are: HDL, high density lipoprotein; Cyp7a1, cholesterol 7α-hydroxylase; SR-BI, scavenger receptor class B type I; LDL, low density lipoprotein; VLDL, very low density lipoprotein; C4, 7α-hydroxy-4-cholesten-3-one.*

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adapt to the animal housing for approximately 2 weeks and fed a standard mouse chow (R66, Lactamin, Kimstad, Sweden). All experiments were started when animals were 10 weeks old. The lights were on between 7 a.m. and 7 p.m. The experiments were approved by the regional animal care and use committee.

Treatments—For cholesterol feeding experiments, ob/ob and their wild-type controls were given either standard mouse chow or standard mouse chow supplemented with 2% cholesterol plus 10% oil (w/w) for 7 days. Each of the four groups consisted of four animals. At 9 a.m. the animals were anesthetized by ether inhalation, and blood was collected by cardiac puncture, after which they were killed by cervical dislocation. Livers were removed and frozen in liquid nitrogen.

For the leptin treatment experiments, leptin (Peprotech, London, UK) was dissolved in sodium citrate buffer according to the manufacturer’s instructions. Leptin was administered intraperitoneally in a volume of 200 μl at 6 p.m. for 2 days at the indicated doses. Controls received sodium citrate buffer. The experiment was ended on the third day by sacrificing the animals at 10 a.m., 16 h after the last injection. Each group in the leptin treatment experiment consisted of five ob/ob mice, except the control group, which consisted of six ob/ob mice.

Plasma Lipids and Lipoproteins—Blood was centrifuged, and the plasma was analyzed for total cholesterol and triglycerides using the IL Test™ cholesterol 181618-10 and triglyceride 181610-60 kits (IL). Plasma was analyzed for total cholesterol and triglycerides using the IL manufacturer’s instructions. Leptin was administered intraperitoneally in a volume of 200 μl at 6 p.m. for 2 days at the indicated doses. Controls received sodium citrate buffer. The experiment was ended on the third day by sacrificing the animals at 10 a.m., 16 h after the last injection. Each group in the leptin treatment experiment consisted of five ob/ob mice, except the control group, which consisted of six ob/ob mice.

Plasma Leptin and Insulin—Plasma leptin levels were determined by a mouse leptin enzyme-linked immunosorbent assay kit (R&D Systems, Oxon, UK) according to the manufacturer’s instructions. Plasma insulin levels were analyzed using a rodent insulin RIA kit according to manufacturer’s instructions (Linco, St. Charles, MI).

Plasma Tα-Hydroxy-4-cholesten-3-one Assay—As an indirect measurement of cholesterol-Tα-hydroxylation activity, the plasma levels of Tα-hydroxy-4-cholesten-3-one (C4) were analyzed from pooled plasma samples by high pressure liquid chromatography as described elsewhere (13).

Liver Preparations—Pooled livers (0.5 g) were homogenized in 50 mM Tris, pH 6.8, 2 mM CaCl₂, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM leupeptin. Samples were sonicated for 15 s and then centrifuged at 14,000 rpm for 10 min. The supernatant was centrifuged in an Airfuge® (Beckman Coulter AB, Bromma, Sweden) for 7 min, aliquoted, and stored at −80 °C until further analysis. Protein concentrations were determined with Lowry DC kit (Bio-Rad). All work was performed on ice.

Protein Blot Analysis—LDL receptor expression was assayed using liver membrane proteins that were electrophoresed under non-reducing conditions on 6% SDS-PAGE (14). Proteins were transferred to nitrocellulose membranes and incubated with 125I-labeled rabbit β-VLDL. The LDL receptor bands (~120 kDa) were analyzed and quantified using a Fuji BAS 1800 analyzer (Fuji Photo Film Co.) and Image Gauge software (Science Lab, 98, version 3.12, Fuji Photo Film Co.). SR-BI was assayed by immunoblot using liver membrane proteins and a rodent polyclonal antibody (Novus Biologicals Inc., Littleton, CO), as described previously (15).

The Cyp7a1 antibody was generated by immunizing rabbits with the peptide NH₂-KLH-Cys-Tyr-Lys-Leu-Lys-His-COOH, as used previously (16), corresponding to the last five amino acids in the mouse and rat Cyp7a1 proteins. The peptide was coupled to an affinity column to purify Cyp7a1 antibodies from rabbit serum. A Cyp7a1 Western blot was performed on hepatic microsomal proteins prepared as described previously (17). In brief, 25 μg of microsomal protein was electrophoresed on a 10% Criterion Tris-HCl gel (Bio-Rad) and then transferred to a polyvinylidene difluoride membrane (Bio-Rad). The membrane was blocked for 1 h at room temperature in blocking solution (phosphate-buffered saline, 0.2% Tween, and 5% fat-free dry milk powder) and then incubated with the Cyp7a1 antibody (1 μg/ml) for 1 h at room temperature. The membrane was washed with 1% TBS (Tris buffered saline) and 0.05% Tween for 1 h at room temperature followed by washing in phosphate-buffered saline plus 0.2% Tween. Secondary antibody (anti-rabbit, anti-goat) was then added to the membrane and further incubated with 1% TBS and 0.05% Tween for 1 h at room temperature. Labeled complexes were visualized on a Fuji BAS 1800 analyzer (Fuji Photo Film Co.) with an exposure time of 30 s. An immunoblot analysis of Cyp7a1 expression was performed on hepatic microsomal proteins prepared as described previously (17), corresponding to the last five amino acids in the mouse and rat Cyp7a1 proteins. The peptide was coupled to an affinity column to purify Cyp7a1 antibodies from rabbit serum. A Cyp7a1 Western blot was performed on hepatic microsomal proteins prepared as described previously (17).

Quantitative Real Time PCR—Total RNA was extracted from snap frozen livers with Trizol reagent (Invitrogen) according to the manufacturer’s instructions. The RNA was DNase-treated with RQ1 DNase (Promega, Madison, WI). Briefly, 40 μg of total RNA was incubated with RQ1 DNase for 20 min at 37 °C; the enzyme was then inactivated at 70 °C for 15 min, and, after the addition of stop solution (Promega, Madison, WI), at 25 °C for 10 min. For cDNA synthesis, 4 μg of DNase treated total RNA was used. cDNA synthesis was carried out with Superscript II (Invitrogen) according to the manufacturer’s instructions, employing 200-ng random hexamers (Promega) per 20-μl cDNA reaction. Quantitative real time PCR was performed with TaqMan™ assay on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) following the guidelines in the user bulletin 2 (Applied Biosystems) on the “comparative Cₘ̇ method.” An 18 S mRNA was used as an endogenous control.

For cholesterol feeding experiments, ob/ob and their wild-type controls were given either standard mouse chow or standard mouse chow supplemented with 2% cholesterol plus 10% oil (w/w) for 7 days. Each of the four groups consisted of four animals. At 9 a.m. the animals were anesthetized by ether inhalation, and blood was collected by cardiac puncture, after which they were killed by cervical dislocation. Livers were removed and frozen in liquid nitrogen.

Effect of cholesterol feeding on total cholesterol and triglycerides in plasma of ob/ob mice compared to their controls

TABLE I

| Group       | Body weight | Cholesterol | Triglycerides |
|-------------|-------------|-------------|---------------|
| Controls    | 28 ± 0.8    | 2.8 ± 0.1   | 1.2 ± 0.1     |
| Controls + 2% cholesterol | 29 ± 0.7    | 2.4 ± 0.4   | 0.7 ± 0.1     |
| ob/ob       | 45 ± 1.6    | 5.0 ± 0.4   | 1.1 ± 0.1     |
| ob/ob + 2% cholesterol | 45 ± 1.2    | 6.6 ± 0.2   | 1.1 ± 0.1     |

* Significantly different from control, p < 0.01.

RESULTS

Plasma Lipids and Lipoprotein Profiles of Cholesterol-challenged ob/ob Mice—Table I displays plasma total cholesterol and triglyceride concentrations together with body weights at the end of the experiment when ob/ob mice and controls were fed either a normal diet or 2% cholesterol for 7 days. As expected (4, 5), the ob/ob mice were markedly hypercholesterolemic, which was due to increased LDL and HDL levels (Fig. 1A).

As expected (12), cholesterol feeding to control mice increased VLDL and intermediate density lipoprotein cholesterol but reduced HDL cholesterol. However, when ob/ob mice were fed the high-cholesterol diet they responded with a marked elevation of cholesterol in all fractions (Fig. 1A).

Decreased Expression of Hepatic LDL Receptors and SR-BI in ob/ob Mice—Because the increased LDL and HDL cholesterol seen in ob/ob mice could be due, at least in part, to a reduced expression of the hepatic receptors for LDL and HDL (18–20), we next examined the expression of the LDL receptor in the liver by ligand blot assay with 125I-labeled rabbit β-VLDL on liver membranes from ob/ob and control mice (Fig. 1B).

The LDL receptor expression was clearly reduced in both groups of ob/ob mice as compared with controls (Fig. 1B). In both controls and ob/ob mice, the expression of LDL receptors tended to increase in response to cholesterol feeding. Analysis of the mRNA expression of the LDL receptor with quantitative real time PCR revealed that the transcripts were more abundant in the ob/ob mice (p < 0.01), although both groups responded with a reduction when fed cholesterol (Fig. 1C).

The expression of the hepatic HDL receptor, SR-BI, was thereafter analyzed by Western blot of liver membranes (Fig.
There was a marked reduction of the SR-BI protein in the livers of ob/ob mice compared with the control mice, and cholesterol feeding did not alter this (Fig. 1, D and E). Also, the hepatic mRNA levels of SR-BI were reduced in ob/ob mice (p < 0.01), both on the normal and the cholesterol-enriched diet (Fig. 1E).

Reduced Cyp7a1 Response to Cholesterol Challenge in ob/ob Mice—Because the formation of bile acids from hepatic cholesterol greatly influences cholesterol metabolism, we examined the rate-limiting enzyme in the bile acid synthesis pathway, Cyp7a1. Fig. 2 shows the enzyme activities for control and ob/ob mice on normal and 2% cholesterol diet, respectively. A normal Cyp7a1 response could be found in the control animals, wherein the activity increased by 3-fold following dietary cholesterol. Notably, in the ob/ob livers this response of Cyp7a1 to cholesterol was weaker (Fig. 2). Both ob/ob groups also displayed markedly lower activities than their respective control group (p < 0.05). This pattern was indeed similar when the mRNA levels of Cyp7a1 were measured, suggesting that this alteration in the ob/ob mice is at the transcriptional level.

Short Term Treatment with Leptin of ob/ob Mice—We then wanted to investigate the metabolic effects of leptin treatment in the absence of leptin-induced weight changes of the animals. For this purpose, we set up a dose-response experiment in which ob/ob animals received one injection per day for 2 days, with doses ranging from 0.25 to 7.2 μg per gram of body weight per injection. During the course of the experiment, animal body weights did not differ significantly between the vehicle-treated and the leptin-treated groups. Plasma total cholesterol or triglycerides were not affected by leptin treatment, which was verified with fast protein liquid chromatography analysis in which no change was found in any of the lipoprotein fractions (not shown).

The plasma levels of leptin in the treated animals increased with increasing doses of recombinant leptin administered (Fig. 3A). To monitor the biological activity of the injected leptin, we then analyzed plasma insulin levels, because previous studies have shown that leptin rapidly increases the peripheral insulin sensitivity in ob/ob mice, thereby leading to lowered plasma insulin levels (1, 9–11). It was found that plasma insulin levels decreased with increasing leptin dosage (Fig. 3A). However, at higher plasma levels of leptin, there were no effects on plasma insulin levels.

Short Term Treatment with Leptin Enhances Hepatic Expression of SR-BI but Not Cyp7a1—Fig. 3B displays a representative Western blot of SR-BI on liver membranes from the ob/ob mice injected with increasing amounts of leptin. The
hepatic SR-BI protein expression pattern correlated strikingly well to the plasma insulin levels (Fig. 3, A and B). Quantitative real time PCR analysis confirmed that the mRNA levels of liver SR-BI were also changed to a similar extent as the SR-BI protein levels (Fig. 3, B and C). Hence, short-term treatment with leptin up-regulates the hepatic SR-BI expression in ob/ob mice under non-weight-reducing conditions.

From the cholesterol feeding experiments, it was evident that ob/ob mice have a lower Cyp7a1 activity, presumably due to a reduced transcription of the cyp7a1 gene. Because leptin treatment has been reported to increase the Cyp7a1 mRNA levels in ob/ob mice (8), we characterized the effects of leptin on bile acid synthesis in this experiment. First, we analyzed the plasma levels of C4, which is a sensitive marker of hepatic Cyp7a1 activity (13). Surprisingly, we could not observe any increased C4 levels, but rather a reduced concentration in all groups treated with leptin (Fig. 4A). A similar pattern was observed when the hepatic microsomal Cyp7a1 protein was assayed by Western blot (Fig. 4B). The highest level of Cyp7a1 protein expression was observed in the vehicle-treated ob/ob mice. Quantitative real time PCR analysis of hepatic mRNA from these ob/ob animals showed a clear reduction ($p < 0.05$ or better) of the mRNA levels of Cyp7a1 (Fig. 4C). Thus, it is evident that leptin treatment does not induce but instead reduces Cyp7a1 when administered to ob/ob mice in a short-term, non-weight-modifying regimen.

**DISCUSSION**

The regulation of cholesterol and lipoprotein metabolism in animal models of obesity is complex. The contribution of abnormalities occurring in response to insulin resistance, fat accumulation in the liver, and increased food intake through interaction with the nuclear receptors controlling genes responsible for lipogenesis and lipolysis make the interpretation of studies in obesity models difficult. The leptin-deficient ob/ob mouse is a suitable model for characterizing the influence of leptin. The present work describes abnormalities in hepatic cholesterol metabolism in ob/ob mice, some of which are reversed by leptin.

The ob/ob mice displayed increased plasma cholesterol as the result mainly of an increase in HDL-size particles but also in larger LDL-like particles. Compared with controls, the ob/ob mice had a reduced expression of hepatic LDL receptors. Similar findings have been seen in Zucker rats (21) and db/db mice,2 which display abnormal leptin receptor function (22–25). Interestingly, the induction of LDL receptor observed in ro-

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dents following dietary cholesterol challenge (26, 27) was less pronounced in ob/ob mice, whereas mRNA levels for the LDL receptor were higher in ob/ob mice and reduced by cholesterol challenge to a similar degree as that found in wild-type controls. The amount of the transcription factor called steroid regulatory element-binding protein 1c (SREBP-1c) has been shown to increase in ob/ob mice compared with wild type controls, whereas that of SREBP-2 is not changed (25). Our finding of a moderate increase in LDL receptor mRNA levels is consistent with the report of Shimomura et al. (25) and may indicate a discrepancy between mRNA levels and protein expression for the LDL receptor, suggestive of posttranscriptional regulation of the LDL receptor (28, 29).

Both by Western blot analysis and quantitative real time PCR analysis of hepatic SR-BI expression we found a clear decrease in ob/ob mice compared with wild-type animals. This finding is in line with the observed increase in plasma HDL cholesterol and is also in concert with the results reported by Silver et al. (6) describing decreased recycling, degradation, and selective lipid uptake from HDL particles in ob/ob hepatocytes. In a previous publication (5), these authors were not able to demonstrate any difference in hepatic SR-BI expression between ob/ob and wild-type livers using Western and Northern blot analysis. We have no explanation for this discrepancy but have confirmed our results in repeated experiments. Also, in db/db mice we have found that the hepatic expression of SR-BI is reduced.3

After 2 days of leptin treatment there was a very clear dose-dependent increase in hepatic SR-BI expression, both measured as protein and as mRNA. The decrease in plasma insulin, indicating improvement of insulin sensitivity, paralleled the increase in SR-BI. This experiment further strengthens the concept that SR-BI is indeed sensitive to leptin deficiency. Although we cannot exclude that there is direct stimulation by insulin, we would interpret the data as further support for the concept that SR-BI down-regulation is an important explanatory mechanism for the increased HDL cholesterol in ob/ob mice. It is worth mentioning that the changes in SR-BI expression were observed at an earlier stage than when the changes in HDL cholesterol could be observed. Thus, in an additional experiment (not shown) wherein a new set of five ob/ob mice were leptin-treated (1.2 μg per gram of body weight per day) for 8 days, a 30% reduction of total plasma cholesterol was observed (from 4.4 ± 0.3 to 3.0 ± 0.2 mmol, p < 0.01), which is in line with the results of Silver et al. (5).

Cyp7a1 enzyme activity and mRNA were reduced in ob/ob mice, and there was a blunted Cyp7a1 response to dietary cholesterol. Surprisingly, we found that Cyp7a1 was reduced in response to leptin treatment in ob/ob mice by using three different methods of assay, i.e., Cyp7a1 protein, mRNA, and plasma C4. These experiments were performed at a stage where there was no difference in body weight between the treated and untreated animals, so the degree of obesity was not different. By using suppression-subtractive hybridization in combination with microarray analysis followed by Northern blot, Liang and Tall have reported an increase in Cyp7a1 mRNA after 2 days of leptin treatment of ob/ob mice (8). On the other hand, in line with our results, Cyp7a1 activity and fecal bile acid excretion were not stimulated by leptin treatment for 14 days, when partial weight reduction was present.

Thus, it appears unlikely that leptin is a direct regulator of Cyp7a1. Increased insulin sensitivity induced by leptin could be an explanation to our finding, because insulin has been shown to down-regulate Cyp7a1 (30, 31). The hormonal regulation of Cyp7a1 in vivo appears to be complex, and further studies on the influence of leptin on bile acid metabolism are warranted.

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