Leptin inhibits apolipoprotein M transcription and secretion in human hepatoma cell line, HepG2 cells.

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LUNDS UNIVERSITET

January 20, 2005

Prof. F. Spener
Executive Editor, BBA-Molecular and Cell Biology of Lipids

Dear Prof. Spener,

Enclosed please find the manuscript “Leptin Inhibits Apolipoprotein M Transcription and Secretion In Human Hepatoma Cell Line, HepG2 Cells” by Guanghua Luo, Maria Hurtig, Xiao-Ying Zhang, Peter Nilsson-Ehle and myself to be considered for publication in the BBA-Molecular and Cell Biology of Lipids as a regular paper. All authors have read and approved the manuscript. There has been no duplicate publication or submission elsewhere of any part of the work. There are no financial or other relations that could lead to a conflict of interest.

Sincerely yours,

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Leptin Inhibits Apolipoprotein M Transcription and Secretion In Human Hepatoma Cell Line, HepG2 Cells

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Short title: Leptin decreases apoM synthesis in HepG2 cells

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ABSTRACT

Apolipoprotein M (apoM) is a novel apolipoprotein presented mostly in high-density lipoprotein (HDL) in human plasma. Previously we have reported that both leptin and leptin receptor are essential for apoM expression *in vivo*. Expression of apoM is lower in the leptin deficient (*ob/ob*) mouse and leptin receptor deficient (*db/db*) mouse than in the normal mouse. In the present study, however, we demonstrated that supra-physiological concentrations of recombinant leptin significantly inhibited apoM transcription and secretion in the human hepatoma cell line, HepG2 cells. Both northern blotting and real-time RT-PCR were applied into the analyses of apoM mRNA levels, and compatible data were obtained. The inhibitory effect of leptin on apoM mRNA levels in HepG2 cells is dose dependent, i.e. 100 ng/mL of leptin decreased apoM mRNA levels by 30%, and 500 ng/mL of leptin decreased apoM mRNA levels about 50%. Even at a physiological concentration of leptin (10 ng/mL), apoM expression was decreased, and in parallel, secretion of apoM into the medium was also decreased. Furthermore, we examined apoAI, apoB and apoE by northern blotting analyses. The results demonstrated that leptin does not significantly influence expressions of apoAI, apoB and apoE in HepC2 cells, suggesting that leptin has a specific regulatory effect on hepatic apoM transcription and secretion *in vitro*. The mechanism on the contradictory effects of leptin on apoM expression *in vivo* and *in vitro* needs further investigation.

Key words: Lipoprotein; Apolipoprotein M; Leptin; and HepG2 cell line.

INTRODUCTION

Apolipoprotein M (apoM) is a recently discovered human apolipoprotein that is associated mainly with high-density lipoprotein (HDL) in human plasma, it is also present in triglyceride-rich lipoproteins (TGRLP) and low-density lipoprotein (LDL) [1, 2]. *In situ* hybridization experiments demonstrated that apoM is exclusively expressed in hepatocytes in liver and in tubular cells in kidney [3, 4]. Previous studies suggest that apoM might be involved in lipid and/or lipoprotein metabolism *in vivo*. For example, the proportion of apoM in TGRLP has been shown to be increased in the postprandial phase [1]. Recently we demonstrated that both leptin and leptin receptor are essential for the apoM synthesis *in vivo* [5]. In the leptin deficient (*ob/ob*) mouse and leptin receptor deficient (*db/db*) mouse the apoM mRNA levels and plasma apoM concentrations were greatly reduced when compared to the normal mouse. Furthermore, it is also reported that plasma apoM concentration is positively correlated to leptin level in obese subjects [6]. Liang and Tall reported that leptin treatment in *ob/ob* mice leads to an increase in mRNA levels of apoAI, apoAII, apoH and apoM, and a decrease in apoAIV in the liver [7]. In the present study, we investigated the effects of leptin on the transcription and secretion of apoM in HepG2 cells. We chose HepG2 cells as these cells have a known leptin receptor [8]. Also, the HepG2 cells can synthesize and secrete lipoprotein including very lower density lipoprotein (VLDL), intermediate density lipoprotein (IDL) and HDL, as well as a variety of plasma apolipoproteins including apoB, apoE, apoAs, apoCs and apoM.

MATERIALS AND METHODS

Materials.

Recombinant leptin was purchased from SIGMA Chemical Company (St. Louis, MO, USA) and from Research Diagnostics, Inc (Flanders, NJ, USA). Oleic acid was from Larodan Fine Chemicals, Sweden. The established hepatoblastoma cell line, HepG2, was from American Type Culture Collection, Manassas, VA. [^{32}P]dCTP was purchased from Amersham Pharmacia Biotech. Six-well cell culture clusters and 25-cm^2 vented cell culture flasks were purchased from Costar. Real-time RT-PCR reagents and control probe G3PDH were purchased from PE Biosystems.

Cell cultures.

HepG2 cells were maintained in RPMI 1640 with 10% fetal calf serum (FCS) in the presence of benzylpenicillin (100 U/mL) and streptomycin (100 μg /mL) under standard culture conditions (5% CO_2, 37°C). Cells were seeded in 25-cm^2 cell culture flasks or in 6-well cell culture clusters, and allowed growing to 50-70% confluence. Prior to the experiment, cells were washed twice with phosphate buffered saline (PBS), once with serum free RPMI 1640, and then the experimental medium (RPMI 1640+ 0.5% human serum albumin (HSA) + antibiotics) containing different leptin concentrations. Cells were incubated at 37°C for 24 hrs.

Preparation of northern blotting probes.

A commercial G3PDH cDNA fragment was used as a control probe and a full-length human apoM cDNA was used as a probe for apoM mRNA hybridization [1]. A 651 bp apoAI exon 3 DNA fragment was amplified from human genomic DNA (primers: 5'-ctgggacagcgtgacctc and 5'-tctgagcaccgggaaggg) as a probe for apoAI hybridization, and

a 317 bp DNA fragment of the apoE gene was amplified from human genomic DNA (primers: 5'-ctgatggacgagaccatgaa and 5'-tcgcgggccccggcctggta) for apoE hybridization. A 385 bp DNA fragment corresponding to the apoB LDL-R binding domain was amplified (primers: 5'-gctgtactctaccgctaaagga and 5'-gacatttgccatggagagagtt) from human genomic DNA, and used as the human apoB northern blotting probe [9]. All PCR reactions were performed using Ampli Taq DNA polymerase with buffers and dNTPs supplied by manufacturer (Perkin Elmer, Roche Molecular Systems Inc., USA) according to the manufacture's directions, on a GeneAmp PCR System 2400 (Perkin Elmer, Applied Biosystems). All probes were radiolabeled with [^{32}P]dCTP using the random primer method (RediPrime, Amersham Pharmacia Biotech).

Isolation of total RNA from HepG2 cells and northern blotting analysis.

Total RNA of HepG2 cells was isolated by the guanidinium thiocyanate method [10] and northern blots were hybridized with the probes described above and eventually exposed to X-ray film. Hybridizations were carried out at 65 °C in a hybridization solution (Clontech). The blots were washed several times in 2 X SSC / 0.1% SDS solution at room temperature for 2 hrs and twice in 0.1 X SSC / 0.05% SDS at 50 °C for 40 min. The washed blots were exposed to X-ray film at -70 °C from 1-3 days. The autoradiographs were analyzed with a scanner (Epson-1600). The membrane was stripped with boiled water in the presence of 0.5% SDS for 10 min and then rehybridized with the next probe. The relative mRNA levels were calculated with a Macintosh computer using the software of Quantity One (Version 4.2.1, Bio-Rad Laboratories) and presented as volume (intensity x mm^2).

Real time RT-PCR.

Primer Express software (Applied Biosystems) was used to design human apoM primers and probe used in the TaqMan assay. In order to avoid amplify DNA template the apoM specific primers that are spanned an 81 bp –intron were designed to amplify a 66-bp product. The primers were 5'-tgccccggaaatggatcta and 5'-cagggcggccttcagtt, and the probe was 5'-FAM-cacctgactgaagggagcacagatctca-TAMRA. A G3PDH primer/probe set (PE Biosystems) was used in separate tubes as control for the input cDNA. Relative standard curve was performed to compensate the efficiency of the PCR. In brief, a serial dilution of human apoM cDNA was used to generate a standard curve by plotting the cycle threshold versus the log of input cDNA. Both G3PDH standard curve and apoM standard curve were linear. Relative quantification of mRNA was performed on an ABI PRISM 7700 Sequence Detector (Applied Biosystems), as described previously [11]. Real time RT-PCR of apoM was performed in two-steps. It was carried out in a 25 μl reaction mixture containing 1x TaqMan Universal PCR Master Mix, 22.5 pmol of both forward and reverse primers, 5 pmol probe and 50 ng of the total RNA templates. Thermal cycling conditions included the following steps: 25 °C 10 min, 48 °C 30 min and 95 °C 5 min to do reverse transcription, and then the reaction mixture was preheated for 2 min at 50 °C and for 10 min at 95 °C to activate Taq polymerase. After that, a 40-cycle two-step PCR was performed consisting of 15 sec at 95 °C and 1 min at 60 °C. Samples were amplified simultaneously in triplicates in one-assay run. Real-time RT-PCR of G3PDH was performed according to the manufacturer's instruction. The threshold cycle (C_T) is defined as the fractional cycle number at which the reporter fluorescence reaches a certain level.

ApoAI, apoB, apoE and apoM protein masses determinations.

ApoAI, apoB, apoE and apoM levels were determined by western blotting analysis [9]. Rabbit antiserum against a synthetic peptide corresponding to amino acid residues 103-122 of apoM, or truncated apoM conjugated to keyhole limpet hemocyanin (LKH) was raised. Cell culture medium without FCS was fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and then incubated with polyclonal rabbit against human apoAI, apoB, apoE or apoM antibodies. Alkaline phosphatase-conjugated swine anti rabbit IgG was used as the secondary antibody. Bands corresponding to the individual apolipoproteins were visualized by an AP Conjugate Substrate Kit (Bio-Rad Laboratories), and quantified by a scanner using the software of Quantity One.

Statistics.

Results are expressed as mean ± SD. Comparisons among groups were statistically evaluated by the one-way ANOVA and followed by the unpaired student *t*-test. Significance was established at a p value less than 0.05.

RESULTS

Effect of leptin on apoAI, apoB, apoE and apoM secretion

The contents of apoM in the medium secreted from HepG2 cells exposed to leptin for 24 hrs at different concentrations are shown in Fig. 1. At leptin concentrations of 100 and 500 ng/ml, apoM levels decreased by 20% and 23%, respectively. There were no significant changes in the secretion of apoAI, apoB and apoE into the medium after any leptin administration in HepG2 cell cultures (data not shown).

Effects of leptin on apoAI, apoB, apoE and apoM expression detected by northern blotting analysis

Fig 2 demonstrates that leptin significantly decreased apoM mRNA levels in HepG2 cells. The effects were dose dependent. At the highest dosage of leptin (500 ng/ml), the reduction of apoM mRNA levels was 58% compared to the control cells, but already at physiological concentration of leptin (10 ng/mL) apoM expression was slightly inhibited. There were no obvious changes in mRNA levels of apoAI, apoB, apoE or control probe, G3PDH, during the administration of leptin (Fig 2).

Measurement of apoM mRNA levels by Real-time RT-PCT

Fig 3 demonstrated that the relative apoM mRNA levels were significantly decreased after administrations of leptin at both 100 and 500 ng/mL, as well as at physiological concentrations of leptin (10 ng/ml). The effects of leptin on apoM expression in HepG2 cells were dose-dependent. The Fig. 3 represents one of four similar experiments.

DISCUSSION

ApoM is a newly discovered human HDL associated apolipoprotein. The patho-physiological function of apoM is not yet understood. Recently we demonstrated that both leptin and leptin receptor are essential for the apoM expression *in vivo* [5]. Expression of apoM was lower in the leptin deficient (*ob/ob*) mouse and leptin receptor deficient (*db/db*) mouse than in the normal mouse. We have also reported that apoM concentration in plasma was positively correlated to leptin level in obese subjects [6]. In the present study, we further examined the effects of leptin on apoM synthesis in HepG2 cell cultures. It is demonstrated that leptin inhibited apoM expression and secretion in the HepG2 cells. Two different methods were applied to measure the relative apoM expressions, i.e., northern blotting and real-time RT-PCR. Both analyses display similar data demonstrating that supra-physiological concentrations of leptin (ten to fifty-times physiological concentration) significantly inhibited apoM transcription in HepG2 cell cultures. The inhibitory effect of leptin on apoM mRNA transcription and secretion in HepG2 cells was dose dependent, whereas leptin did obviously influence neither secretion nor expression of apoAI, apoB and apoE in HepC2 cells. This indicates that leptin has a specific regulatory effect on hepatic apoM transcription and secretion *in vitro*.

Leptin is a cytokine-like peptide secreted by adipose tissue, known to regulate hepatic lipid and lipoprotein metabolism [12-15]. Mice with genetic defects in leptin exhibit hyperlipidemia, hyperinsulinemia, hyperglycemia and resistance to insulin [16-18]. Administration of leptin could partially or fully reverse these abnormal phenotypes [12,

13, 15, 19-21]. It is still unknown why leptin has contradictory effects on apoM expression *in vivo* and *in vitro*. One possible explanation is that leptin is a multi-functional hormone that regulates many metabolic aspects *in vivo*.

ApoM is mainly associated with HDL with a small proportion present in TGRLP and LDL in human plasma [1]. On a human tissue-array blotting analysis, apoM mRNA was expressed exclusively in liver and in kidney, with a minor expression found in fetal liver and in fetal kidney [22]. The synthesis of apoM in liver may be associated with the production of HDL, whereas the physiological importance of the apoM mRNA in kidney is less obvious. Apart from the hypothetical involvement of apoM in the synthesis of HDL, it is possible that apoM serves a specific function in the metabolism and transfer of cholesterol and/or other bioactive molecules in plasma. Molecular modeling methods demonstrated that apoM is a member of the lipocalin super-family [23]. Most members of this super-family have the ability to bind and transport hydrophobic molecules in plasma. Alternatively, apoM could have the ability to transport hydrophobic low molecular weight compounds, the nature of which remains to be determined.

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LEGEND TO FIGURES

Fig 1.

Effect of leptin on apoM secretion in HepG2 cells. ApoM concentration was determined by western blotting analysis as described in the material and methods. Data are expressed as the volume that was analyzed by the software of Quantity One. Data are means ± SD (n=6 for each sample group). Lanes 1-3, control group, lanes 4-12, leptin concentrations at 10, 100 and 500 ng /mL, respectively. * $P< 0.05$ vs. control group.

Fig 2.

Northern blotting analysis of G3PDH, apoAI, apoB, apoE and apoM mRNA levels after administration of leptin. Total RNA was extracted, and mRNA levels of G3PDH, apoAI, apoB, apoE and apoM were determined. Aliquots of 10 μg total RNA isolated from control cells (without leptin) or cells treated with different amounts of leptin (10 – 500 ng /mL), were used. The same membrane was blotted with ^{32}P-labeled probes. Data are means ± SD (n=5 for each sample group). ** $P< 0.01$ vs. control group.

Fig 3.

Real –time RT-PCR determination of apoM levels in control cells and in leptin treated cells. ApoM mRNA levels were determined by real-time RT-PCR analyses as described in the materials and methods. Each experimental group contains 6 replicates and real-time RT-PCT was run triplicates. Data are means ± SD (n=6 for each sample group). Control group represented 100%. * $P<0.05$; ** $P< 0.01$ vs. control group. The data is one of four similar experiments.

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Figure 1

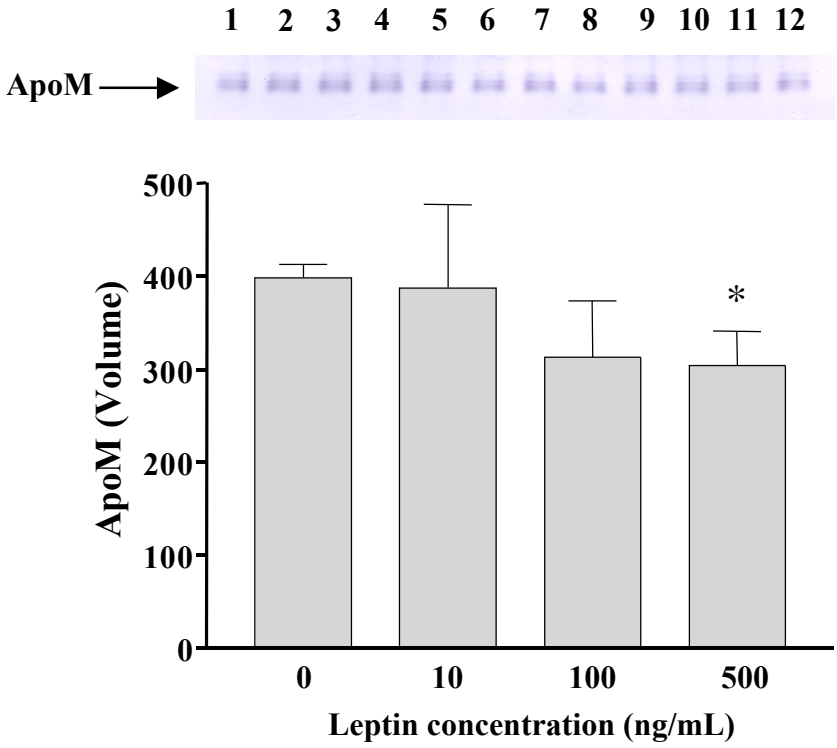

1 2 3 4 5 6 7 8 9 10 11 12
ApoM
500
400
300
200
100
0
ApoM (Volume)
*
0
10
100
500
Leptin concentration (ng/mL)

Figure 2

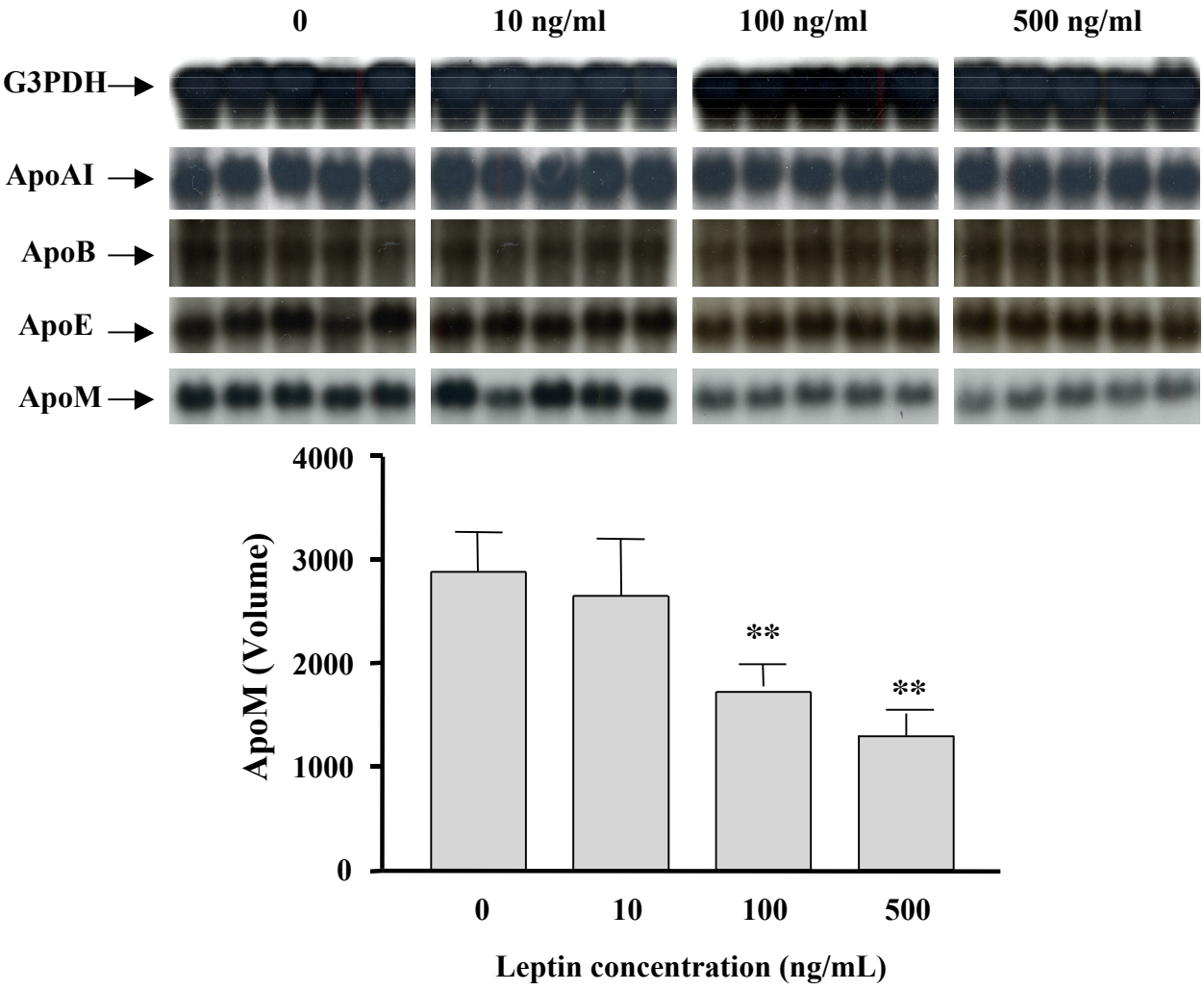
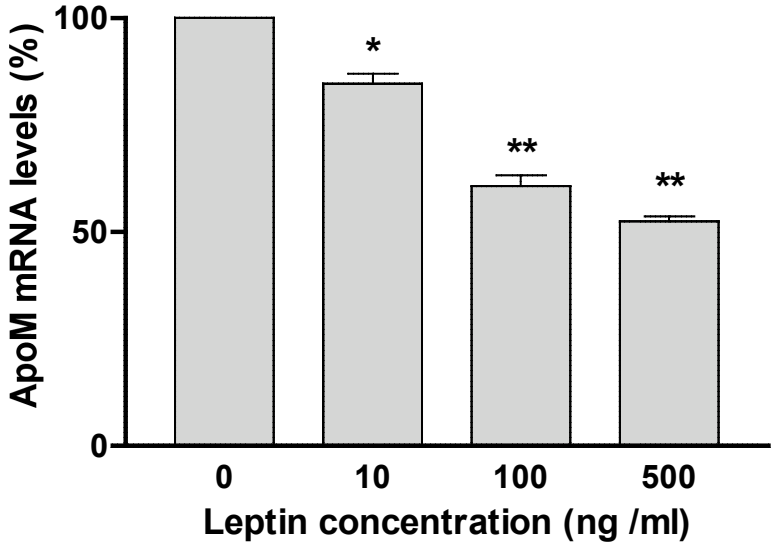

0
10 ng/ml
100 ng/ml
500 ng/ml
G3PDH
ApoAI
ApoB
ApoE
ApoM
4000
3000
2000
1000
0
ApoM (Volume)
**
**
0
10
100
500
Leptin concentration (ng/mL)

Figure 3


ApoM mRNA levels (%)
100
50
0
*
**
**
0
10
100
500
Leptin concentration (ng /ml)

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