Expression of apolipoprotein M and its association with adiponectin in an obese mouse model

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Abstract. The aim of the present study was to explore the association between apolipoprotein M (ApoM) and adiponectin, and the underlying mechanism, via observation of ApoM expression in an obese mouse model. For in vivo experiments, mice were randomly distributed into four groups: Control group, obese group, obese group treated with adiponectin, and normal group treated with adiponectin. Body weight, plasma adiponectin, blood glucose and fasting insulin were measured and visceral adipose tissue was weighed at the end of the experiment. ApoM and transcription factor forkhead box A2 (Foxa2) mRNA expression in the mouse liver was evaluated and the protein level of ApoM detected. For in vitro experiments, an insulin-resistant (IR) hepatic cell model was established by inducing the HepG2 cell line with a high concentration of insulin. Following treatment with adiponectin, changes in ApoM and Foxa2 mRNA expression and ApoM protein expression were evaluated in the control and IR HepG2 cells. Results demonstrated that compared with the control group, body weight, visceral adipose tissue weight, blood glucose, fasting insulin and insulin-resistance index (HOMA-IR) were significantly increased in the obese group, whilst plasma adiponectin, ApoM mRNA expression, Foxa2 mRNA expression and ApoM protein in the mouse liver were all significantly decreased. Following intervention with adiponectin on IR HepG2 cells, ApoM and Foxa2 mRNA expression and ApoM protein expression were significantly increased. However, the intervention did not have any effect on HepG2 cells. In conclusion, intervention with adiponectin elevated ApoM mRNA expression, potentially via relieving IR and upregulating Foxa2 mRNA expression.

Introduction

Obesity has become a major global healthcare issue with increasing prevalence across the world. The condition is highly associated with dyslipidemia, metabolic syndrome, type 2 diabetes, hypertension, hyperuricemia and many other diseases, and is an independent risk factor for mortality and lower quality of life for patients (1,2). Obesity is often considered to result in decreased high-density lipoprotein-cholesterol (HDL-C), whilst the level of HDL-C is negatively correlated with the incidence of coronary heart disease (3). Numerous studies have demonstrated that HDL has a protective effect on the arterial wall therefore apolipoproteins related to the metabolism of HDL are increasingly gaining attention. A recently discovered apolipoprotein, ApoM, is an important part of HDL and contributes considerably to the metabolism of HDL-C (4). Currently, it remains unclear whether HDL-C deficiency in the blood is associated with ApoM level. Furthermore, obesity is typically accompanied by hyperleptinemia and hyperadiponectinemia, and certain studies have revealed that leptin is associated with ApoM. However, it has not yet been reported whether there is an association between adiponectin and ApoM. Therefore, the present study investigated the underlying mechanism of blood HDL-C deficiency during obesity via detection of ApoM expression in obese mice, and also the possible association between ApoM and adiponectin.

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Insulin resistance (IR) is closely associated to obesity (5,6) and can aggravate the progress of the condition whilst promoting the development of diabetes, hyperlipidemia and hypertension. IR is characterized by the insensitivity of the target tissue to insulin, and also decreased uptake and utilization of glucose in peripheral tissues. The liver and peripheral tissues (fat and muscle) are the main sites of IR. HepG2 cells are derived from hepatocytes and retain many of the properties of hepatocytes. Following co-incubation with high concentrations of insulin, HepG2 cells exhibit the functional defects of insulin receptors and post-receptors (7-10). IR induced by a high concentration of insulin in HepG2 cells is an ideal cell model for studying this condition (11-14). Presently, adiponectin is the only adipocyte-derived cytokine found to have a protective effect on humans and can significantly improve IR (15). In order to explore the mechanism of adiponectin on ApoM, the effects of adiponectin on IR in obese mice and IR HepG2 cells were investigated.

Materials and methods

Materials and reagents. C57BL/6N male mice (at 3 weeks of age) were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences. The human hepatoblastoma cell line HepG2 was kindly provided by the Xiangya School of Medicine, Central South University (Changsha, China). Fluorescent quantitative polymerase chain reaction (PCR) kit was purchased from Promega Corporation. The antibody for ApoM detection was obtained from Abcam (cat. no. ab66379) and an insulin ELISA kit (cat. no. 10-1247-01) was obtained from Mercodia. All PCR primers were synthesized by Aoke Biotechnology Company, Ltd. (Table I). TRIzol® reagent was purchased from Invitrogen (Thermo Fisher Scientific, Inc.). A plasma insulin detection kit was purchased from Merckodia. Recombinant mouse adiponectin was obtained from Abcam (cat. no. ab54483).

Animal grouping and treatment. The Ethics Committee of the Xiangya Hospital of Central South University reviewed and approved this study. A total of 32 C57BL/6N male mice (3 weeks of age), initially weighing ~9.5 g, were observed for 1 week whilst housed in single-cages (12-h light/dark cycle; temperature 24-28°C, relative humidity 60-75%) and fed individually. Mice were then randomly and evenly distributed into four groups: The control group where mice were fed with a regular diet for 12 weeks; the obese group where mice were fed with a fat-rich diet (from Research Diets D12492) for 12 weeks; the obese group with intervention where mice were fed with a fat-rich diet (Research Diets D12492) for 12 weeks then treated with adiponectin for a further 7 days; and the normal group with intervention where mice were fed with a regular diet for 12 weeks followed by 7 days of treatment with adiponectin. The recombinant mouse adiponectin was dissolved in PBS reaching a final concentration of 0.5 μg/μl. Adiponectin was administered to mice intraperitoneally (IP) at 1.5 mg/kg body weight once per day for 7 days. It was administered at a speed of 10 μl/min from 17:00. Mice had free access to food and water. At the end of the experiment, body weight, fasting blood glucose and plasma adiponectin levels were measured following which all mice were sacrificed under anesthesia to minimize suffering. Mice were anesthetized by IP injection of sodium pentobarbital (2%, 40 mg/kg) and executed by cardiac puncture. Liver tissue was isolated and preserved in liquid nitrogen. Visceral adipose tissue weight was obtained by measuring the wet weight of the epididymis and adipose tissue surrounding the kidney with an electronic balance (the adhering tissue fluid was removed by suction with filter paper). Plasma insulin and adiponectin levels were determined by ELISA. Mice were fasted for 6-8 h then blood samples were obtained from the caudal vein to determine fasting plasma glucose (FPG) level with a One-Touch blood glucose meter (LifeScan, Inc.) The formula for calculation of IR index was: IR index [Homeostatic model assessment (HOMA)-IR]=FPG x fasting insulin/22.5.

Animal diets. The basal diet was prepared by the Animal Laboratory of Xiangya School of Medicine, providing energy of 15 kJ/g, with a contribution of 23% protein, 65% carbohydrates and 12% fat. The fat-rich diet (D12492) was provided by Research Diets Company, providing energy of 22 kJ/g, with a contribution of 20% protein, 20% carbohydrates and 60% fat.

Maintenance and subculture of the HepG2 cell line. The HepG2 cell line was cultured with Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% fetal bovine serum (Tian Hang Biotechnology Company) and 1.0x10^5 U/l penicillin-streptomycin and incubated under standard conditions (37°C, 5% CO₂). Medium was replaced every other day and subculture was performed using 0.25% trypsin to detach cells from the culture vessel.

The liver is the organ with highest metabolic activity in humans and is responsible for a variety of important functions, such as lipid metabolism, therefore it is reasonable to employ hepatic cells in studies on lipid metabolism in vitro. It is extremely difficult to obtain normal hepatic cells under physiological conditions, and normal human hepatic cells usually exhibited marked individual differences that make it hard to achieve stable subcultures. For example, human hepatic cell line HL-7702, which is derived from normal human hepatic tissue, shows dramatic biological differences to primary hepatic cell cultures. Human hepatic cell lines, L-02, LX-1 and LX-2 could be regarded as good representatives of the human liver (16); however, they possess defective regeneration abilities which makes them less suitable options for in vitro experiments (17). The HepG2 cell line, derived from human hepatoblastoma, possesses a strong regenerative ability as well as the majority of all liver-related functions (18,19), and could effectively mimic the in vivo environment for lipid metabolism. Therefore, the HepG2 cell line was selected to perform all in vitro experiments.

Establishment of IR in HepG2 cell model. Studies typically use glucose consumption as a criterion to determine whether IR is successful (20-22). HepG2 cells at logarithmic growth stage were digested, the cell density adjusted to 5x10^4 cells/ml using serum-containing medium, then 200 μl cell suspension was added to each well of a 96-well culture plate. Samples were divided into either control group (no insulin stimulation, normal cultured cells) or IR model group. Following monolayer adherence, serum-containing medium was discarded and...
cells washed with PBS. Serum-free medium was added to the control group and serum-free medium containing 10^{-7} mol/l insulin was added to the model group. The supernatant was removed from the culture medium 24 h following treatment, then glucose content was measured using the glucose oxidase assay kit (Zhongbei Biotechnology Co., Ltd.). The glucose consumption was determined by calculating the difference between glucose content in the cell culture medium without cells and glucose content in the experimental group.

**Cell groups and interventions.** Adiponectin was used as an intervention in IR HepG2 cells and HepG2 cells for 24 h. All chemicals were dissolved in dimethyl sulphoxide (DMSO) and the final concentration of DMSO in media was maintained at 0.1% (v/v). The treatment groups were as follows: Control group, which was treated with 0.1% DMSO; IR group, which was treated with 10^{-7} mol/l insulin; adiponectin intervention IR group, which was treated with a combination of 10^{-7} mol/l insulin and 30 µg/ml adiponectin; and adiponectin intervention group, which was treated with 30 µg/ml adiponectin. All treatments lasted 24 h, then glucose consumption and ApoM and forkhead box A2 (Foxa2) gene expression were evaluated in each group.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from mouse liver and other tissues using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA was inspected under ultraviolet light following formaldehyde-denatured agarose gel electrophoresis for 10 min (stained with ethidium bromide, buffered with 1X MOPS, applied with constant voltage of 5 V/cm). The synthesis of cDNA was performed according to the Avian Myeloblastosis Virus reverse transcriptase protocol (Promega Corporation), and purified via the PAGE method. GAPDH mRNA was employed as the loading control to ensure even loading of all RNA samples. The buffer for SYBR Green Real Time PCR and reaction conditions were selected based on the manufacturer’s protocol for the Real Time PCR Master kit (Promega Corporation): 50˚C for 2 min, 95˚C for 10 min for the first cycle, and 95˚C for 15 sec, 61˚C for 45 sec and 61˚C for 10 sec for 40 cycles, until the reaction ended. The transcription level of target genes (Table I) in all samples was normalized to the internal reference gene GAPDH and analyzed via the 2^{-ΔΔCq} method (23).

**Detection of ApoM expression by western blot analysis.** Proteins were extracted using RIPA lysis buffer (Shanghai Biyuntian Biotechnology Co., Ltd.) from cultured cells or mouse livers. Proteins were then determined via a BCA assay. For each sample, 50 µg of total protein was diluted in loading buffer, boiled in a 100˚C water bath for 10 min then stored on ice. Protein samples were separated by 6% SDS-PAGE then transferred onto polyvinylidene difluoride membranes and blocked with 5% skimmed milk for 2 h. Then the membrane was incubated with primary antibody ApoM (1:500) at 4˚C overnight. Membranes were washed in a mixture of tris-buffered saline and Polysorbate (TBST) three times (10 min/wash) then incubated in horseradish peroxidase-conjugated secondary antibody (1:2,000) for 1 h at RT then washed in TBST three more times. X-ray films were used to capture the signals from membranes that had been processed using a western blot chemiluminescence detection reagent kit (Pierce; Thermo Fisher Scientific, Inc.) The bands on films were analyzed with TINA 2.09 image processing software (Raytest). Densitometry analysis was performed with β-actin used as loading control.

**Statistical analysis.** All experimental data are presented as the mean ± standard deviation and analyzed with the software SPSS 15.0 (SPSS, Inc.). Comparisons among groups were evaluated using one-way analysis of variance and Student-Newman-Keuls test. P<0.05 was considered to indicate significant difference.

| Gene  | Resource | Primer | Sequence                  |
|-------|----------|--------|---------------------------|
| ApoM  | Mu       | Forward| 5’-CAGTGCCCTGAGCACAGTCAA-3’ |
|       |          | Reverse| 5’-GCTGCTCCGCAATAAAGTACC-3’ |
| ApoM  | Hs       | Forward| 5’-CTGACAACCTCTGGGCGTGGA-3’ |
|       |          | Reverse| 5’-CAGAGCCAGACAGCATGAA-3’ |
| Foxa2 | Mu       | Forward| 5’-GTCTGCCAGCGACAGCAAC-3’ |
|       |          | Reverse| 5’-GGGGTJAGTCATGACCTGTTCGTAG-3’ |
| Foxa2 | Hs       | Forward| 5’-GGGCTTCCAGCTGCTTCA-3’ |
|       |          | Reverse| 5’-CCATGTTTCCTATGGTGTTGAA-3’ |
| GAPDH | Mu       | Forward| 5’-ACAGCAACAGGTGTTGAGAC-3’ |
|       |          | Reverse| 5’-TGGGAGGTTGCACGGAACCT-3’ |
| GAPDH | Hs       | Forward| 5’-CCCAAGGCGAGGAGGATG-3’ |

ApoM, apolipoprotein M; Foxa2, forkhead box A2; Mu, Mus musculus; Hs, Homo sapiens.
Table II. Comparisons of body weight, visceral adipose tissue weight, levels of blood glucose, insulin, HOMA-IR and plasma adiponectin.

| Variables                             | Control              | Obesity               | Obesity with intervention | Control with intervention |
|---------------------------------------|----------------------|-----------------------|---------------------------|---------------------------|
| Body weight by the end of 12th week (g) | 29.21±1.51           | 42.37±1.42<sup>a</sup> | 42.67±1.33<sup>a</sup>    | 28.92±1.32                |
| Visceral adipose tissue weight (g)     | 0.55±0.02            | 1.81±0.50<sup>a</sup> | 1.79±0.32<sup>a</sup>     | 0.54±0.03                 |
| Blood glucose (mmol/l)                | 8.69±1.13            | 13.66±1.27<sup>a</sup> | 11.23±1.23<sup>a,b</sup>  | 8.50±1.17                 |
| Plasma insulin (ng/ml)                | 0.35±0.02            | 0.75±0.05<sup>a</sup> | 0.60±0.07<sup>a,b</sup>   | 0.33±0.02                 |
| HOMA-IR                               | 0.13±0.01            | 0.45±0.03<sup>a</sup> | 0.30±0.05<sup>a,b</sup>   | 0.12±0.01                 |
| Adiponectin (mg/l)                    | 2.11±0.03            | 1.32±0.07<sup>a</sup> | 1.97±0.03<sup>b</sup>     | 3.19±0.07<sup>a</sup>     |

<sup>a</sup>P<0.05 vs. control group; <sup>b</sup>P<0.05 vs. obese group. HOMA-IR, homeostatic model assessment-insulin resistance.

Results

Comparisons of body weight, visceral adipose tissue weight, blood glucose, insulin, HOMA-IR and plasma adiponectin amongst groups. At the end of week 12, body weight and visceral adipose tissue weight of the obese group and the obese group with intervention were significantly higher compared with the control group (P<0.05; Table II). The levels of blood glucose, insulin and HOMA-IR were all elevated compared with the control group which indicated that the continuous fat-rich diet could lead to elevated blood glucose, hyperinsulinemia and IR. The adiponectin level in the obese group was markedly decreased when compared with the control group (P<0.05; Table II). Intervention with adiponectin in obese mice was associated with lower levels of blood glucose, insulin and HOMA-IR compared with the obese group (P<0.05; Table II). Plasma adiponectin in the obese group with intervention was significantly higher compared with the obese group, however there was no significant difference in visceral adipose tissue weight (P>0.05; Table II). Intervention in the control group led to elevated plasma adiponectin level compared with the control group without intervention, however no significant differences were observed in terms of visceral adipose tissue weight, levels of blood glucose, insulin and HOMA-IR (P>0.05; Table II).

Levels of ApoM mRNA and protein in mouse livers. ApoM mRNA expression (Fig. 1) and protein levels (Fig. 2) in the obese group were markedly decreased when compared with the control group (P<0.05; Table II). Following intervention with adiponectin, ApoM mRNA expression (Fig. 1) and protein levels (Fig. 2) were elevated in the obese group with intervention compared with the obese group. There was no significant difference between the control group with intervention and the control group (P>0.05).

Foxy2 mRNA expression levels in mouse livers. Compared with the control group, the Foxo2 mRNA expression levels were significantly lower in the obese group (P<0.05; Fig. 3). Following intervention with adiponectin, the Foxo2 mRNA expression level was markedly elevated in the obese group with intervention compared with the obese group (P<0.05; Fig. 3). However, there was no significant difference in Foxo2 mRNA expression levels between the control group and the control group with intervention (P>0.05; Fig. 3).

Levels of ApoM mRNA and protein in HepG2 cells. Compared with the control group, the levels of ApoM mRNA expression and protein were significantly decreased in the IR group (P<0.05; Figs. 4 and 5). Following intervention, the levels of ApoM mRNA expression and protein were both significantly increased in the adiponectin intervention IR group compared with the IR group (P<0.05; Figs. 4 and 5).
were no significant differences in ApoM mRNA expression or protein levels between the control group and the adiponectin intervention group (P>0.05; Figs. 4 and 5).

**Fossa2 mRNA expression levels in HepG2 cells.** Compared with the control group, the Fossa2 mRNA expression levels were significantly lower in the untreated obese group. Following intervention with adiponectin, the Fossa2 mRNA expression levels were markedly elevated compared with the untreated obese group. There was no significant difference in Fossa2 mRNA expression levels between the control group and the control group with intervention. *P<0.05 vs. control; †P<0.05 vs. obese group. Fossa2, forkhead box A2.

**Glucose consumption in HepG2 cells.** Glucose consumption in the IR group was significantly lower than the control group (P<0.05; Fig. 7). This suggested that the glucose consumption capacity of the cells in the IR group was decreased, and the glucose uptake and utilization was impaired, which resulted in IR. Glucose consumption of the adiponectin intervention IR group was higher than the IR group (P<0.05; Fig. 7) which suggested that adiponectin could increase the glucose consumption of IR HepG2 cells. There was no significant difference in glucose consumption between adiponectin intervention group and control group (P>0.05; Fig. 7).

**Discussion**

Obesity is closely associated with cardiovascular diseases, and is an independent risk factor for mortality and lower quality of life. Obesity is often correlated with disorders in carbohydrate and fat metabolism, the most significant of which is HDL-C deficiency, a risk factor for coronary heart disease. However, the mechanism by which HDL-C deficiency occurs in obesity remains unclear. Current evidence indicates that HDL-C deficiency could occur in obese patients because its particle size is small enough to easily penetrate the filter structure of the kidney and due to an overactive catabolism (24). ApoM is a recently discovered apolipoprotein that is closely associated with HDL-C. Increased HDL-C in plasma was observed in ApoM over-expressing mice whilst silencing of the ApoM gene resulted in a significant decrease in plasma HDL-C levels and a complete abolishment of pre-β-HDL, the primary form of HDL during its maturation as well as an important cholesterol receptor in somatic cells. The clearance rate of HDL from plasma and the uptake rate by somatic cells lacking ApoM were significantly increased (4). It has also been demonstrated that ApoM is the receptor for sphingosine-1-phosphate (SIP) in HDL particles with function primarily achieved via the ApoM/SIP axis (25-27). Furthermore, studies have identified that ApoM gene polymorphism is related to the metabolism of HDL in obese Korean male adults (28). The aforementioned
regulator for ApoM expression (30,31). Studies have reported that an adipocytokine, leptin, is an important factor associated with cardiovascular diseases via a series of cellular signaling pathways. Both adiponectin and leptin are adipocytokines (29). Both adiponectin and leptin are closely associated with cardiovascular diseases via a series of cellular signaling pathways. A series of cellular signaling pathways is activated by adiponectin and leptin, which collectively suggest that the decreased ApoM expression in obesity was correlated with decreased adiponectin levels. Therefore, elevated adiponectin level may upregulate ApoM expression, indicating that adiponectin is another adipocytokine responsible for ApoM expression regulation. It is notable that the regulatory role of adiponectin on ApoM expression was only observed in IR HepG2 cells and not in normal HepG2 cells, suggesting that IR is required as a pre-condition for the regulatory effects of adiponectin. This was supported by the observation that adiponectin intervention in normal mice failed to upregulate ApoM expression.

In summary, ApoM gene expression in obese mice was significantly decreased in the IR group compared with the control group. This was supported by the observation that adiponectin intervention in obese mice failed to upregulate ApoM expression. Furthermore, the decreased ApoM expression was positively correlated with HDL-C (32,33). Therefore, the present study speculated that the metabolism of HDL might be related to adiponectin and the effect of adiponectin on ApoM was investigated.

Adiponectin is mainly secreted by adipose tissue and is currently the only known protective adipocytokine effectively alleviating IR and demonstrating anti-inflammatory and anti-atherosclerosis properties (34,35). The present study demonstrated that obese mice exhibited an elevated plasma adiponectin level and ApoM expression was upregulated following adiponectin-treatment. Adiponectin intervention IR cells also exhibited marked upregulation of ApoM expression, which collectively suggested that the decreased ApoM expression in obesity was correlated with decreased adiponectin levels. Therefore, elevated adiponectin level may upregulate ApoM expression, indicating that adiponectin is another adipocytokine responsible for ApoM expression regulation. It is notable that the regulatory role of adiponectin on ApoM expression was only observed in IR HepG2 cells and not in normal HepG2 cells, suggesting that IR is required as a pre-condition for the regulatory effects of adiponectin. This was supported by the observation that adiponectin intervention in normal mice failed to upregulate ApoM expression.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LY, TL, SPZ and SDZ conceived and designed the experiments. LY and TL performed the experiments. LY and TL collected and analyzed the data. LY and TL wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Animal care and handling were completed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Ethics Committee of the Xiangya Hospital of Central South University (Changsha, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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