Adipose tissue regulates hepatic cholesterol metabolism via adiponectin

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A B S T R A C T

Aims: Lipid metabolic disorder involves multiple tissues and organs. Hepatic cholesterol metabolism is an important physiological process, which is tightly related to obesity and lipid metabolic disorders. In this study, we examined the direct effects of adipocytes on hepatic cholesterol metabolic factors and investigated the role of potential adipocytokines in it.

Main methods: Male SD rats were induced by a high-fat diet (HFD) and hepatic cholesterol metabolic factors, 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) and ATP-binding cassette transporter A1 (ABCA1) were measured by immunoblotting. Then the effects of adipocytes on the expressions of hepatic cholesterol metabolism proteins were examined in the co-culture system. Finally, the concentrations of several adipocytokines were detected by ELISA and the effect of adiponectin (APN) on hepatic cholesterol metabolism was confirmed by short interference RNA (siRNA) in vitro.

Key findings: Our results showed that adipocytes significantly increased ABCA1 and decreased HMGR in hepatocytes after co-culture. Lipopolysaccharide (LPS) treatment in this co-culture system reversed cholesterol metabolism compared with the untreated group. APN, which also decreased in obese rats, had a significant positive correlation with ABCA1 and an inverse correlation with HMGR in vitro. Co-culturing with APN-silenced adipocytes partially restored ABCA1 and HMGR levels.

Significance: The present study demonstrates that adipocytes regulate hepatic cholesterol metabolism partly via APN.

Introduction

Recently, nonalcoholic fatty liver disease, hyperlipidemia and other lipid metabolic disorders are on the rise among the general population. It has been demonstrated that lipid metabolic diseases are systemic disorders affecting many organs such as the liver, muscle, and adipose tissue. Cholesterol homeostasis is presumed to be primarily responsible for the development of many lipid metabolic disorders. The liver plays an important role in cholesterol homeostasis, such as biosynthesis, via 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGR), reverse cholesterol transport through ATP-binding cassette transporter A1 (ABCA1), (Weber et al., 2004). The major precursor of cholesterol synthesis is acetyl-CoA which gives rise to hydroxyl methylglutaryl-CoA (HMG-CoA). The rate limiting step in the cholesterol biosynthetic pathway is the conversion of HMG-CoA to mevalonic acid (MVA) by HMGR, and ABCA1 plays a crucial role in the efflux of cellular cholesterol (Espenshade and Hughes, 2007; Segatto et al., 2011). ABCA1 is a critical factor in the maintenance of plasma HDL-C levels, and is important for the transfer of cellular cholesterol, phospholipids, and other molecules to lipid-poor apolipoproteins (Ye et al., 2011).

According to recent surveys, obesity is a major risk factor for metabolic syndrome and strong relationship exists between obesity and cholesterol metabolic disorder (Fabbri et al., 2010; Oda, 2012). One characteristic of obesity is the enlargement of adipose tissue, and scores of studies have reported that adipose tissue can be regarded as a major secretory and endocrine organ. Further, numerous proteins secreted by adipocytes potentially play physiological roles in metabolism (Galic et al., 2010). Leptin, adiponectin (APN), resistin and several other adipocytokines have been thought to be involved in the regulation of lipid metabolism (Lago et al., 2009). Studies have clearly demonstrated that APN signaling have beneficial effects on lipid and glucose metabolism. Current research has indicated that APN dramatically promoted reverse cholesterol transport in the liver by increasing high density lipoprotein assembly (Matsuura et al., 2007). APN exerts its effects by two receptors, AdipoR1 which involved in AMP-activated kinase (AMPK) pathway, and AdipoR2 which is involved in PPARα pathway (Yamauchi et al., 2014). Many clinical studies pointed out the association between APN and serum lipoprotein (Chan et al., 2009; Park et al., 2010; Tsuchiya et al., 2009), and patients with high cholesterol level revealed significantly elevated levels of pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α).

Abbreviations: APN, adiponectin; ABCA1, ATP-binding cassette transporter A1; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; MVA, mevalonic acid; siRNA, short interference RNA; siRNA, short interference RNA; LPS, lipopolysaccharide; PPARα, peroxisome proliferator-activated receptor α; AMPK, AMP-activated kinase; IL-6, interleukin 6; TNF-α, tumor necrosis factor α.
Although direct actions of some adipocyte-secreted factors on cholesterol metabolism have been reported (e.g., IL-1β and TNF-α up-regulated cholesterol influx and down-regulated cholesterol efflux in vivo and in vitro), the effects of adipocytes on hepatic cholesterol metabolism are unclear.

In this study, the association between adipocytes and hepatic cholesterol metabolism was verified by a co-culture system in vitro. Then we detected the adipocytokines in the co-culture system and obese rats. Furthermore, we tried to find out which adipocytokine plays a role between adipocytes and hepatic cholesterol metabolism. The results demonstrate the direct involvement of adipocytes in hepatic cholesterol metabolism partly via APN.

Material and methods

Culture of 3T3-L1 cells

Mouse embryonic fibroblast-adipose like cell line 3T3-L1 (ATCC) were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 U/ml penicillin, streptomycin and 10% newborn bovine serum. Cells were incubated at 5% CO2 and 37 °C and differentiated into adipocytes according to the previously described methods (Kim et al., 2002). Briefly, cells were propagated and allowed to reach 100% of confluence. After 2 days, the medium was changed to IDIX I (DMEM containing 10% fetal bovine serum and 150 nmol/L insulin, 250 nmol/L dexamethasone, and 0.5 mmol/l 3-isobutyl-1-methylxanthine). Two days later, the medium was switched to IDIX II (DMEM containing 10% fetal bovine serum and 150 nmol/L insulin). After another 2 days, the cells were switched back to DMEM supplemented with 10% fetal bovine serum and cultured for an additional 4 days. Cells in two differentiation periods (0 and 8 days after the differentiation, respectively) were fixed with p-formaldehyde and stained with 0.5% Red-Oil O (Sigma), in 60% isopropanol (Miki et al., 2001).

Isolation and culture of primary hepatocytes (Wang et al., 2006)

Primary hepatocytes were isolated from adult male Sprague–Dawley rats (250 to 300 g). In brief, the liver was initially perfused with Ca2+-free Hank’s buffer and then dissociated with collagenase type IV (GIBCO) in Hank’s buffer plus 5 mmol/L CaCl2. Isolated hepatocytes were resuspended in Hank’s buffer containing 1.0 mmol/L CaCl2 and 0.6 mmol/L MgSO4. Cells were then filtered through a 90 μm nylon mesh, counted, and tested for viability using a trypan blue (GIBCO) exclusion. Isolated cell pellets were resuspended in DMEM supplemented with 10% fetal bovine serum.

Co-culture (Suganami et al., 2007; Yamashita et al., 2007)

Co-culture of adipocytes and hepatocytes was conducted using a transwell system (Millipore) and transwell insert has a 0.4 μm PET membrane to separate upper and lower chambers. Briefly, 3T3-L1 cells (2 × 10^5 cells/well) were seeded in six-well plates (lower chamber) and differentiated into mature adipocytes as described above. Then the hepatocytes (2 × 10^5 cells/insert) were plated onto the transwell insert (upper chamber) and transferred to plate wells and incubated with mature adipocytes. The medium in the transwell system was changed to DMEM without serum; this resulted in an assembly of the two cell types sharing the same culture medium but being separated by the membrane of the insert.
To examine the crosstalk between the adipose tissue and the liver, male Sprague–Dawley rats (200 to 240 g) were randomly assigned to four groups (n = 6). The control group was fed a normal diet; all other three groups were fed high-fat diet containing 2% cholesterol and 10% lard for 1, 2 and 8 weeks, respectively. All rats were housed under identical conditions in an aseptic facility and given free access to water and food. At the end of each time period, rats were fasted for 16 h, then euthanized and blood samples were collected. IL-6, TNF-α, resistin and APN, in plasma, were quantified using ELISA kits (Uscn Life). And then the AdipoR2 in the liver was analyzed by immunostaining. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China Pharmaceutical University (Permit Number: PMY33069N). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Western blotting

Samples were lysed in a buffer containing 50 mM Tris/HCl (pH 7.4), 1% (v/v) NP-40, 0.25% (v/v) sodium-deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na3VO4, and a complete protease inhibitors cocktail (Roche). After incubation for 2 h at 4 °C, the suspension was centrifuged at 10000 g for 10 min. Thereafter, 20 μg of lysates was separated by SDS-PAGE using 10% horizontal gels and transferred to polyvinylidene fluoride filters in a semidry blotting apparatus. For detection, membranes were blocked with TBS containing 0.1% Tween 20 and 5% non-fat dried milk and subsequently incubated overnight with a 1:1000 dilution of appropriate antibodies (Abcam). After extensive washing, filters were incubated with a secondary HRP-coupled antibody (Sigma) and processed for enhanced chemiluminescence detection (Thermo). Signals were visualized and evaluated on an Imager workstation using Image Analysis software (Quantity One, Bio-Rad).

Animal studies

To examine the crosstalk between the adipose tissue and the liver, male Sprague–Dawley rats (200 to 240 g) were randomly assigned to four groups (n = 6). The control group was fed a normal diet; all other three groups were fed high-fat diet containing 2% cholesterol and 10% lard for 1, 2 and 8 weeks, respectively. All rats were housed under identical conditions in an aseptic facility and given free access to water and food. At the end of each time period, rats were fasted for 16 h, then euthanized and blood samples were collected. IL-6, TNF-α, resistin and APN, in plasma, were quantified using ELISA kits (Uscn Life). And then the AdipoR2 in the liver was analyzed by immunostaining. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China Pharmaceutical University (Permit Number: PMY33069N). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

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Table 1

<table>
<thead>
<tr>
<th></th>
<th>IL-6 (ng/mL)</th>
<th>TNF-α (ng/mL)</th>
<th>Adiponectin (pg/mL)</th>
<th>Resistin (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocytes</td>
<td>119.32 ± 13.41</td>
<td>7.25 ± 0.10</td>
<td>1672.49 ± 20.93</td>
<td>12.96 ± 1.05</td>
</tr>
<tr>
<td>Adipocytes (LPS)</td>
<td>315.13 ± 10.41</td>
<td>177.64 ± 2.20</td>
<td>585.23 ± 27.92</td>
<td>18.63 ± 2.74</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>NA</td>
<td>NA</td>
<td>1745.28 ± 13.21</td>
<td>13.07 ± 1.26</td>
</tr>
<tr>
<td>Hepatocytes (LPS)</td>
<td>327.88 ± 18.96</td>
<td>181.45 ± 11.43</td>
<td>526.39 ± 19.99</td>
<td>19.50 ± 3.27</td>
</tr>
</tbody>
</table>

Results are shown as means ± S.D. NA means below the detection limit.
ELISA

TC, IL-6, TNF-α, resistin and APN, in cell culture medium or plasma, were quantified using ELISA kits (Uscn Life). The assays were performed following the manufacturer’s instructions using duplicate samples for all determinations.

RNA interference

The APN-specific siRNA (5′-CUACGACCAGUAUCAGGAATT-3′) was purchased from Ambion. The siRNA was transfected into cells by Lipo-fectamine 2000 (Invitrogen), which were grown in six-well culture plates or transwell inserts with DMEM. 48 h after transfection, cells and supernatants were collected for ELISA and immunoblotting detection.

Real time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated by TRIzol (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed by using Superscript First strand synthesis kit (Fermentas) to synthesize cDNA. Gene transcript level of AdipoR2 was amplified using SYBR Green Master Mix (Qiagen) with specific primer for AdipoR2 in an IQ5 (Bio-Rad). Primers for rat AdipoR2 were: forward 5′-ATAGTCCCAGTGGGACATG-3′, reverse 5′-AGGATCCGGGCAGCATACA-3′.

Presentation of data and statistics

Data obtained from different experiments were presented as means ± S.D. (standard of deviation) from at least three independent experiments. Statistical analysis was performed with one-way ANOVA followed by Tukey’s post hoc test for multiple comparisons tests. Significant differences were accepted when P < 0.05. All graphs were treated with GraphPad Prism 5.0 statistical software.

Results

Adipocytes regulate hepatic cholesterol metabolic factors in vitro

We examined the direct effects of adipocytes on hepatic cholesterol metabolic factors by the co-culture system. 3T3-L1 cell line is a widely used cell type that has been proven to be valuable for the elucidation of many processes relevant to primary adipocytes in vitro (Green and Kehinde, 1975, 1976). In the present study, we used the classical “differentiation cocktail” containing insulin, dexamethasone and 3-isobutyl-1-methylxanthine to induce adipogenesis in 3T3-L1 cells incubated in a medium containing 25 mmol/L glucose (Oda, 2012). Using this protocol, hepatocytes were co-cultured with mature adipocytes for 24, 48, and 72 h. Hepatic ABCA1 and HMGR were analyzed by immunoblotting. As indicated in Fig. 1, co-culture with adipocytes for 24, 48, and 72 h could significantly enhance ABCA1 and suppress HMGR expressions in hepatocytes, respectively. Moreover, no significant difference was found between different co-culture groups.

However, recent investigations have revealed that adipose tissue in obesity is characterized by a chronic low-grade inflammatory state (Greenberg and Obin, 2006). So we used LPS-induced adipocytes as an inflammatory model co-cultured with hepatocytes for 48 h. In contrast, in the co-culture system, LPS treatment markedly impaired cholesterol metabolism, as reflected by rising HMGR and declining ABCA1 expression in hepatocytes (Fig. 2).

Secretion of adipocytokines in vitro

To identify the major mediator of cholesterol metabolism in the co-culture model, efforts were made to quantify IL-6, TNF-α, APN and REN concentrations in a cell culture supernatant of separated hepatocytes, adipocytes (LPS-induced or not), and the medium of co-culture model (co-cultured for 48 h) by ELISA. As shown in Table 1, APN secretion was inhibited, IL-6, TNF-α and REN secretions were increased by LPS in adipocytes. The cytokine concentrations in the co-culture
supernatant were observed to be similar to the individual adipocytes, suggesting that hepatocytes did not influence the cytokine secretion of adipocytes, and these changing cytokines may play roles in the crosstalk between adipocytes and hepatocytes.

Secretion of adipocytokines in obese rats

As shown in Fig. 3, at the end of 8 weeks, the TC in HFD-fed rats was 113% greater than that in normal-diet fed rats. Compared to the control group, the plasma concentrations of IL-6, TNF-α, and REN were significantly increased in HFD group, however, the content of plasma APN and the expression of hepatic AdipoR2 were decreased. This result was further confirmed by immunostaining of AdipoR2 in the liver (Fig. 4, arrow heads indicate AdipoR2 protein expression). As APN has beneficial effects on lipid metabolism, these results mentioned above showed that HFD decreased the expression of APN and its receptor AdipoR2 in the liver, which may be a cause of lipid metabolic disorders in vivo.

Adipocytes regulate the hepatic cholesterol metabolic factors partly via APN

In view of the data in vitro and vivo, we assumed that APN may play an important role in hepatic cholesterol metabolism. In order to confirm our hypothesis, we incubated hepatocytes with 0, 400, 800, 1600 pg/ml APN for 48 h, and then ABCA1, HMGR expressions were analyzed. As indicated in Fig. 5, APN significantly increased ABCA1 and inhibited HMGR level with a dose-dependent manner.

Then, we validated our hypothesis by APN genes silencing under co-culture condition. First we detected the APN in the transfected cells for the indicated time period (0–72 h), as presented in Fig. 6, the siRNA transfection efficiency was the highest at 93.2% when cells were transfected with siRNA for 48 h.

As presented in Fig. 7, siRNA-mediated APN silencing reversed ABCA1 and HMGR expressions compared with vehicle group. These results suggested that APN could significantly influence the hepatic cholesterol synthesis and efflux factors in vitro.

Discussion

Lipid metabolic disorder induced by obesity involves multiple target tissues. Hepatic cholesterol metabolism is an important physiological process in lipid metabolic disorder. It has been suggested that hepatic cholesterol metabolism can directly relate to visceral adiposity (Koska et al., 2008; Palmieri et al., 2006). Theoretically, visceral fat is anatomically close to the liver, and substances secreted from the cells of this fatty tissue enter directly into the portal circulation, which may lead to an interaction between the adipose tissue and the liver, with a potential involvement of some adipocytokines. Our data demonstrated that hepatic cholesterol efflux and synthesis proteins are correlated with adipocytes in vitro. In order to evaluate the effects of adipocytes on hepatic cholesterol metabolic factors, we developed a co-culture system of adipocytes and hepatocytes. Co-culture technique is generally an acceptable approach for studying the crosstalk between two cell types (Honda et al., 1994; Shillabeer et al., 1996). We report here, for the first time, that there is an interaction between adipocytes and hepatocytes in regulating cholesterol metabolic factors. This involved the decline of HMGR and the ascension of ABCA1 in hepatocytes. However, LPS treatment reversed all these changes under the co-culture condition. These data showed that adipose tissue is a potential physiologic target of hepatic cholesterol metabolism.

Some evidences indicate that adipocytokines, as the major cellular component of white adipose tissue, contribute to hepatic cholesterol metabolism via adipocytokines (Al-Daghri et al., 2011). APN, leptin, resistent, IL-6, TNF-α are most widely reported in this context. We found that APN secretion was inhibited, IL-6, TNF-α and REN secretions were increased in the co-culture model (LPS-induced) and HFD group, the liver AdipoR2 expression was also decreased in HFD rats. These results indicated that adipocytokines play roles in hepatic cholesterol metabolism. We observed the effects of APN on the expressions of cholesterol efflux and synthesis proteins in hepatocytes, and then used siRNA to silence the APN gene in adipocytes to confirm the effect. It is inferred that adipocytes regulate hepatic cholesterol metabolic factors partly via APN,

![Fig. 5](image1)

Fig. 5. The effects of APN on the expressions of ABCA1 and HMGR in hepatocytes. (A): Hepatocytes were treated in the presence of 0, 400, 800, 1600 pg/mL APN for 48 h. (B): Densitometric scanning of band intensities obtained from three separate experiments were sent to quantify the change of proteins expressions (control value taken as one-fold in each case) are showed. Bars indicate means ± S.D. *P < 0.05, **P < 0.01 compared with the corresponding group, #P < 0.05, ##P < 0.01 compared with the control group.

![Fig. 6](image2)

Fig. 6. The silencing efficiency of siRNA for APN in adipocytes. Adipocytes were transfected with siRNA for APN (siAPN) or control (siCtrl) and incubated for 24, 48, and 72 h. The expression of APN and the silencing efficiency in adipocytes were measured by ELISA.
which is responsible for up-regulation of ABCA1 and down-regulation of HMGR. This result suggested the involvement of APN in the hepatic cholesterol metabolism.

Other adipocytokines may also take part in the crosstalk between adipocytes and hepatocytes. Previous studies reported that leptin also modulates cholesterol efflux from human macrophages (Hongo et al., 2009), and regulates cholesterol biosynthesis in human monocytes (Balogh et al., 2011). Furthermore, serum resistin is related to plasma HDL-C and it is inversely correlated with LDL-C in diabetic and obese humans (Owecki et al., 2010). Thus, the effects of all adipocytokines, especially pro-inflammatory adipocytokines secreted by normal or inflammatory adipose tissue, on lipid metabolic disorder should be investigated in depth.

However, it has been shown that with increased adiposity there is a profound macrophage infiltration into adipose tissue. In both humans and rodents, macrophages accumulate in the adipose tissue with an increased body weight and disappear again with weight loss (Cancello et al., 2005, 2006). Moreover, adipocyte CD40 may contribute to obesity-related inflammation; T lymphocytes regulate adipocytokine production through both the release of soluble factors and heterotypic contact with adipocytes involving CD40 (Poggi et al., 2009). In order to gain insight into the crosstalk between the adipose tissue and the liver, further study on a mixed culture of adipocytes, macrophages and hepatocytes is a part of our ongoing project.

Conclusion

In summary, our results confirmed the crosstalk between obesity and liver cholesterol metabolism and found that adipocytokines regulated hepatic cholesterol metabolic factors HMGR and ABCA1 partly via APN. Our research gave the evidence about the interaction between the adipocyte and hepatocyte in hepatic cholesterol metabolism. Identification in future studies of other specific adipocytokines that are involved in hepatic cholesterol metabolism will shed light on the etiology of lipid metabolic disorder.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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