学位論文

HDL promotes adiponectin gene expression via the CAMKK/CAMKIV pathway

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HDL promotes adiponectin gene expression via the CAMKK/CAMKIV pathway

Toshihiro Kobayashi, Hitomi Imachi, Kensaku Fukunaga, Jingya Lyu, Seisuke Sato, Takanobu Saheki, Tomohiro Ibata, Mari Matsumoto, Salimah B Japar and **Koji Murao**

Department of Endocrinology and Metabolism, Faculty of Medicine, Kagawa University, Miki-cho, Kita-gun, Kagawa, Japan

Correspondence should be addressed to K Murao: mkoji@med.kagawa-u.ac.jp

Abstract

Adiponectin (APN) is an adipokine that protects against diabetes and atherosclerosis. High-density lipoprotein (HDL) mediates reverse cholesterol transport, which also protects against atherosclerosis. In this process, the human homolog of the B class type I scavenger receptor (SR-BI/CLA-1) facilitates the cellular uptake of cholesterol from HDL. The level of circulating APN is positively correlated with the serum level of HDL-cholesterol. In this study, we investigated whether HDL stimulates the gene expression of APN through the Ca^{2+}/calmodulin (CaM)-dependent protein kinase IV (CaMKIV) cascade. APN expression was examined using real-time PCR and western blot analysis in 3T3-L1 cells incubated with HDL. CaMKIV activity was assessed by the detection of activation loop phosphorylation (at Thr^{196} residue), and the effect of the constitutively active form, CaMKIVc, on APN promoter activity was investigated. Our results showed that HDL stimulated APN gene expression via hSR-BI/CLA-1. Furthermore, we explored the signaling pathways by which HDL stimulated APN expression in 3T3-L1 cells. The stimulation of APN gene expression by HDL appears to be mediated by CaMKK, as STO-609, a specific inhibitor of CaMKK2, prevents this effect. We revealed that CaMKIVc increased APN gene transcriptional activity, and the CaMKIV-dominant negative mutant blocked the effect of HDL on APN promoter activity. Finally, knockdown of hSR-BI/CLA-1 also canceled the effect of HDL on APN gene expression. These results suggest that HDL has an important role to improve the function of adipocytes by activating hSR-BI/CLA-1, and CaMKK/CaMKIV pathway is conceivable as one of the signaling pathways of this activation mechanism.

Key Words

- adiponectin
- HDL
- SR-BI
- CaMKK/CaMKIV pathway

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Introduction

One of the main roles of adipocytes is to store energy as triglycerides during feeding and to provide free fatty acids to other tissues during fasting. It also plays an important role as an endocrine organ by secreting adipokines that contribute to the regulation of energy metabolism in the body. Adiponectin (APN), an adipokine, is an adipocyte-derived vasoactive peptide with anti-inflammatory and insulin-sensitizing peptides. A number of clinical trials have shown that subjects with high levels of circulating APN tend to be protected against type 2 diabetes and myocardial infarction (Kadowaki *et al.* 2006). It has been reported that higher concentrations of APN protect against the later development of type 2 diabetes (incidence rate ratio 0.63; 95% CI, 0.43–0.92, $P = 0.02$) (Dekker *et al.* 2008).

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Furthermore, participants in the highest are compared with the lowest quintile of APN levels and have been reported to have a significantly decreased risk of myocardial infarction (relative risk, 0.39; 95% CI, 0.23–0.64; $P < 0.001$) (Pischon *et al.* 2004).

APN has been correlated with various parameters of lipoprotein metabolism. Specifically, APN is negatively correlated with serum triglycerides and positively correlated with HDL-C (Christou & Kiortsis 2013). HDL exerts several effects on metabolic homeostasis and thereby beneficially impacts glucose homeostasis, inflammation, thrombosis, and atherosclerosis. HDL plays an important role in removing excess cholesterol from peripheral tissues and cells. This process of peripheral cholesterol efflux to HDL and delivery to the liver is termed reverse cholesterol transport and is important for the anti-atherogenic properties of HDL. Several clinical studies reported that circulating APN levels are positively correlated with HDL-C serum levels (Christou & Kiortsis 2013). HDL-C itself has been shown to be positively correlated with high-molecular weight (HMW) APN, which is considered the most biologically active fraction of APN (Kangas-Kontio *et al.* 2010). HMW APN has a high correlation coefficient, independent of various factors of adiposity and insulin sensitivity (Im *et al.* 2006).

The mouse scavenger receptor class B type I (SR-BI) mediates the selective uptake of HDL-C ester in transfected Chinese hamster ovary cells. This finding provides an important link between a specific cell surface receptor and a pathway involved in the uptake of HDL-C (Acton *et al.* 1996). Our previous studies show that CD36, and LIMPII analogous-1 (hSR-BI/CLA-1), like mouse SR-BI, functions as a human receptor for HDL (Murao *et al.* 1997, Imachi *et al.* 2003). Adipose tissue contains a very large pool of free cholesterol (Zhang *et al.* 2010). In fact, adipocytes are known to support *in vitro* cholesterol efflux to HDL and apoA-I (Prattes *et al.* 2000). Recent literature shows that SR-BI is expressed in mature adipocytes, and adipocyte cholesterol homeostasis may be regulated in a cell-specific manner (Prattes *et al.* 2000, Le Lay *et al.* 2003). SR-BI is a 509-amino acid glycoprotein with two cytoplasmic C-terminal and N-terminal domains, separated by a large extracellular domain. The extracellular domain of SR-BI is not only important in binding HDL particles but is also required for the efficient and selective cholesterol uptake of HDL-C ester (Linton *et al.* 2017). Additionally, the C-terminal tail of hSR-BI/CLA-1 might play an important role in HDL-mediated signal transduction mechanisms, including the PI3K/Akt signaling pathway and the calcium-mediated signal transduction pathway (Cao *et al.* 2004*a*, Linton *et al.* 2017).

However, the underlying mechanism that regulates APN expression via HDL receptor has yet to be revealed. In this study, we have examined the effect of HDL-hSR-BI/CLA-1 signal transduction mechanism on APN gene expression.

Materials and methods

Cell culture

The 3T3L1 cells originated from a mouse adipose cell line (American Type Culture Collection). The undifferentiated 3T3L1 cells were cultured in Dulbecco's Modified Eagle's Medium with 25 mM glucose (DMEM, Gibco-BRL, Tokyo, Japan) and supplemented with 10% newborn fetal bovine serum (FBS; Thermo Electron Co., Melbourne, Australia), 4 mM L-glutamine, 1 mM sodium pyruvate, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. To introduce differentiation, 3T3L1 cells were grown in DMEM supplemented with 10% FBS and the standard differentiation inducing mix: insulin (10 µg/mL, Sigma), dexamethasone (2.5 µM, Sigma), and isobutylmethylxanthine (IBMX, 0.5 mM, Sigma) for the first 2 days. For the next 2 days, the medium was supplemented only with 100 nM insulin. From day 5, the cells were switched back to plain DMEM with 10% FBS. The cells would be fully differentiated on day 10 and could be used until days 15–17. Y-1 and COS-7 cells (American Type Culture Collection) were grown in DMEM supplemented with 10% FBS. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

Western blot analysis

Cells were washed, scraped in PBS, and lysed as described previously (Murao *et al.* 2008). The proteins (10 µg) were separated on a 7.5% SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride membranes for immunoblot assay. The membranes were incubated for 1 h at 4°C with 0.2% Tween 20 in PBS containing anti-APN antiserum (dilution, 1:250; Abcam) or anti-hSR-BI/CLA-1 antiserum (dilution, 1:2000) as described previously (Murao *et al.* 1997). The binding of each antibody was visualized using a chemiluminescence detection kit (enhanced chemiluminescence; Amersham Pharmacia Biotech). An antibody for GAPDH (diluted 1:1000; Trevigen, Gaithersburg, MD, USA) was used as the internal standard for the cytosolic extract. The membranes were then washed with PBS-T and incubated for 1 h at room temperature in PBS-T containing a second antibody linked

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to horseradish peroxidase. The signal was visualized using an enhanced chemiluminescence detection kit (ECL; GE Healthcare) under Luminescent image analyzer LAS-1000 Plus (Fujifilm). Band intensities were quantified using Multi Gauge V 3.2 software (Fujifilm).

Quantitative real-time PCR

Total RNA was extracted with RNA-Bee-RNA isolation reagent (Tel-Test Inc., Friendswood, TX, USA) from the mouse adipose and liver tissue, and RT was performed using Superscript II (Invitrogen) and 6 μg of total RNA; cDNA was used for quantitative real-time PCR (qPCR) using the Fast Start DNA Master SYBR Green I kit (Roche) in a CFX96 Real-time PCR Detection system (Bio-Rad) as previously described (Lyu *et al.* 2018). GAPDH was used as the housekeeping gene. The qPCR was performed using the following specific primer sequences: GAPDH forward: 5'-TGAACGGGAAGCTCACTGG-3' and reverse: 5'-TCCACCACCCTGTTGCTGTA-3'; APN forward: 5'-AA GGACAAGGCCGTTCTC-3' and reverse: 5'-AGAGTCGTTGA CGTTATCTGCATAG-3'.

Transfection of small interfering RNA

The small interfering RNAs (siRNAs) designed to target SR-BI or scramble were purchased from Santa Cruz. Transfection of SR-BI siRNA was performed using siPORT Amine (Ambion) as described previously (Fukata *et al.* 2014).

Cholesterol content assay

For cholesterol content measurement, we employed a method developed by Shahnaz *et al.* (1998), which is a completely automated fluorimetric method for the determination of cellular cholesterol, consisting of enzymatic hydrolysis of cholesteryl ester to free cholesterol and enzymatic oxidation of free cholesterol in the presence of an indicator substrate to produce a fluorescent product.

Phosphorylation of Ca^{2+}/calmodulin-dependent protein kinase IV at Thr^{196}

3T3L1 cells were treated with 100 μg/mL HDL (Merck Millipore) for 2 min and harvested at predetermined time intervals. The cells were lysed, and endogenous Ca^{2+}/calmodulin (CaM)-dependent protein kinase (CaMK)IV was immunoprecipitated using the anti-CaMKIV antibody by the following procedure. Antibodies directed against CaMKIV (dilution, 1:2000; Abcam) were pre-incubated with protein G agarose for 30 min with rotation in 500 μL harvesting buffer and collected after centrifugation. Following aspiration, 1 mL of clarified cell extract was added to the immobilized antibody–protein G complex and tumbled overnight at 4°C. Following centrifugation, supernatants were removed and analyzed by western blotting. Western blotting analysis was carried out using anti-phospho-Thr196 antibody. The total cell lysate was also subjected to western blotting analysis using CaMKIV antibody as control. Anti-phospho-CaMKIV Thr^{196} monoclonal antibodies were generated against the synthetic phosphopeptides corresponding to residues 189–203 of rat CaMKIV (CEHQVLMKT(p)VCGTPGY) (Tokumitsu *et al.* 2004). Peptide was conjugated using keyhole limpet hemocyanin through the N-terminus cysteine and was injected into BALB/c mice as described previously (Kimura *et al.* 1994).

Transfection of 3T3L1 cells and luciferase reporter gene assay

To confirm the transcriptional regulation of the APN promoter by HDL, we used a plasmid construct containing the mouse APN promoter obtained by PCR amplification, cloned in front of the luciferase reporter gene as previously described (Li *et al.* 2010*a*). WT (−984 WT LUC) contains the mouse APN gene sequences spanning the region from −984 to +1, linked to the luciferase reporter gene (Li *et al.* 2010*a*). Purified reporter plasmid was co-transfected with a CaMKIV-expressing plasmid or an empty vector and a β-galactosidase expression plasmid (for determining transfection efficiency into the 3T3L1 cells at 70% confluence) using Lipofectamine (Life Technologies). Both cDNA of Ca^{2+}/CaM-independent mutant of CaM-KIV (CaM-KIVc, 305 HMDT to DEDD) and CaM-KIV kinase-negative mutant (CaM-KIVdn, 305 HMDT to DEDD, K71E) were constructed as described previously (Kimura *et al.* 1994, Tokumitsu *et al.* 1994, Tokumitsu & Soderling 1996, Li *et al.* 2010*a*). Transfected cells were maintained in control media for 24 h as previously described (Lyu *et al.* 2020). Transfected cells were harvested, and β-galactosidase activity was measured in an aliquot of the cytoplasmic preparation. Twenty-microliter aliquots were taken for the luciferase assay, which was performed according to the manufacturer's instructions (ToyoInk, Tokyo, Japan).

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Statistical analysis

Data were expressed as mean ± S.E.M. Data were analyzed statistically using SPSS Statistics V17.0 software, and the significance of the differences between the corresponding groups was determined by ANOVA followed by one-tailed multiple *t*-tests. *P* value <0.05 was considered as statistically significant.

Results

Expression of SR-BI/CLA-1 in differentiated and undifferentiated 3T3-L1 cells

SR-BI/CLA-1 expression has been confirmed in mouse-derived Y-1 adrenal cells. Using Y-1 cells as a positive control, SR-BI/CLA-1 expression was examined in mouse-derived undifferentiated 3T3-L1 cells, differentiated 3T3-L1 cells, and mouse adipose tissue. As shown in Fig. 1, SR-BI/CLA-1 was expressed in mouse adipose tissue as well as in undifferentiated and differentiated 3T3-L1 cells.

Effect of HDL on adiponectin gene expression in 3T3-L1 cells

The effect of lipoproteins on APN expression in 3T3-L1 cells was examined by exposing the cells to 100 μg/mL of both LDL and HDL for 24 h. APN expression was examined by qPCR analysis to assess the level of mRNA transcription in the cells. The results showed an induction of the APN gene in response to HDL, but not LDL (Fig. 2A). We hypothesized that the transcriptional activity of the APN promoter is regulated by HDL in 3T3-L1 cells. In order to examine this hypothesis, we measured the APN promoter activity using the luciferase reporter gene assay in differentiated 3T3-L1 cells in the presence of HDL (Fig. 2B). HDL was found to have a stimulatory effect on the activity of the APN promoter. The results showed that HDL treatment stimulated the promoter activity of APN.

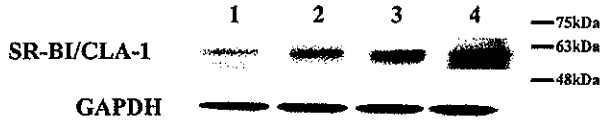

Figure 1
Expression of SR-BI/CLA-1 in 3T3L1 cells. Total cell protein (10 μg) extracted from the adipose tissue and 3T3L1 cells was blotted with anti-SR-BI/CLA-1 or GAPDH antibody, respectively. Abundance of GAPDH served as a control. Lane 1, Y-1 cell; lane 2, mouse adipose tissue; lane 3, undifferentiated 3T3L1 cell; lane 4, differentiated 3T3L1 cell.

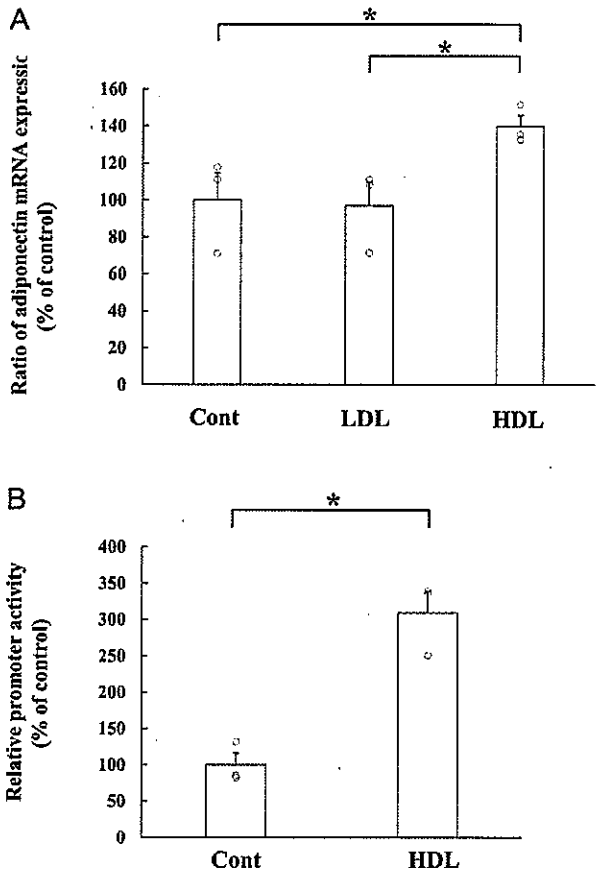

Figure 2
(A) HDL-stimulated APN gene expression. The effect of HDL on mRNA expression of APN by real-time PCR methods as described in the methods section. The ratio is shown as percent of control. A graph showing the mean ± S.E.M. (n = 3) of separate experiments for each treatment group is indicated. (B) HDL-stimulated APN promotor activity. 3T3L1 cells were transfected with 1 μg of pAPN-LUC and treated with 100 μg/mL HDL for 24 h prior to cell harvesting. All assays were corrected for β-galactosidase activity, and the total amount of protein in every reaction was identical. The results were expressed as relative luciferase activity compared with the control cells arbitrarily set at 100. Each data point shows the mean ± S.E. of three separate transfections that were performed on separate days. *P < 0.05 compared to control. The differences between the corresponding groups were determined by ANOVA followed by one-tailed multiple *t*-tests.

HDL increases adiponectin promoter activity in 3T3-L1 cells via the CaMKK pathway

To test whether the interaction of HDL and its membrane receptor is involved in a signaling pathway, we used known inhibitors of signaling pathways to disrupt HDL induction of ANP promoter activity. Thus, we treated pANP-LUC-

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transfected 3T3-L1 cells with compounds known to inhibit PI3-K (LY294002, 10 μmol/L), extracellular-signal-regulated kinase (PD98059, 10 μmol/L), protein kinase C (PKC) (bisindolylmaleimide I, 1 μmol/L), and p38-mitogen-activated protein kinase (p38-MAPK) (SB203580, 1 μmol/L) before exposing them to HDL. The results showed that all aforementioned inhibitors had no effect on the action of HDL; however, the STO-609 (1 μM), inhibitor for Ca^{2+}/calmodulin (CaM)-dependent protein kinase kinase (CaMKK2), abrogated the ability of HDL to induce APN promoter activity (Fig. 3A). The STO-609 treatment therefore also inhibited the expression of APN (Fig. 3B). These results suggest that the effects of HDL are mediated via the CaMKK cascade. It is well known that HDL promotes cholesterol efflux from cells. Therefore, we investigated the effect of STO-609 on cholesterol efflux in HDL-treated 3T3L1 cells. To assess the effect of STO-609 on cholesterol efflux, cholesterol content in HDL-treated 3T3L1 cells was measured with or without STO-609 treatment. The results showed that the addition of STO-609 increased the cholesterol content of 3T3L1 cells (Supplemental data). This showed that STO-609 inhibited the efflux of cholesterol from cells.

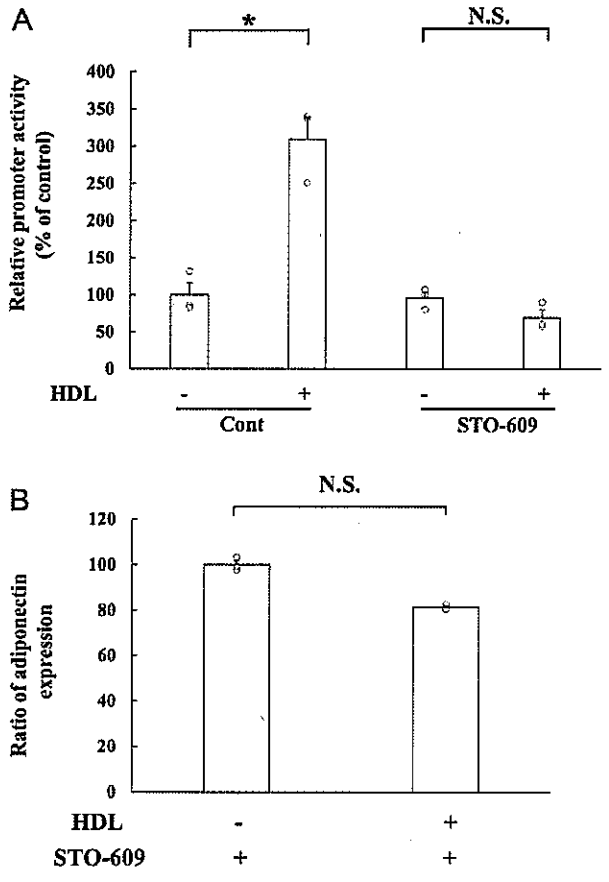

Figure 3
(A) STO-609, inhibitor for Ca^{2+}/calmodulin (CaM)-dependent protein kinase kinase (CaMKK2), abrogates the effect of HDL on APN expression. The effect of STO-609 on HDL-induced APN transcriptional activity. Cont; without STO-609, STO-609; with STO-609 (1 μM). All assays were corrected for β-galactosidase activity, and the total amount of protein in every reaction was identical. The results were expressed as relative luciferase activity compared with the control cells arbitrarily set at 100. Each data point shows the mean ±S.E.of four separate transfections that were performed on separate days. (B) STO-609, inhibitor for CaMKK2, abrogates the effect of HDL on APN expression. The effect of STO-609 on HDL-induced APN mRNA expression by real-time PCR methods. The ratio is shown as percentage of control. A graph showing the mean ± S.E.M. ($n = 3$) of separate experiments for each treatment group is indicated. *$P < 0.05$ compared to control. The differences between the corresponding groups were determined by ANOVA followed by one-tailed multiple *t*-tests. N.S., no significance.

CaMKK/CaMKIV cascade mediates HDL-induced ANP gene expression

Phosphorylation of CaMKIV by HDL in 3T3-L1 cells

Our study indicated that the CaMKK pathway might be involved in the enhancement of HDL-mediated APN gene expression in 3T3-L1 cells. It has been reported that CaMKK is capable of activating multiple protein kinases including CaMKI, CaMKIV, PKB, AMP-kinase, and SAD-B kinase. To determine which downstream effector kinase of CaMKK is involved in this mechanism, we first examined the ability of HDL to stimulate CaMKIV activity in 3T3-L1 cells, since CaMKIV has shown to be localized in nucleus and involved in the transcription, through the phosphorylation of transcriptional factors such as cAMP response element-binding (CREB) protein. We confirmed that CaMKK/CaMKIV was expressed in 3T3-L1 cells. As shown in Fig. 4A, it was found that CaMKK/CaMKIV was expressed in differentiated 3T3-L1 cells in the same manner as the positive control, in INS-1 cells. Previous studies showed that the phosphorylation of Thr^{196} residues in the activation loop of CaMKIV by CaMKK greatly induced its kinase activity. Therefore, we examined Thr^{196} phosphorylation of CaMKIV in response to HDL in 3T3-L1 cells. 3T3-L1 cells were treated with HDL and harvested at predetermined time intervals. Cells were then analyzed using western blotting with either anti-phospho-Thr^{196} antibodies (Fig. 4B, upper panel) or anti-CaMKIV antibodies (Fig. 4B, lower panel). 3T3-L1 cells treated with HDL showed an increase in CaMKIV phosphorylation at Thr^{196}. The upregulation of CaMKIV phosphorylation peaked after 15 min of HDL treatment.

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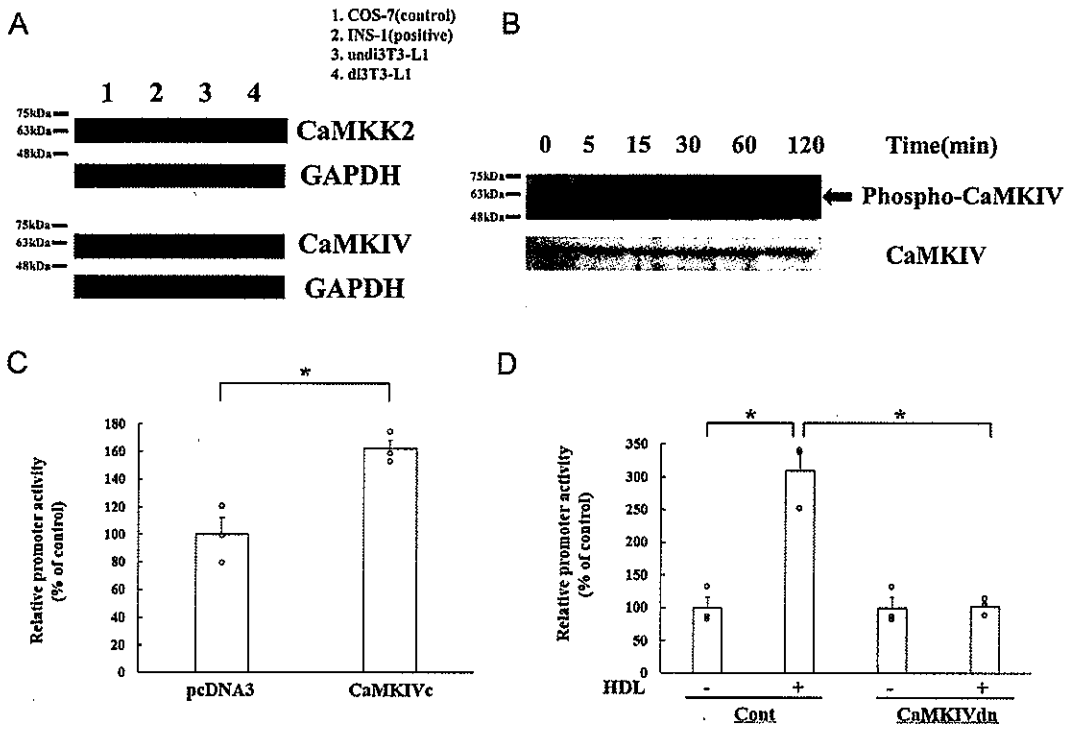

Figure 4

The role of CaMKK/CaMKIV pathway on HDL-induced APN expression. (A) The expression of CaMKK2 or CaMKIV in 3T3L1 cells. Total cell protein (10 μg) extracted from 3T3L1 cells or INS-1 cells was blotted with anti-CaMKK2 or anti-CaMKIV antibody, respectively. Abundance of GAPDH served as a control. COS-7 (control), INS-1 (positive control), undi3T3L1 (undifferentiated 3T3L1 cells), di3T3L1 (differentiated 3T3L1 cells). (B) Phosphorylation of CaMKIV by HDL in 3T3L1 cells. Post-confluent 3T3-L1 preadipocytes treated with differentiation medium, which contains a mixture of IBMX, dexamethasone, and insulin, initiated adipogenic differentiation and intracellular lipid accumulation was marked by day 10, suggesting that 3T3-L1 preadipocytes differentiated into mature adipocytes. 3T3L1 cells were exposed to 100 μg/mL HDL for 2 min before harvest at the predetermined time interval. The total cell extracts (10 μg) were subjected to immunoprecipitation using anti-CaMKIV antibodies and SDS-PAGE, followed by western blotting analysis using anti-phospho-Thr196 antibodies (upper panel). Total cell lysates (10 μg) were also blotted using anti-CaMKIV antibodies as a control (lower panel). An identical experiment independently performed yielded similar results. (C) Effect of the CaMKIV on APN promoter activity. The cells were transfected with APN-LUC and empty vector (pcDNA3) or CaMKIVc expression vectors. The results are expressed as relative luciferase activity compared with control cells arbitrarily set at 100. Each data point shows the mean ± S.E.M. ($n = 3$) of separate transfections. The asterisk denotes the significant difference ($P < 0.05$). (D) CaMKIV-dominant negative (CaMKIVdn) mutant inhibits the upregulation of APN promoter activity by HDL. 3T3L1 cells were transfected with APN-LUC and CaMKIVdn for 24 h prior to measuring luciferase activity. Cont, APN-LUC and pcDNA3, CaMKIVdn; APN-LUC and CaMKIVdn expression vector with or without HDL. The results are expressed as relative luciferase activities compared with control cells arbitrarily set at 100. Each data point shows the mean ± S.E.M. ($n = 3$) of separate transfections. The differences between the corresponding groups were determined by ANOVA followed by one-tailed multiple *t*-tests.

Role of CaMKIV in HDL-induced APN gene expression

The results of this experiment so far identified the components of the CaMKK cascade in HDL-induced APN promoter activity. The lack of information on whether this signal transduction cascade mediates the action of HDL in 3T3-L1 cells led us to examine whether CaMKIV plays a role in the HDL-induced transcription of the APN gene. Therefore, we examined the potential role of CaMKIV by co-transfecting 3T3-L1 cells with the APN promoter gene and CaMKIV to determine whether CaMKIV affected APN gene transcription. Results showed that CaMKIVc stimulated APN promoter activity in 3T3-L1 cells (Fig. 4C). Furthermore, when 3T3-L1 cells were co-transfected with a dominant negative mutant of CaMKIV (CaMKIVdn), the increase in HDL-induced APN promoter activity was entirely suppressed (Fig. 4D). Together, these findings support the notion that the CaMKK/CaMKIV cascade is required for APN expression induced by HDL in 3T3-L1 cells.

Role of SR-BI/CLA-1 in HDL-induced APN expression

To further characterize the role of SR-BI/CLA-1 in the HDL-mediated signaling that enhances APN expression, we used siRNA to block SR-BI/CLA-1-expression. 3T3-L1 cells were exposed to SR-BI/CLA-1-specific siRNA and scramble siRNA and then treated with HDL. As shown in Fig. 5,

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APN protein expression was increased in cells exposed to scrambled siRNA following stimulation with HDL. In contrast, the increase in HDL-induced APN expression was significantly suppressed in cells treated with SR-BI-siRNA. The above-mentioned findings support the idea that SR-BI/CLA-1 plays a role in HDL-induced APN expression.

Discussion

In this report, we have summarized the results of our studies, showing that HDL regulated APN gene expression via hSR-BI/CLA-1. Furthermore, we have explored the signaling pathways by which HDL stimulated APN expression in 3T3-L1 cells. This effect appears to be mediated by CaMKK due to a specific inhibitor of this kinase, STO-609, which prevents HDL-stimulated APN expression. To further define the actions of HDL, we used a transient transfection assay to clarify the signal transduction pathway that mediated HDL-induced APN gene expression. We revealed that CaMKIVc increased APN gene transcriptional activity. Finally, knockdown of hSR-BI/CLA-1 canceled the effect of HDL on APN gene expression. In this study, our results suggest that HDL improves the function of adipocytes by activating hSR-BI/CLA-1, and CaMKK/CaMKIV pathway is conceivable as one of the signaling pathways of this activation mechanism.

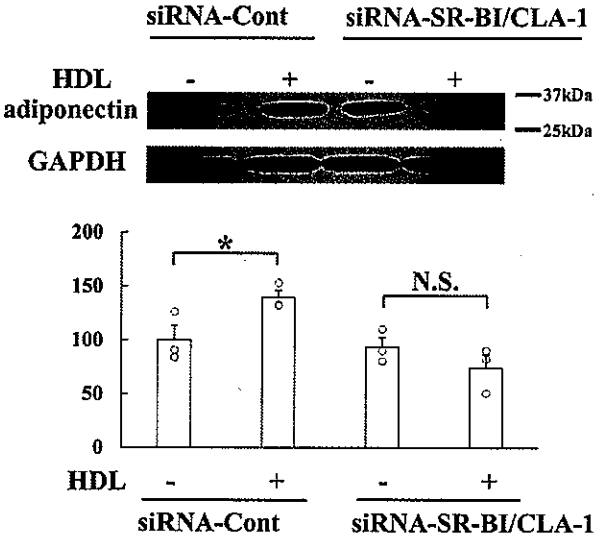

Figure 5
Effects of SR-BI/CLA-1-knockdown on APN expression by HDL. SR-BI/CLA-1 (siRNA-SR-BI/CLA-1) siRNA or scrambled siRNA (siRNA-Cont) was transfected into 3T3L1 cells with or without HDL. The upper panel shows that the abundance of APN protein level was measured using western blot analysis at 48 h after transfection. The ratio of APN to GAPDH is represented as a percentage of the control. Each data point shows the mean ± S.E.M. ($n = 3$) of separate experiments. The asterisk denotes a significant difference ($P < 0.05$). The differences between the corresponding groups were determined by ANOVA followed by one-tailed multiple *t*-tests. N.S., no significance.

The cellular uptake of cholesterol in HDL is facilitated by SR-BI. This protein was identified as an HDL receptor in rodents (Acton *et al.* 1996). Human CD36 and LIMPII Analogous-1 (CLA-1) share 81% sequence homology with hamster SR-BI (Calvo & Vega 1993). Our previous reports show that human SR-BI (hSR-BI/CLA-1), like mouse SR-BI, functions as a receptor for HDL (Murao *et al.* 1997, Imachi *et al.* 1999, 2003, Cao *et al.* 2004*a*,*b*). Human SR-BI/CLA-1 is also similar to the mouse homolog because it can mediate selective uptake of cholesterol ester and is expressed in the liver and steroidogenic tissues. Several reports indicated that SR-BI is expressed in adipocytes and adipose tissues (Zhang *et al.* 2010). Adipose tissues harbor a large depot of free cholesterol. HDL has an important role to remove excess cholesterol from peripheral tissues and cells. The process of peripheral cholesterol efflux to HDL and delivery to the liver was also observed in adipocytes (Wu & Zhao 2009, Mostafa *et al.* 2015). It was revealed that adipocytes support the transfer of cholesterol to HDL *in vivo* as well as *in vitro* and implicate ABCA1 and SR-BI cholesterol transporters (Zhao *et al.* 2006). However, the role of SR-BI in adipocytes has not yet been fully investigated. Although SR-BI is the receptor that mediates cholesterol transport in HDL metabolism, in this report we have revealed that it may be the sensing receptor for HDL-dependent signal transduction.

In endothelial cells, a series of studies have established that SR-BI interaction with HDL prevents endothelial dysfunction by stimulating eNOS activity (Mineo & Shaul 2013). Regarding its mechanisms, we previously reported that the C-terminal tail of hSR-BI/CLA-1 might play an important role in HDL-mediated signal transduction mechanisms, including the PI3K/Akt signaling pathway (Yu *et al.* 2007). The PDZ domain-containing protein 1 (PDZK1), also known as CLAMP, was first identified as an SR-BI-associated protein in affinity chromatography studies of rat liver membrane extracts (Kocher & Krieger 2009). PDZK1 contains four PDZ domains. The N-terminal domain interacts with the extreme C-terminal residues of SR-BI and PDZK1, which is linked to the PI3K/Akt signaling pathway. It was previously reported that increased HDL-C following human apoA-I transfer affects the expression of genes involved in fatty acid and triglyceride metabolism in adipose tissue. It was also reported that increased HDL-C potently attenuates the increase of triglycerides and free fatty acids induced

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by LPS administration, whereas HDL elevates plasma APN concentrations *in vivo* and increases APN expression in adipocytes in a PI3K-dependent manner (Van Linthout *et al.* 2010).

In addition to the previously mentioned signaling pathways activated by HDL via SR-BI, other signaling events have also been reported in HDL-treated cells. These include the activation of phosphatidylinositol and phosphatidylcholine-specific phospholipases (PI-PLC, PC-PLC, and PC-PLD), protein kinase C (PKC), heterotrimeric G proteins, the production of 3',5'-cAMP and ceramide, and the stimulation of intracellular calcium release (Saddar *et al.* 2010). The importance of calcium signaling in HDL-stimulated gene expression was reported previously (Romero *et al.* 2006). Preincubation with nifedipine, a calcium channel blocker, completely blocked HDL stimulation of *CYP11B2* in adrenal cells, whereas KN93, a calmodulin kinase inhibitor, inhibited 80% of the stimulation.

Our study identified the role of the CaMKK/CaMKIV cascade in APN gene expression in response to HDL. Numerous studies have demonstrated that the CaMKK/CaMKIV cascade is present and functional in various cell types, including pancreatic β-cells (Li *et al.* 2010*b*, Chen *et al.* 2011). We previously reported that pancreatic β-cells incorporate the CaMKK/CaMKIV cascade and that it plays an important role in glucose-upregulated transcriptional activation of the insulin gene (Yu *et al.* 2004). In this study, we confirmed that this pathway also exists in adipocytes, and HDL induced the phosphorylation of CaMKIV at Thr^{196} by CaMKK. We also confirmed that the expression of CaMKIVdn canceled the effect of HDL on HDL-induced APN expression, raising the possibility that activated CaMKIV mediates the stimulatory effect of HDL-dependent APN gene expression. Several studies have reported that APN is transcriptionally regulated by various transcription factors, such as peroxisome proliferator-activated receptor γ (PPARG, also known as PPARγ; (Iwaki *et al.* 2003)), CCAAT/enhancer-binding protein-α (CEBPA, also known as C/EBPα; (Park *et al.* 2004)), nuclear factor of activated T cells 4 (NFAT4), and activating transcription factor three (ATF3; (Kim *et al.* 2006)) through cis-regulatory elements in its proximal promoter region. The active, phosphorylated form of CaMKIV has been shown to translocate to the nucleus, where it regulates the transcriptional activity of several targets that play crucial roles in immune response, inflammation, and cellular proliferation (Chow *et al.* 2005). Activation of CaMKIV has been associated with increased phosphorylation of CREB, NFAT, and serum response factor protein (Jensen *et al.* 1991). The APN promoter region has the binding sites for these transcription factors. Further studies are needed to investigate the mechanism of CaMKIV-mediated transcriptional activation of the APN gene.

Of the reviewed studies, almost all reported that circulating APN is positively correlated with the serum level of HDL-C (Christou & Kiortsis 2013). HDL-C has also been shown to be positively correlated with HMW APN. Low levels of APN are associated with type 2 diabetes, NASH, and atherosclerosis (Phillips & Kung 2010). Although the underlying mechanisms responsible for hypoadiponectinemia in obesity have not been discovered, increased fat deposits result in adipose tissue hypoxia, thereby increasing endoplasmic reticular stress. Obesity also induces macrophage filtration into adipocytes, resulting in a low-grade chronic inflammatory state accompanied by an increase in the production of pro-inflammatory cytokines, such as TNF-α, IL-6, and IL-18 (Su *et al.* 2011). These factors that induce hypoadiponectinemia may also be involved in the onset of type 2 diabetes. Previously, we reported that the hyperglycemic state inhibits the expression of SR-BI/CLA-1 via the p38MAPK-Sp1 pathway (Murao *et al.* 2008). Hyperglycemia is also related to hypoadiponectinemia, raising the possibility that a lower expression of SR-BI/CLA-1 suppresses HDL-dependent APN gene expression in adipocytes. Clinical studies suggest that hypoadiponectinemia contributes to insulin resistance, and elevated serum APN levels are associated with increased insulin sensitivity and reduced cardiovascular injury (Kadowaki *et al.* 2006). Circulating APN levels are increased by many commonly used drugs, such as statins, thiazolidinediones (TZDs), and angiotensin-converting enzyme inhibitors (Su *et al.* 2011). We previously reported that angiotensin II (Ang II) inhibited the expression of SR-BI/CLA-1 via PI3K/Akt/FoxO1 pathway, and angiotensin II receptor type 1 antagonist rescued the effect of Ang II, resulting in the upregulation of SR-BI/CLA-1. The upregulation of Ang II-inhibited hSR-BI/CLA-1 expression might represent a novel mechanism contributing to the anti-hypoadiponectinemia of AT type 1 receptor blockade in adipose tissue (Yu *et al.* 2007). Further investigation is needed to determine the detailed mechanisms involved in the regulation of APN gene by the HDL-SR-BI/CLA-1 pathway.

In summary, we examined the role of the CaMKK/CaMKIV cascade in HDL-induced APN gene expression via SR-BI/CLA-1. The results indicated that HDL has an important role to improve the function of adipocytes by activating hSR-BI/CLA-1, and CaMKK/CaMKIV pathway is conceivable as one of the signaling pathways of

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this activation mechanism, but we could not fully explain about direct connection between CaMKK/CaMKIV pathway and hSR-BI/CLA-1. Further consideration might be needed in the future research.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/JME-20-0211.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH & Kriegert M 1996 Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* **271** 518–520. (https://doi.org/10.1126/science.271.5248.518)

Calvo D & Vega MA 1993 Identification, primary structure, and distribution of CLA-1, a novel member of the CD36/LIMPII gene family. *Journal of Biological Chemistry* **268** 18929–18935. (https://doi.org/10.1016/s0021-9258(17)46716-0)

Cao WM, Murao K, Imachi H, Yu X, Abe H, Yamauchi A, Niimi M, Miyauchi A, Wong NCW & Ishida T 2004*a* A mutant high-density lipoprotein receptor inhibits proliferation of human breast cancer cells. *Cancer Research* **64** 1515–1521. (https://doi.org/10.1158/0008-5472.CAN-03-0675)

Cao WM, Murao K, Imachi H, Yu X, Dobashi H, Yoshida K, Muraoka T, Kotsuna N, Nagao S, Wong NCW, *et al.* 2004*b* Insulin-like growth factor-I regulation of hepatic scavenger receptor class BI. *Endocrinology* **145** 5540–5547. (https://doi.org/10.1210/en.2004-0330)

Chen K, Yu X, Murao K, Imachi H, Li J, Muraoka T, Masugata H, Zhang GX, Kobayashi R, Ishida T, *et al.* 2011 Exendin-4 regulates GLUT2 expression via the CaMKK/CaMKIV pathway in a pancreatic β-cell line. *Metabolism: Clinical and Experimental* **60** 579–585. (https://doi.org/10.1016/j.metabol.2010.06.002)

Chow FA, Anderson KA, Noeldner PK & Means AR 2005 The autonomous activity of calcium/calmodulin-dependent protein kinase IV is required for its role in transcription. *Journal of Biological Chemistry* **280** 20530–20538. (https://doi.org/10.1074/jbc.M500067200)

Christou GA & Kiortsis DN 2013 Adiponectin and lipoprotein metabolism. *Obesity Reviews* **14** 939–949. (https://doi.org/10.1111/obr.12064)

Dekker JM, Funahashi T, Nijpels G, Pilz S, Stehouwer CDA, Snijder MB, Bouter LM, Matsuzawa Y, Shimomura I & Heine RJ 2008 Prognostic value of adiponectin for cardiovascular disease and mortality. *Journal of Clinical Endocrinology and Metabolism* **93** 1489–1496. (https://doi.org/10.1210/jc.2007-1436)

Fukata Y, Yu X, Imachi H, Nishiuchi T, Lyu J, Seo K, Takeuchi A, Iwama H, Masugata H, Hoshikawa H, *et al.* 2014 17β-Estradiol regulates scavenger receptor class BI gene expression via protein kinase C in vascular endothelial cells. *Endocrine* **46** 644–650. (https://doi.org/10.1007/s12020-013-0134-5)

Im JA, Kim SH, Lee JW, Shim JY, Lee HR & Lee DC 2006 Association between hypoadiponectinemia and cardiovascular risk factors in nonobese healthy adults. *Metabolism: Clinical and Experimental* **55** 1546–1550. (https://doi.org/10.1016/j.metabol.2006.06.027)

Imachi H, Murao K, Sato M, Hosokawa H, Ishida T & Takahara J 1999 CD36 LIMPII analogous-1, a human homolog of the rodent scavenger receptor B1, provides the cholesterol ester for steroidogenesis in adrenocortical cells. *Metabolism: Clinical and Experimental* **48** 627–630. (https://doi.org/10.1016/S0026-0495(99)90061-6)

Imachi H, Murao K, Cao W, Tada S, Taminato T, Wong NCW, Takahara J & Ishida T 2003 Expression of human scavenger receptor B1 on and in human platelets. *Arteriosclerosis, Thrombosis, and Vascular Biology* **23** 898–904. (https://doi.org/10.1161/01.ATV.0000067429.46333.7B)

Iwaki M, Matsuda M, Maeda N, Funahashi T, Matsuzawa Y, Makishima M & Shimomura I 2003 Induction of adiponectin, a fat-derived antidiabetic and antiatherogenic factor, by nuclear receptors. *Diabetes* **52** 1655–1663. (https://doi.org/10.2337/diabetes.52.7.1655)

Jensen KF, Ohmstede CA, Fisher RS & Sahyoun N 1991 Nuclear and axonal localization of Ca2+/calmodulin-dependent protein kinase type Gr in rat cerebellar cortex. *PNAS* **88** 2850–2853. (https://doi.org/10.1073/pnas.88.7.2850)

Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K & Tobe K 2006 Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. *Journal of Clinical Investigation* **116** 1784–1792. (https://doi.org/10.1172/JCI29126)

Kangas-Kontio T, Huotari A, Ruotsalainen H, Herzig KH, Tamminen M, Ala-Korpela M, Savolainen MJ & Kakko S 2010 Genetic and environmental determinants of total and high-molecular weight adiponectin in families with low HDL-cholesterol and early onset coronary heart disease. *Atherosclerosis* **210** 479–485. (https://doi.org/10.1016/j.atherosclerosis.2009.12.022)

Kim HB, Kong M, Kim TM, Suh YH, Kim WH, Lim JH, Song JH & Jung MH 2006 NFATc4 and ATF3 negatively regulate adiponectin gene expression in 3T3-L1 adipocytes. *Diabetes* **55** 1342–1352. (https://doi.org/10.2337/db05-1507)

Kimura K, Nozaki N, Saijo M, Kikuchi A, Ui M & Enomoto T 1994 Identification of the nature of modification that causes the shift of DNA topoisomerase IIβ to apparent higher molecular weight forms in the M phase. *Journal of Biological Chemistry* **269** 24523–24526. (https://doi.org/10.1016/S0021-9258(17)31419-9)

Kocher O & Krieger M 2009 Role of the adaptor protein PDZK1 in controlling the HDL receptor SR-BI. *Current Opinion in Lipidology* **20** 236–241. (https://doi.org/10.1097/MOL.0b013e32832aee82)

Le Lay S, Robichon C, Le Liepvre X, Dagher G, Ferre P & Dugail I 2003 Regulation of ABCA1 expression and cholesterol efflux during adipose differentiation of 3T3-L1 cells. *Journal of Lipid Research* **44** 1499–1507. (https://doi.org/10.1194/jlr.M200466-JLR200)

Li J, Murao K, Imachi H, Yu X, Muraoka T, Kim JB & Ishida T 2010*a* Prolactin regulatory element-binding protein involved in cAMP-mediated suppression of adiponectin gene. *Journal of Cellular and Molecular Medicine* **14** 1294–1302. (https://doi.org/10.1111/j.1582-4934.2009.00752.x)

Li J, Murao K, Imachi H, Masugata H, Iwama H, Tada S, Zhang GX, Kobayashi R, Ishida T & Tokumitsu H 2010*b* Exendin-4 regulates pancreatic ABCA1 transcription via CaMKK/CaMKIV pathway. *Journal of Cellular and Molecular Medicine* **14** 1083–1087. (https://doi.org/10.1111/j.1582-4934.2009.00955.x)

Linton MF, Tao H, Linton EF & Yancey PG 2017 SR-BI: a multifunctional receptor in cholesterol homeostasis and atherosclerosis. *Trends in*

Endocrinology and Metabolism **28** 461–472. (https://doi.org/10.1016/j.tem.2017.02.001)

Lyu J, Imachi H, Fukunaga K, Sato S, Ibata T, Kobayashi T, Dong T, Yoshimoto T, Yonezaki K, Nagata H, *et al.* 2018 Angiotensin II induces cholesterol accumulation and impairs insulin secretion by regulating ABCA1 in beta cells. *Journal of Lipid Research* **59** 1906–1915. (https://doi.org/10.1194/jlr.M085886)

Lyu J, Imachi H, Fukunaga K, Sato S, Kobayashi T, Dong T, Saheki T, Matsumoto M, Iwama H, Zhang H, *et al.* 2020 Role of ATP-binding cassette transporter A1 in suppressing lipid accumulation by glucagon-like peptide-1 agonist in hepatocytes. *Molecular Metabolism* **34** 16–26. (https://doi.org/10.1016/j.molmet.2019.12.015)

Mineo C & Shaul PW 2013 Regulation of signal transduction by HDL. *Journal of Lipid Research* **54** 2315–2324. (https://doi.org/10.1194/jlr.R039479)

Mostafa AM, Hamdy NM, El-Mesallamy HO & Abdel-Rahman SZ 2015 Glucagon-like peptide 1 (GLP-1)-based therapy upregulates LXR-ABCA1/ABCG1 cascade in adipocytes. *Biochemical and Biophysical Research Communications* **468** 900–905. (https://doi.org/10.1016/j.bbrc.2015.11.054)

Murao K, Terpstra V, Green SR, Kondratenko N, Steinberg D & Quehenberger O 1997 Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. *Journal of Biological Chemistry* **272** 17551–17557. (https://doi.org/10.1074/jbc.272.28.17551)

Murao K, Yu X, Imachi H, Cao WM, Chen K, Matsumoto K, Nishiuchi T, Wong NCW & Ishida T 2008 Hyperglycemia suppresses hepatic scavenger receptor class B type I expression. *American Journal of Physiology: Endocrinology and Metabolism* **294** E78–E87. (https://doi.org/10.1152/ajpendo.00023.2007)

Park SK, Oh SY, Lee MY, Yoon S, Kim KS & Kim JW 2004 CCAAT/enhancer binding protein and nuclear factor-Y regulate adiponectin gene expression in adipose tissue. *Diabetes* **53** 2757–2766. (https://doi.org/10.2337/diabetes.53.11.2757)

Phillips SA & Kung JT 2010 Mechanisms of adiponectin regulation and use as a pharmacological target. *Current Opinion in Pharmacology* **10** 676–683. (https://doi.org/10.1016/j.coph.2010.08.002)

Pischon T, Girman CJ, Hotamisligil GS, Rifai N, Hu FB & Rimm EB 2004 Plasma adiponectin levels and risk of myocardial infarction in men. *JAMA* **291** 1730–1737. (https://doi.org/10.1001/jama.291.14.1730)

Prattes S, Horl G, Hammer A, Blaschitz A, Graier WF, Sattler W, Zechner R & Steyrer E 2000 Intracellular distribution and mobilization of unesterified cholesterol in adipocytes: triglyceride droplets are surrounded by cholesterol-rich ER-like surface layer structures. *Journal of Cell Science* **113** 2977–2989. (https://doi.org/10.1242/jcs.113.17.2977)

Romero DG, Plonczynski MW, Gomez-Sanchez EP, Yanes LL & Gomez-Sanchez CE 2006 RGS2 is regulated by angiotensin II and functions as a negative feedback of aldosterone production in H295R human adrenocortical cells. *Endocrinology* **147** 3889–3897. (https://doi.org/10.1210/en.2005-1532)

Saddar S, Mineo C & Shaul PW 2010 Signaling by the high-affinity HDL receptor scavenger receptor B type I. *Arteriosclerosis, Thrombosis, and Vascular Biology* **30** 144–150. (https://doi.org/10.1161/ATVBAHA.109.196170)

Shahnaz B, Tada S, Kajikawa T, Ishida T & Kawanishi K 1998 Automated fluorimetric determination of cellular cholesterol. *Annals of Clinical Biochemistry* **35** 665–670. (https://doi.org/10.1177/000456329803500511)

Su H, Lau WB & Ma XL 2011 Hypoadiponectinaemia in diabetes mellitus type 2: molecular mechanisms and clinical significance. *Clinical and Experimental Pharmacology and Physiology* **38** 897–904. (https://doi.org/10.1111/j.1440-1681.2011.05606.x)

Tokumitsu H & Soderling TR 1996 Requirements for calcium and calmodulin in the calmodulin kinase activation cascade. *Journal of Biological Chemistry* **271** 5617–5622. (https://doi.org/10.1074/jbc.271.10.5617)

Tokumitsu H, Brickey DA, Glod J, Hidaka H, Sikela J & Soderling TR 1994 Activation mechanisms for Ca2+/calmodulin-dependent protein kinase IV. Identification of a brain CaM-kinase IV kinase. *Journal of Biological Chemistry* **269** 28640–28647. (https://doi.org/10.1016/s0021-9258(19)61953-8)

Tokumitsu H, Hatano N, Inuzuka H, Yokokura S, Nozaki N & Kobayashi R 2004 Mechanism of the generation of autonomous activity of Ca 2+/calmodulin-dependent protein kinase IV. *Journal of Biological Chemistry* **279** 40296–40302. (https://doi.org/10.1074/jbc.M406534200)

Van Linthout S, Foryst-Ludwig A, Spillmann F, Peng J, Peng Y, Meloni M, Van Craeyveld E, Kintscher U, Schultheiss HP, De Geest B, *et al.* 2010 Impact of HDL on adipose tissue metabolism and adiponectin expression. *Atherosclerosis* **210** 438–444. (https://doi.org/10.1016/j.atherosclerosis.2010.01.001)

Wu ZH & Zhao SP 2009 Niacin promotes cholesterol efflux through stimulation of the PPARγ-LXRα-ABCA1 pathway in 3T3-L1 adipocytes. *Pharmacology* **84** 282–287. (https://doi.org/10.1159/000242999)

Yu X, Murao K, Sayo Y, Imachi H, Cao WM, Ohtsuka S, Niimi M, Tokumitsu H, Inuzuka H, Wong NCW, *et al.* 2004 The role of calcium/calmodulin-dependent protein kinase cascade in glucose upregulation of insulin gene expression. *Diabetes* **53** 1475–1481. (https://doi.org/10.2337/diabetes.53.6.1475)

Yu X, Murao K, Imachi H, Cao WM, Li J, Matsumoto K, Nishiuchi T, Ahmed RAM, Wong NCW, Kosaka H, *et al.* 2007 Regulation of scavenger receptor class BI gene expression by angiotensin II in vascular endothelial cells. *Hypertension* **49** 1378–1384. (https://doi.org/10.1161/HYPERTENSIONAHA.106.082479)

Zhang Y, McGillicuddy FC, Hinkle CC, O'Neill S, Glick JM, Rothblat GH & Reilly MP 2010 Adipocyte modulation of high-density lipoprotein cholesterol. *Circulation* **121** 1347–1355. (https://doi.org/10.1161/CIRCULATIONAHA.109.897330)

Zhao SP, Wu ZH, Hong SC, Ye HJ & Wu J 2006 Effect of atorvastatin on SR-BI expression and HDL-induced cholesterol efflux in adipocytes of hypercholesterolemic rabbits. *Clinica Chimica Acta: International Journal of Clinical Chemistry* **365** 119–124. (https://doi.org/10.1016/j.cca.2005.08.006)

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