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Palmitic acid suppresses apolipoprotein M gene expression via the pathway of PPARβ/δ in HepG2 cells

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ABSTRACT

It has been demonstrated that apolipoprotein M (APOM) is a vasculoprotective constituent of high density lipoprotein (HDL), which could be related to the anti-atherosclerotic property of HDL. Investigation of regulation of APOM expression is of important for further exploring its pathophysiological function in vivo. Our previous studies indicated that expression of APOM could be regulated by platelet activating factor (PAF), transforming growth factors (TGF), insulin-like growth factor (IGF), leptin, hyperglycemia and etc., in vivo and/or in vitro. In the present study, we demonstrated that palmitic acid could significantly inhibit APOM gene expression in HepG2 cells. Further study indicated neither PI-3 kinase (PI3K) inhibitor LY294002 nor protein kinase C (PKC) inhibitor GFX could abolish palmitic acid induced down-regulation of APOM expression. In contrast, the peroxisome proliferator-activated receptor beta/delta (PPAR_β/δ) antagonist GSK3787 could totally reverse the palmitic acid-induced down-regulation of APOM expression, which clearly demonstrates that down-regulation of APOM expression induced by palmitic acid is mediated via the PPAR_β/δ pathway.

Keywords: Apolipoprotein M; Palmitic acid; Peroxisome proliferator-activated receptor beta/delta; PI-3 kinase; Protein kinase C
1. Introduction

Human APOM is mainly found in hepatocytes of the liver and tubular epithelial cells in kidney [1], and it’s also expressed weakly in the colorectal tissues [2]. It has been demonstrated that APOM is important for the formation of preβ-HDL and cholesterol efflux to HDL, which could attenuate atherosclerotic process [3]. Previous study has revealed that elevated level of palmitic acid might contribute to the development of atherosclerosis through enhanced uptake of oxLDL via upregulation of LOX-1 in macrophages [4]. Moreover, enriched dietary palmitic acid can augment the cholesterol-induced increases in total and LDL-cholesterol by both suppression of LDL receptor activity and further stimulation of CETP activity [5]. It has been demonstrated that APOM mRNA levels could be regulated by many intracellular and extracellular factors, including platelet activating factor (PAF), insulin, leptin, transforming growth factor-beta (TGF-β), epidermal growth factor (EGF), hepatic growth factor (HGF), and etc. [6,7,8,9,10,11,12]. However, so far, the effect of palmitic acid on APOM gene expression has not been clearly defined. In the present study we demonstrated that palmitic acid could significantly inhibit APOM expression and further investigated the regulating pathway of palmitic acid induced down-regulation of APOM in HepG2 cell cultures.

2. Materials and methods

2.1 Cell cultures

HepG2 cells (American type culture collection, ATCC) were cultured in 25-cm²
vented flasks containing DMEM with 20% fetal calf serum (FCS) in the presence of benzylpenicillin (100 U/ml) and streptomycin (100μg/ml) under standard culture conditions (5% CO₂, 37 °C). Cells were seeded in six-well cell culture clusters, and were grown to 50-70% confluence. Prior to experiments, cells were washed twice with phosphate buffered saline (PBS), and once with serum-free DMEM without antibiotics. The experimental medium contained DMEM with 1.5% FFA-free human serum albumin (HSA) and one or more additives, i.e., palmitic acid, PI-3 kinase (PI3K) inhibitor LY294002, protein kinase C (PKC) inhibitor GFX and the peroxisome proliferator-activated receptor beta/delta (PPARβ/δ) antagonist GSK3787, at different concentrations as described in the legends to figures.

2.2 Reverse transcription, real-time RT-PCR and PCR array

Total RNA in cultured cells was extracted according to the manufacturer’s instructions using a total RNA purification kit (Omega Bio-Tek). The quality of the RNA samples was determined by the absorbance measurements at 260/280 nm. Using the first strand cDNA synthetic kit (Qiagen) according to the manufacturer’s instructions, 2μg total RNA was reverse transcribed to cDNA. The mRNA levels of the target and reference genes were measured under real-time PCR using TaqMan technology. The PCR primer sets were designed according to the information of GenBank, as listed in Table 1. GAPDH was used as reference genes. Relative standard curves were produced to compensate for the efficiency of the PCRs. Quantification of target genes mRNA levels was relative to GAPDH mRNA level.
The real-time PCR reaction for each gene was performed in a 25μL volume, in a glass capillary containing 0.1μL 100mM each primer and probe, 2μL cDNA, 2.5μL 10xbuffer, 1.5μL MgCl₂ (25mM), 0.5μL dNTP (10mmol/L), and Taq DNA polymerase 0.5μL. Thermal cycling conditions included the following steps: initial denaturation at 95 ºC for 3 min, followed by 40 cycles at 95 ºC for 5 sec and 60 ºC for 15 sec. All PCRs were performed on the LightCycler (Roche, Switzerland) real-time PCR system.

To scan genes of interest in HepG2 cells treated with palmitic acid, we used PCR Array analyses for Human Insulin Signaling Pathway (PAHS-030Z) according to the manufacturer's instructions. PCR array data were calculated by the comparative cycle threshold method, normalized against multiple housekeeping genes. Genes were expressed as mean fold change in 1 mM palmitic acid group (n=5) relative to 0 mM palmitic acid group (n=6).

2.3 Statistics

Data are expressed as means ± SEM. Statistical analyses were performed with the GraphPad Prism 6.0 software (GraphPad Software, Inc., San Diego, California, USA). Multiple comparisons were performed with one-way ANOVA, and comparisons between two groups were statistically evaluated by the unpaired t-test. Cross interaction was analyzed by two-way ANOVA. P-values less than 0.05 were considered significant.
3. Results

3.1 Effects of palmitic acid on expressions of APOM gene and genes that are related to insulin signaling pathway in HepG2 cells

In the present study we selected palmitic acid, one of the major FFAs in human plasma to mimic the effect of FFAs on the expressions of APOM gene and genes that are related to the insulin signaling pathway in HepG2 cells. Palmitic acid could significantly inhibit APOM expression with a dose dependent manner (Fig. 1). Table 2 summarizes 44 genes significantly altered by 1 mM palmitic acid. Genes related to the PI3K, mitogen-activated protein kinase (MAPK), and peroxisome proliferator-activated receptor gamma (PPARG), insulin or sterol regulatory element-binding protein-1 (SREBP1) pathways were significantly increased, whereas only the secondary effector target gene for insulin signaling, solute carrier family 2 (SLC2A1), corresponding to the glucose transporter type 4 (GLUT4) gene, was significantly decreased.

3.2 Effects of palmitic acid, PI3K inhibitor LY294002 (LY) and PKC inhibitor GF109203X (GFX) on APOM mRNA expression in HepG2 cells

As shown in Fig. 2A, 1 mM palmitic acid significantly reduced APOM mRNA levels in hepatocytes cultures (P=0.0070). LY alone had no effect on expression of APOM in HepG2 cells (P=0.2863). Two-way ANOVA indicated that there was no interaction between palmitic acid and LY on APOM expression in HepG2 cells (P=0.4723). GFX at 2μM significantly decreased APOM mRNA levels (P=0.0248).
but it did not reverse the palmitic acid-induced down-regulation of \textit{APOM} expression (Fig. 2B).

3.3 Effects of palmitic acid on \textit{PPARB/D} mRNA expression

Palmitic acid significantly increased \textit{PPARB/D} mRNA levels in HepG2 cells (Fig. 3A, \(P=0.0015\)). In HepG2 cell cultures, the \textit{PPAR}\(_{\beta/\delta}\) antagonist, GSK3787, had no effect on \textit{APOM} mRNA levels (\(P=0.2484\)), whereas GSK3787 almost totally reversed the palmitic acid-induced down-regulation of \textit{APOM} expression (Fig. 3B). As estimated by two-way ANOVA analysis, the interaction between palmitic acid and GSK3787 on \textit{APOM} mRNA levels was statistically significant (\(P=0.0390\)).

4. Discussion

It has been suggested that \textit{APOM} plays important roles involved in the anti-atherosclerotic effects of HDL particles, although the underlying mechanisms are not fully understood. \textit{APOM} may function as the acceptor of HDL-carrying S1P [13], \textit{APOM} can enhance the HDL-mediated anti-oxidation effect [14], and \textit{APOM} has an important role for the pre-\(\beta\) HDL formation [3]. In the present study, we demonstrated that palmitic acid could decrease the expression of \textit{APOM} gene in HepG2 cells, which may also possibly be related to its anti-atherosclerotic properties. In order to explore the mechanism of down-regulation of \textit{APOM} by palmitic acid, we monitored the expressions of 84 genes related to the insulin response. In cells treated with 1 mM palmitic acid, certain genes associated with the \textit{PI3K}, \textit{MAPK}, \textit{PPARG}, \textit{insulin} and
SREBP1 pathways were significantly increased, whereas SLC2A1 (reflecting the GLUT4 gene), was significantly down-regulated. This finding suggests that glucose transport might be impaired after administration of palmitic acid. We have previously reported [9] that activation of PI3K pathway or PPAR<sub>β/δ</sub>, but not PPARα and PPARγ, might be involved in down-regulation of APOM. Furthermore, down-regulation of APOM expression by insulin could not be blocked by addition of MAPK inhibitor, suggesting that the palmitic acid-induced down-regulation of APOM expression may be mediated via the PI3K and/or PPAR<sub>β/δ</sub> pathway.

Further study indicates that there is no cross interaction between palmitic acid and LY on the APOM expression in HepG2 cells, which suggests that down-regulation of APOM by palmitic acid is not mediated via PI3K activation. As protein kinase C (PKC) can be activated by FFAs [15], we thereafter investigated the effect of the PKC inhibitor, GFX, on APOM mRNA expression. GFX alone significantly decreased APOM mRNA levels; however, GFX could not reverse the palmitic acid-induced down-regulation of APOM expression. To sum up, we concluded that down-regulation of APOM by palmitic acid is not mediated via PI3K, PPARα, PPARγ, MAPK and PKC pathways. Finally, our results showed that palmitic acid significantly increased PPAR<sub>B/D</sub> mRNA levels in HepG2 cells, and PPAR<sub>B/D</sub> antagonist GSK3787 could entirely reverse the palmitic acid-induced down-regulation of APOM expression; thus, it can be concluded that palmitic acid induced down-regulation of APOM expression via the PPAR<sub>β/δ</sub> pathway.
ACKNOWLEDGEMENTS

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References:


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<th>Gene</th>
<th>Primer/Probe</th>
<th>Sequence (5’ to 3’)</th>
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<tr>
<td>Human $APOM$</td>
<td>Forward primer</td>
<td>tgccccgggaatggatcta</td>
</tr>
<tr>
<td>Human $APOM$</td>
<td>Reverse primer</td>
<td>cagggcggccttgcaggtt</td>
</tr>
<tr>
<td>Human $APOM$</td>
<td>Probe</td>
<td>FAM-cacctgactgaagggagcagatctca-TAMRA</td>
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<td>Reverse primer</td>
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<td>Reverse primer</td>
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Table 2: Effects of palmitic acid on genes related to the insulin signaling pathway

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<th>Symbol</th>
<th>Description</th>
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<th>Pathway</th>
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<tr>
<td>AKT1</td>
<td>V-akt murine thymoma viral oncogene homolog 1</td>
<td>1.6*</td>
<td>PI3K</td>
</tr>
<tr>
<td>EIF2B1</td>
<td>Eukaryotic translation initiation factor 2B, subunit 1 alpha</td>
<td>2.4*</td>
<td>PI3K</td>
</tr>
<tr>
<td>MTOR</td>
<td>Mechanistic target of rapamycin</td>
<td>2.7***</td>
<td>PI3K</td>
</tr>
<tr>
<td>PIK3CA</td>
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<td>5.5***</td>
<td>PI3K</td>
</tr>
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<td>Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)</td>
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<td>PI3K</td>
</tr>
<tr>
<td>PIK3R2</td>
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<td>PI3K</td>
</tr>
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<td>PRKCI</td>
<td>Protein kinase C, iota</td>
<td>2.2**</td>
<td>PI3K</td>
</tr>
<tr>
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<td>Protein kinase C, zeta</td>
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<td>PI3K</td>
</tr>
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<td>G6PC</td>
<td>Glucose-6-phosphatase, catalytic subunit</td>
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<td>PI3K</td>
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<tr>
<td>IGFBP1</td>
<td>Insulin-like growth factor binding protein 1</td>
<td>10.6**</td>
<td>PI3K</td>
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<tr>
<td>PCK2</td>
<td>Phosphoenolpyruvate carboxykinase 2</td>
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<td>PI3K</td>
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<td>VEGFA</td>
<td>Vascular endothelial growth factor A</td>
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<td>PI3K</td>
</tr>
<tr>
<td>ANG</td>
<td>Angiogenin, ribonuclease, RNase A family 5</td>
<td>4.7**</td>
<td>MAPK</td>
</tr>
<tr>
<td>BRF</td>
<td>V-raf murine sarcoma viral oncogene homolog B1</td>
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<td>MAPK</td>
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<tr>
<td>ERCC1</td>
<td>Excision repair cross-complementing rodent repair deficiency, complementation group 1</td>
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<td>MAPK</td>
</tr>
<tr>
<td>GRB2</td>
<td>Growth factor receptor-bound protein 2</td>
<td>3.3*</td>
<td>MAPK</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low density lipoprotein receptor</td>
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<td>MAPK</td>
</tr>
<tr>
<td>MAP2K1</td>
<td>Mitogen-activated protein kinase kinase 1</td>
<td>7.4**</td>
<td>MAPK</td>
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<td>V-raf-1 murine leukemia viral oncogene homolog 1</td>
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<td>Son of sevenless homolog 1</td>
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<td>ACOX1</td>
<td>Acyl-CoA oxidase 1</td>
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<td>Adrenergic, beta-3-, receptor</td>
<td>5.7*</td>
<td>PPARG</td>
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<td>Fibroblast growth factor receptor substrate 2</td>
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<td>KRAS</td>
<td>V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog</td>
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<td>NCK1</td>
<td>NCK adaptor protein 1</td>
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<td>Cell Proliferation</td>
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*P<0.05, **P<0.01 and ***P<0.001 compared with 0 mM palmitic acid group
FIGURE LEGENDS

**Fig 1.** Effects of palmitic acid on *APOM* expression in HepG2 cells. *APOM* mRNA levels were determined with real-time RT-PCR in HepG2 cells treated without or with different concentrations of palmitic acid. Data are represented as means ± SEM (n=6 for each group). The control group without palmitic acid is given as 100%. **P <0.01 vs. control group (One-way ANOVA followed by Tukey's multiple comparison test).**

**Fig 2.** Effects of palmitic acid, PI3K inhibitor LY294002 and PKC inhibitor GFX on *APOM* mRNA expression in HepG2 cells. (A) HepG2 cells were treated with experiment medium containing palmitic acid (1 mM) without or with PI3K inhibitor LY294002 at 10μM. Each experimental group contains 6 replicates. Data are presented as means ± SEM. The cells without palmitic acid are given as 100%. **P <0.01 vs. without palmitic acid. (B) HepG2 cells were treated with experiment medium containing palmitic acid (1mM) without or with PKC inhibitor GFX at 2μM. Each experimental group contains 6 replicates. Data are presented as means ± SEM. The cells treated without palmitic acid are given as 100%. *P <0.05 and **P <0.01 vs. without palmitic acid.**

**Fig 3.** Effects of palmitic acid and GSK3738 on mRNA level of *PPARβ/D* and *APOM*, respectively. (A) Effects of palmitic acid on *PPARβ/D* mRNA expression in HepG2 cells. **P <0.01 vs. cells treated without palmitic acid. (B) Effects of *PPARβ/D* antagonist GSK3787 on *APOM* mRNA expression in HepG2 cells treated without or with palmitic acid. **P <0.01 vs. without palmitic acid.