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Published in:
Biochemical and Biophysical Research Communications

DOI:
10.1016/j.bbrc.2014.01.170

2014

Palmitic acid suppresses apolipoprotein M gene expression via the pathway of $PPAR_{\beta/\delta}$ 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 ($PPAR_{\beta/\delta}$) 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 $PPAR_{\beta/\delta}$ 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-cm^2

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% CO_2, 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 ($PPAR_{\beta/\delta}$) 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 $MgCl_2$ (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 *APOM* expression (Fig. 2B).

3.3 Effects of palmitic acid on PPARB/D mRNA expression

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

4. Discussion

It has been suggested that *APOM* plays important roles involved in the anti-atherosclerotic effects of HDL particles, although the underlying mechanisms are not fully understood. *APOM* may function as the acceptor of HDL-carrying S1P [13], *APOM* can enhance the HDL-mediated anti-oxidation effect [14], and APOM has an important role for the pre-β HDL formation [3]. In the present study, we demonstrated that palmitic acid could decrease the expression of *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 *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 *PI3K*, *MAPK*, *PPARG*, *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$_{\beta/\delta}$, 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$_{\beta/\delta}$ 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 *PPARB/D* mRNA levels in HepG2 cells, and *PPARB/D* 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$_{\beta/\delta}$ pathway.

ACKNOWLEDGEMENTS

This research project was supported by the National Natural Science Foundation of China (NSFC) (81071414), the Natural Science Foundation of Jiangsu Province (BK2011245) a research grant from the Changzhou Science & Technology Bureau (CJ20122012) and Jiangsu Provincial 333 High-level Talents Cultivation Project (BRA2013062). Luo GH, Zhang XY, Berggren-Söderlund M, Nilsson-Ehle P and Xu N have involved in the project design and controlled study and revised manuscript; Luo GH performed the statistical analysis and has written the manuscript; Shi YP performed cell culture experiments; Zhang J, Mu FQ, Qin L, Zheng L and Feng YH performed laboratory analyses and commented the manuscript; Zhang XY and Xu N instructed present project and have full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1: Sequences of primers and probes

Gene	Primer/Probe	Sequence (5' to 3')
Human *APOM*	Forward primer	tgccccggaaatggatcta
	Reverse primer	cagggcggccttcagtt
	Probe	FAM-cacctgactgaagggagcacagatctca-TAMRA
Human *GAPDH*	Forward primer	ggaaggtgaaggtcggagtc
	Reverse primer	cgttctcagccttgacggt
	Probe	FAM-tttggtcgtattgggcgcctg-TAMRA
Human *PPARB/D*	Forward primer	tctacaatgcctacctgaaaaacttc
	Reverse primer	acaatgtctcgatgtcgtggatc
	Probe	FAM-acatgaccaaaaagaaggcccgcag-TAMRA

Table 2: Effects of palmitic acid on genes related to the insulin signaling pathway

Symbol	Description	Fold change 1 mM/0 mM	Pathway
AKT1	V-akt murine thymoma viral oncogene homolog 1	1.6*	PI3K
EIF2B1	Eukaryotic translation initiation factor 2B, subunit 1 alpha	2.4*	PI3K
MTOR	Mechanistic target of rapamycin	2.7***	PI3K
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	5.5***	PI3K
PIK3R1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	2.8*	PI3K
PIK3R2	Phosphoinositide-3-kinase, regulatory subunit 2 (beta)	1.6*	PI3K
PRKCI	Protein kinase C, iota	2.2**	PI3K
PRKCZ	Protein kinase C, zeta	1.6*	PI3K
G6PC	Glucose-6-phosphatase, catalytic subunit	14.6***	PI3K
IGFBP1	Insulin-like growth factor binding protein 1	10.6**	PI3K
PCK2	Phosphoenolpyruvate carboxykinase 2	5.7**	PI3K
PDPK1	3-phosphoinositide dependent protein kinase-1	2.7***	PI3K
SERPINE1	Serpin peptidase inhibitor, clade E, member 1	1.8**	PI3K
SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4	4.9*	PI3K
VEGFA	Vascular endothelial growth factor A	1.9*	PI3K
ANG	Angiogenin, ribonuclease, RNase A family, 5	4.7**	MAPK
BRAF	V-raf murine sarcoma viral oncogene homolog B1	2.7*	MAPK
ERCC1	Excision repair cross-complementing rodent repair deficiency, complementation group 1	3.3*	MAPK
GRB2	Growth factor receptor-bound protein 2	3.3*	MAPK
LDLR	Low density lipoprotein receptor	2.0**	MAPK
MAP2K1	Mitogen-activated protein kinase kinase 1	7.4**	MAPK
MAPK1	Mitogen-activated protein kinase 1	2.0**	MAPK
RAF1	V-raf-1 murine leukemia viral oncogene homolog 1	2.4***	MAPK
SOS1	Son of sevenless homolog 1	2.3**	MAPK
ACOX1	Acyl-CoA oxidase 1	3.9*	PPARG
ADRB3	Adrenergic, beta-3-, receptor	5.7*	PPARG
CEBPA	CCAAT/enhancer binding protein (C/EBP), alpha	3.2***	PPARG
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	4.4**	PPARG
PPARG	Peroxisome proliferator-activated receptor gamma	3.6***	PPARG
FRS2	Fibroblast growth factor receptor substrate 2	12.2*	Insulin
FRS3	Fibroblast growth factor receptor substrate 3	2.9*	Insulin
GAB1	GRB2-associated binding protein 1	4.3*	Insulin
IGF1R	Insulin-like growth factor 1 receptor	2.0**	Insulin
INSR	Insulin receptor	4.0***	Insulin
IRS1	Insulin receptor substrate 1	2.0*	Insulin
IRS2	Insulin receptor substrate 2	2.9*	Insulin
PTPN1	Protein tyrosine phosphatase, non-receptor type 1	4.7*	Insulin
SLC2A1	Solute carrier family 2 (facilitated glucose transporter), member 1	0.2**	Insulin
SORBS1	Sorbin and SH3 domain containing 1	2.8**	Insulin
ACACA	Acetyl-CoA carboxylase alpha	1.9*	SREBP1
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	4.5**	Transcription Factors
FBP1	Fructose-1,6-bisphosphatase 1	2.0*	SREBP1
KRAS	V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	4.3*	Cell Cycle
NCK1	NCK adaptor protein 1	3.2**	Cell Proliferation

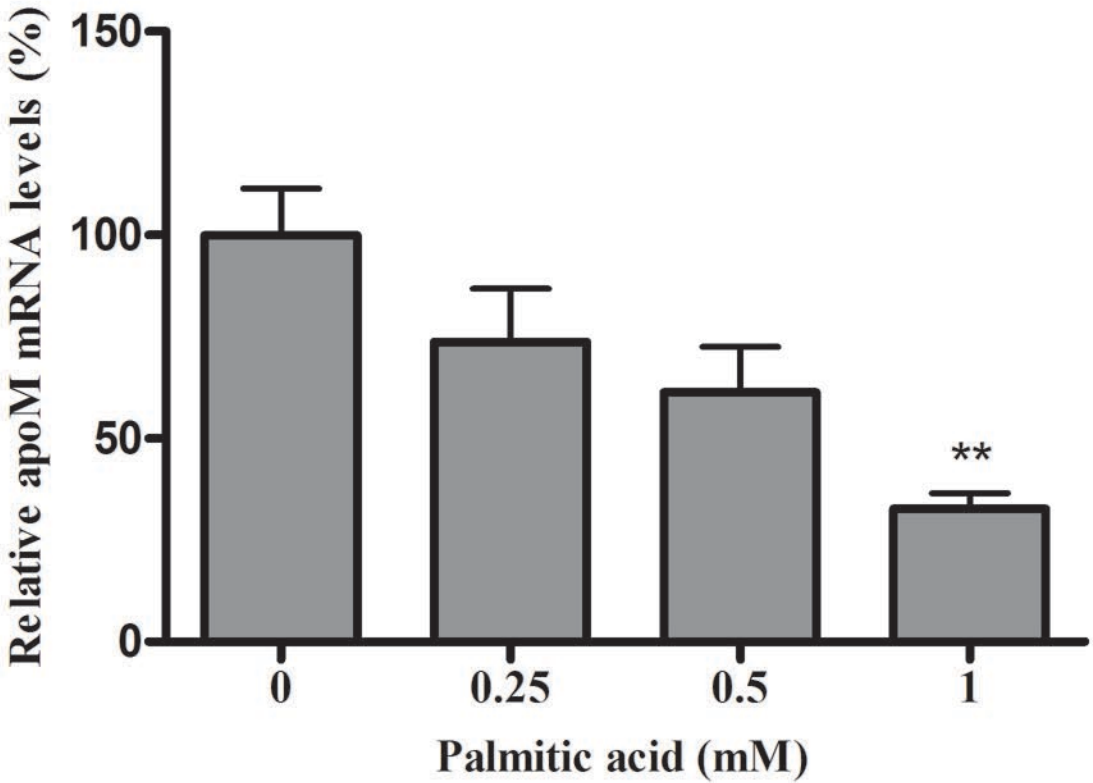
*P<0.05, **P<0.01 and ***P<0.001 compared with 0 mM palmitic acid group

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 *PPARB/D* and *APOM*, respectively. (A) Effects of palmitic acid on *PPARB/D* mRNA expression in HepG2 cells. ***P* <0.01 vs. cells treated without palmitic acid. (B) Effects of *PPARB/D* antagonist GSK3787 on *APOM* mRNA expression in HepG2 cells treated without or with palmitic acid. ***P* <0.01 vs. without palmitic acid.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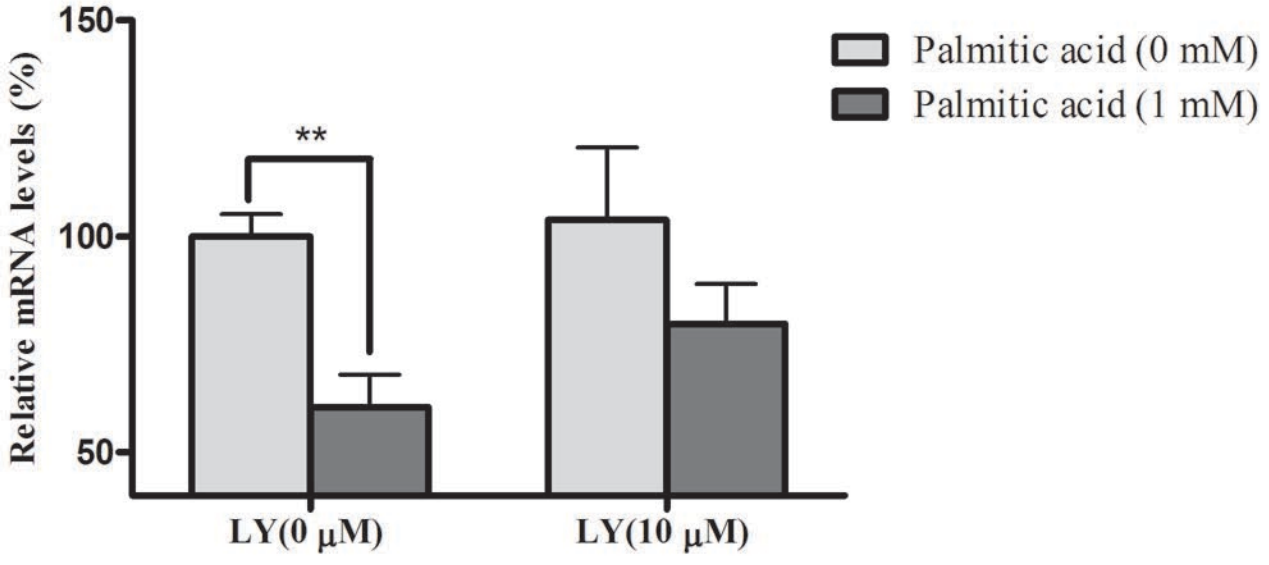
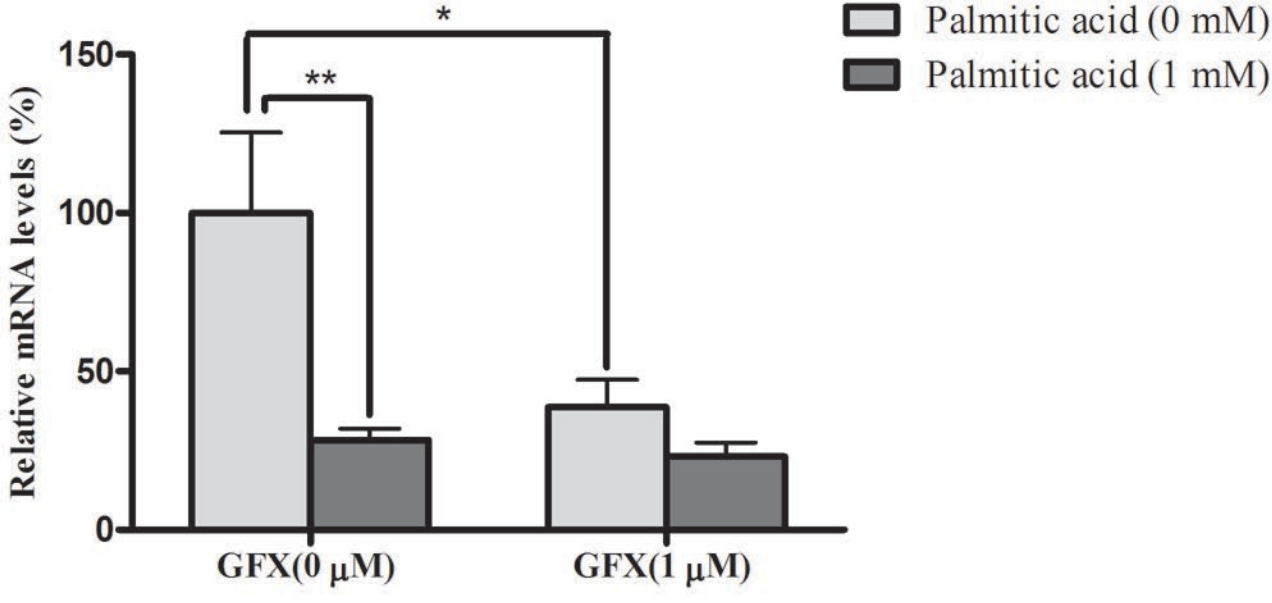
100
50
0
**
0
0.25
0.5
1
Palmitic acid (mM)

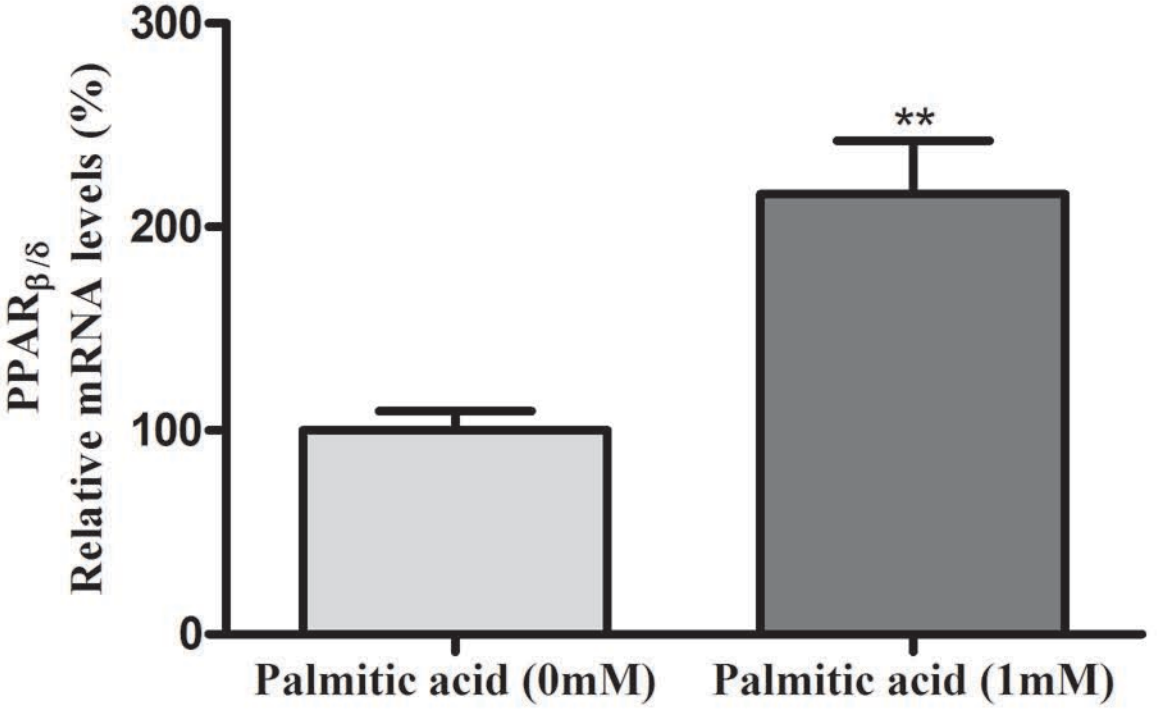
A


150
100
50
Relative mRNA levels (%)
**
Palmitic acid (0 mM)
Palmitic acid (1 mM)
LY(0 μM)
LY(10 μM)

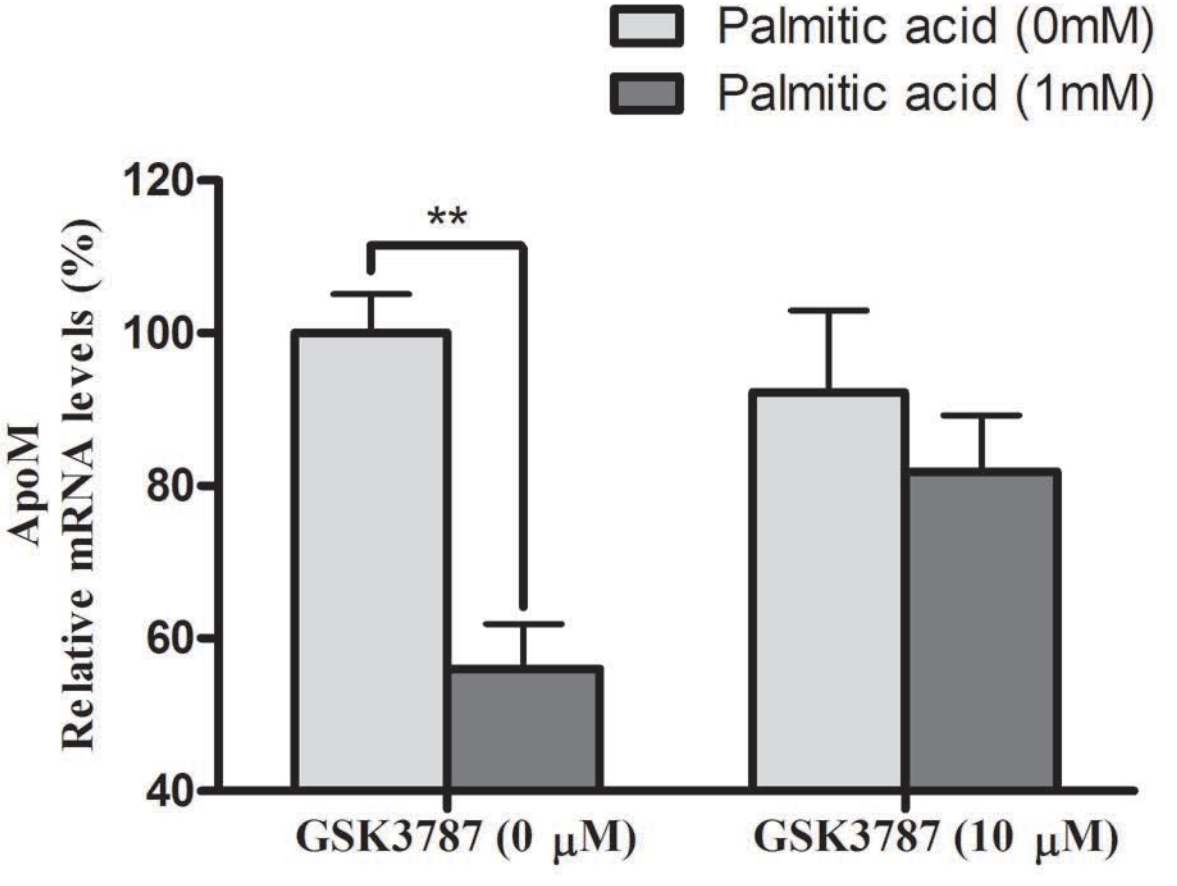
B


150
100
50
0
Relative mRNA levels (%)
*
**
Palmitic acid (0 mM)
Palmitic acid (1 mM)
GFX(0 μM)
GFX(1 μM)

A


300
200
100
0
PPARβ/δ
Relative mRNA levels (%)
**
Palmitic acid (0mM)
Palmitic acid (1mM)

B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Palmitic acid (1mM)
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100
80
60
40
ApoM
Relative mRNA levels (%)
**
GSK3787 (0 μM)
GSK3787 (10 μM)