The FEBS Journal

FEBS PRESS
science publishing by scientists

Adrenoceptor-related decrease in serum triglycerides is independent of PPARα activation

Maria Konstandi[1,2], Kyriakos E. Kypreos[3], Tsutomu Matsubara[2,4], Eva Xepapadaki[3], Yatrik M. Shah[2,5], Kristopher Krausz[2], Christina E. Andriopoulou[1], Aristeidis Kofinas[1] and Frank J. Gonzalez[2]

1 Department of Pharmacology, Faculty of Medicine, University of Ioannina, Greece
2 Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
3 Department of Pharmacology, Faculty of Medicine, University of Patras, Rio, Greece
4 Department of Anatomy and Regenerative Biology, Graduate School of Medicine, Osaka City University, Japan
5 Department of Molecular and Integrative Physiology, Medical School, University of Michigan, Ann Arbor, MI, USA

Keywords
adrenergic receptors; hypertriglyceridemia; PPARα; triglycerides; TRLs

Correspondence
M. Konstandi, Department of Pharmacology, Faculty of Medicine, University of Ioannina, School of Health Sciences, Ioannina GR-451 10, Greece
Tel: +3026510 07554
E-mail: mkonstan@uoi.gr

(Received 7 September 2018, revised 5 April 2019, accepted 19 May 2019)

doi:10.1111/febs.14966

Adrenoceptor (AR)-linked pathways belong to the major components of the stress response system and are associated with the pathophysiology of diseases within the spectrum of metabolic syndrome. In this study, the role of adrenoceptor stimulation in serum triglyceride (TG) regulation in mice was investigated. For this purpose, α_1-ARs were activated with phenylephrine (PH) and $\beta_{1/2}$-ARs with isoprenaline (ISOP). Both AR agonists markedly reduced serum TG levels independently of PPARα activation. These drugs also significantly activated the hormone-sensitive lipase in the white adipose tissue indicating increased mobilization of TGs in this tissue. In addition, PH and ISOP up-regulated Lpl, Nr4A, Dgat1, Mttp, Aadac and Cd36 genes, critical in TG regulation, whereas the observed decrease in serum TG levels was independent of the hepatic very low-density lipoprotein (VLDL)-TG secretion. Interestingly, PH and ISOP also inactivated the hepatic insulin/PI3k/AKT/FoxO1 signaling pathway, holding a critical role in the regulation of genes involved in TG synthesis. Taken together, the findings of the present study indicate that stimulation of α_1- and $\beta_{1/2}$-ARs markedly reduced serum TG steady-state levels as a result of alterations in TG synthesis, uptake, transport, hydrolysis, metabolism and clearance, an effect induced by PPARα independent mechanisms.

Introduction

Hypertriglyceridemia is a major pathological feature of metabolic syndrome, which is associated with the accumulation of triglyceride-rich lipoproteins (TRLs) in circulation. Patients with elevated serum TRLs are

Abbreviations
AADAC, arylacetamide deacetylase; ACADM, acyl-CoA dehydrogenase; ACOT, acyl-CoA thioesterase; ACOX, acyl-CoA oxidase; AKT, protein kinase B; ALT, alanine aminotransferase; ApoE, apolipoprotein E; AR, adrenergic receptor; AST, aspartate aminotransferase; ATGL, adipose triglyceride lipase; BBAT, bile acid CoA; cAMP, cyclic AMP; CD36, cluster of differentiation 36; CES3/TGH, carboxylesterase 3; CREB, cAMP-response element-binding protein; DGAT, diacylglycerol O-acyltransferase; EIA, Elisa; FFA, free fatty acids; FoxO1, forkhead box protein O1; HDL, high-density lipoprotein; HNF4α, hepatocyte nuclear factor 4α; HSL, hormone-sensitive lipase; ISOP, isoprenaline; LDL-r, low-density lipoprotein receptor; LPL, lipoprotein lipase; MTTP, microsomal triglyceride transfer protein; NEFA, non-esterified fatty acids; Nr4A, nuclear receptor 4a; PCR, polymerase chain reaction; PCSK9, proprotein convertase subtilisin/kexin type 9; PH, phenylephrine; PI3k, phosphatidylinositol 3-kinase; PKA, protein kinase A; PPARα, peroxisome proliferator-activated receptor-α; RIA, radioimmunoassay; TG, triglycerides; TRLs, triglyceride-rich lipoproteins; VLDL, very low-density lipoprotein; W.A.T., white adipose tissue.

at high risk for cardiovascular and renal disease, as well as for steatohepatitis and other disorders. To date, treatment with fibrates is the most effective pharmacological approach in clinical practice for the reduction of serum TG levels. Fibrates are used either as monotherapy or in combination with statins and other hypolipidemic drugs [1,2].

Fibrates are ligands for the peroxisome proliferator-activated receptor α (PPARα), which is activated by psychophysiological stress via stimulation of AR-linked pathways and glucocorticoids [3,4]. PPARα acts as a cellular 'lipostat' that transduces alterations in cellular lipid levels to the transcriptional regulation of various target genes, which are critical to the fate of fatty acids [5–7]. In particular, activation of PPARα up-regulates a broad array of genes encoding enzymes that are involved in fatty acid uptake, transport, as well as in mitochondrial and peroxisomal fatty acid β-oxidation and microsomal fatty acid ω-oxidation. In addition, several apolipoproteins are regulated by PPARα including apolipoproteins (Apo) AI, AII, and CIII, a fact that indicates the central role of PPARα in the extracellular transport and metabolism of TG-rich lipoproteins in blood [3]. These PPARα-mediated changes in gene expression result in reduced serum TG-rich lipoproteins and increased high-density lipoprotein (HDL) levels [8,9], although the exact mechanisms that link TG and HDL levels are currently poorly defined.

The apparent causative relationship between serum TRL levels and a wide range of human pathologies has triggered the development of several biological drugs targeting TRL metabolism, such as Volanesorsen, [10], Evinacumab [11–13], and IONIS-ANGPTL3-LRx [14]. Nonetheless, the effective prevention of hypertriglyceridemia requires a deeper understanding of the biochemical mechanisms involved and more precisely, the triggers leading to excess TRL accumulation in serum.

The role of stress in the regulation of lipid homeostasis is well documented. In particular, chronic stress deregulates lipid and carbohydrate homeostasis and is considered as a causative factor of several pathologies related to the metabolic syndrome, such as visceral obesity, insulin resistance, dyslipidemia, dyscoagulation, and hypertension [15–23]. It also has been reported that humans with low-sympathetic nervous system (SNS) activity, reduced beta-adrenergic sensitivity, and lipid mobilizing efficacy of catecholamines display lowered energy expenditure and are at high risk to develop obesity compared to physiological subjects. Therefore, adrenergic receptors, major components of the SNS, have been considered as putative therapeutic targets against obesity [24]. Accumulating evidence also suggests that short-term exposure to stress has a beneficial effect on TG regulation. Specifically, subacute exposure to repeated restraint stress markedly reduces serum TG steady-state levels, predominantly via adrenergic receptor (AR)-linked pathways. In particular, α_1- and $\beta_{1/2}$-ARs appear to hold major roles in this regulation, as blockade of these receptors prior to stress completely inhibited the suppressive effect of stress on serum TG levels [4]. Activation of the hormone-sensitive lipase (HSL) in the white adipose tissue (W.A.T.) by stress or epinephrine, a major effector of the stress response, is potentially responsible, at least in part, for this suppressive effect. In addition, stress via stimulation of α_1- and $\beta_{1/2}$-ARs up-regulated several genes in the W.A.T., which are critical in the synthesis and metabolism of TG depots, such as the diacylglycerol acyltransferase *(Dgat)1* and *2*, lipoprotein lipase *(Lpl)*, adipose triglyceride lipase/patatin-like phospholipase domain containing 2 *(Atgl/Pnpla2)*, arylacetamide deacetylase *(Aadac)*, microsomal triglyceride transfer protein *(Mttp)*, and the orphan nuclear receptor *(Nr4A)* [2,25–32].

To better understand the molecular mediators of the stress-related hypertriglyceridemia, in this study, we investigated the involvement of α_1- and $\beta_{1/2}$-ARs in the regulation of serum TRL homeostasis. For this purpose, pharmacological manipulations of α_1- and $\beta_{1/2}$-AR–linked pathways by phenylephrine (PH) and isoprenaline (ISOP), respectively, were used. The data revealed a strong suppressive effect of the $\beta_{1/2}$-AR agonist and less of the α_1-AR agonist on serum TG steady-state levels, independent of PPARα activation, shedding light in novel-signaling pathways triggered by the adrenergic system with significant roles in TRL homeostasis.

Results

Alterations in serum lipid levels, post-prandial triglyceride kinetics, and hepatic VLDL triglyceride secretion

Pharmacological stimulation of α_1-ARs markedly reduced serum TG, free fatty acid (FFA) and total cholesterol levels in wild-type mice (Fig. 1A–C). Beta-AR stimulation decreased only serum TG and FFA concentration, whereas it had no effect on total cholesterol levels (Fig. 1A–C). Interestingly, stimulation of ARs also suppressed serum TG steady-state levels in *Ppara*-null mice thus indicating a PPARα-independent

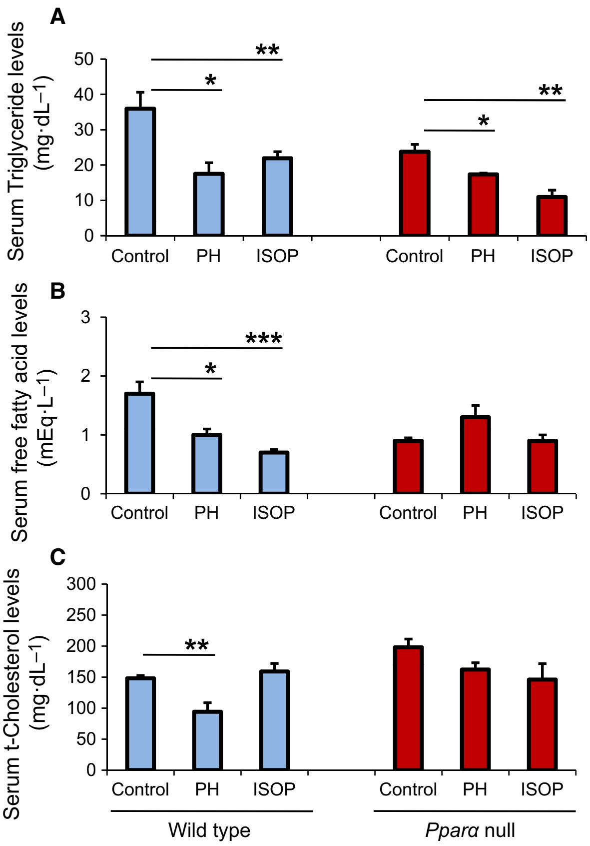

Fig. 1. Effects of PH and ISOP treatment on serum lipid markers. PH: phenylephrine (α_1-AR agonist); ISOP: isoprenaline (β_1/β_2-AR agonist). Values are expressed as mean ± SEM, n: 5 per treatment group and comparisons took place between controls and drug-treated mice; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

mechanism in TG regulation by PH and ISOP (Fig. 1A). In an effort to provide a mechanistic interpretation of the observed reduction in serum TG levels following stimulation of ARs with AR-agonists, the hepatic VLDL-TG secretion kinetics was determined in control and AR-agonist–treated *Ppar*α-null mice. Stimulation of β_1/β_2-ARs with ISOP resulted in a significant increase in the rate of hepatic VLDL-TG secretion in treated mice when compared to controls (Fig. 2A), while PH (α_1-AR agonist) did not have any significant effect (Fig. 2A), suggesting that mobilization and secretion of hepatic TG into VLDL particles could not account for the observed reduction of serum TG levels following PH treatment (Figs 1A and 2A). Similarly, no significant changes in serum AST, ALT and body weight levels were observed following the above mentioned drug treatments (Tables 1 and 2).

In vivo assessment of the role of AR-related pathways in TG regulation

To further elucidate the mechanisms underlying the strong suppressive effect of AR-linked pathways on serum TG steady-state levels, the expression of various genes encoding factors involved in TG synthesis, metabolism, and clearance were determined by qPCR and western blot analysis. Both PH and ISOP increased hepatic *Lpl* mRNA expression (Fig. 3A). *Nr4A* mRNA expression was also increased by PH and ISOP in the liver (Fig. 3). In contrast, the expression of *Atgl/Pnpla2, Hsl,* and *Aadac* mRNAs were suppressed by PH in this tissue (Fig. 3,B). *Dgat2* mRNA transcripts were also increased in the liver of PH- and ISOP-treated mice (Fig. 3B), whereas *Dgat1* mRNA expression was not affected (Fig. 3A). Moreover, *Mttp*, carboxylesterase 3 (*Ces3/tgh)* and cluster of differentiation 36 (*Cd36)* mRNAs were increased to the same extent in the liver of both, PH- and ISOP-treated mice compared to placebo-treated animals (Fig. 3B). No effect was observed on hepatic low-density lipoprotein receptor *(Ldl-r)* mRNA expression (Fig. 3C). Similarly, APOE protein expression was not affected by either AR agonists (Fig. 3C).

It is of interest to note that the AR agonists, PH, and ISOP also up-regulated Nr4A in the W.A.T., which may in turn trigger the up-regulation of *Lpl* (Fig. 4). *Hsl* and *Atgl/Pnpla2* mRNA and protein were not affected by either PH or ISOP in this tissue (Fig. 4A and D). PH, though, induced HSL phosphorylation at Ser563 in the W.A.T. compared to controls (Fig. 4D), whereas ISOP increased HSL phosphorylation at Ser660 in this tissue (Fig. 4D). Notably, total perilipin and specifically, PLIN5 protein levels were not modified by the AR agonists in the W.A.T. (Fig. 4D). *Dgat1* mRNA expression was increased only by ISOP in the W.A.T. (Fig. 4A), whereas Mttp and Cd36 were up-regulated by both drugs (Fig. 4B). *Ces3/tgh* mRNA expression was not affected (Fig. 4B). Interestingly, TG content in the W.A.T. was lower in PH- and ISOP-treated mice compared to controls (Fig. 1C). In contrast, TG levels were higher in the livers of ISOP-treated mice compared to controls and PH-treated animals (Fig. 2C).

In order to determine the role of AR agonists in lipid β-oxidation, the effect of PH and ISOP on mRNA encoding ACADM, the rate-limiting enzyme of this reaction, was assessed in the liver and W.A.T. using qPCR analysis. Only stimulation of α_1-ARs with PH markedly increased *Acadm* mRNA expression in the liver compared to controls; ISOP had no effect (Fig. 3C). In contrast, PH repressed *Acadm* mRNA expression in the W.A.T. (Fig. 4C).

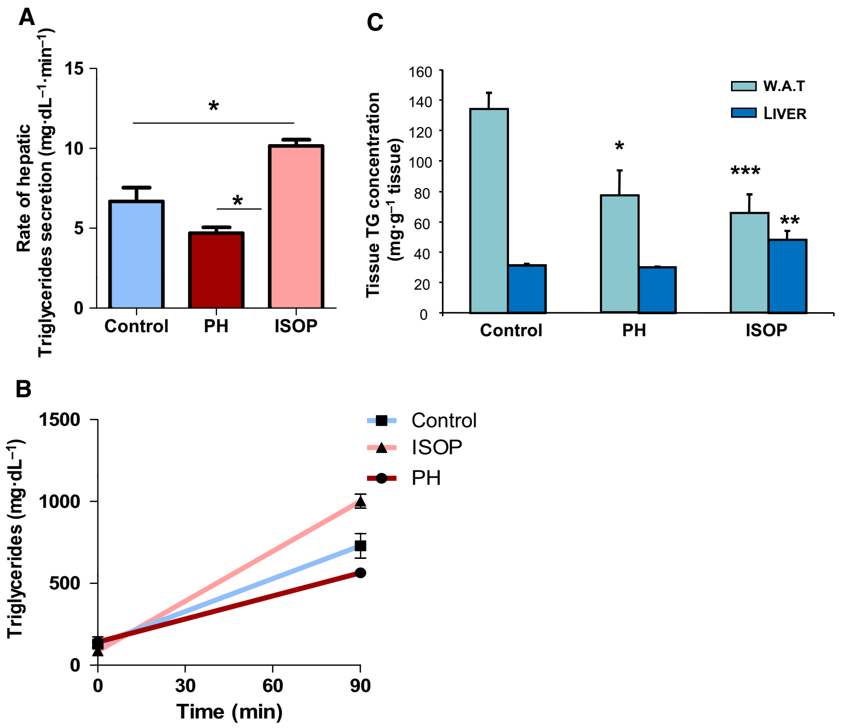

Fig. 2. Effects of PH and ISOP treatment on kinetic parameters of serum triglyceride metabolism. Panel A shows the rate of hepatic VLDL triglyceride secretion of the PH- or ISOP-treated mice and controls and Panel B represents the kinetics of post-prandial triglyceride clearance in PH- and ISOP-treated mice. Panel C shows TG concentration in the liver and white adipose tissue (W.A.T.) of mice following treatment with either PH: phenylephrine (α_1-AR agonist), ISOP: isoprenaline ($\beta_{1/2}$-AR agonist) or normal saline (controls). Values are expressed as mean ± SEM, n: 5 per treatment group and comparisons took place between controls and drug-treated mice. Group differences were calculated by one-way ANOVA, followed by Bonferroni's test. All experiments were performed as described in Materials and Methods. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1. AR-induced alterations in serum ALT and AST concentration. Adrenergic receptor (AR); Alanine aminotransferase (ALT), aspartate aminotransferase (AST), phenylephrine (PH), isoprenaline (ISOP), (wild-type, n = 20; *Ppara*-null, n = 15)

	Wild-type			*Pparα null*		
	Control	PH	ISOP	Control	PH	ISOP
ALT	9.1 ± 0.9	11.3 ± 4.9	7.1 ± 2.0	10.1 ± 2.3	11.4 ± 2.7	8.3 ± 3.4
AST	25.2 ± 4.6	29.5 ± 11.2	15.5 ± 2.0	24.6 ± 5.3	28.2 ± 8.9	18.9 ± 2.7

In vivo and *in vitro* assessment of the AR-induced alterations in Hnf4α regulation

Stimulation of α_1- or $\beta_{1/2}$-ARs with PH or ISOP, respectively, markedly increased *Hnf4a* mRNA and HNF4α protein levels (Fig. 5A). The AR-induced *Hnf4a* expression triggered the up-regulation of the Hnf4α target genes, *Cyp8b1* and bile acid CoA: amino acid N-acyltransferase (*Baat*) (Fig. 5B). Further investigation revealed that the drug-induced up-regulating effect on hepatic Hnf4α is due to a direct effect of the drug on hepatocyte α_{1}- or β_1-ARs, respectively. Treatment of primary hepatocytes with either PH or ISOP markedly induced hepatocyte *Hnf4a* mRNA expression (Fig. 5C). This up-regulating effect on Hnf4α was blocked by pre-treatment of the cells with the PKA inhibitor, H89, and the phosphatase- and ATPase inhibitor, NaOV (Fig. 5C). The ISOP-induced Hnf4α up-regulation was also prevented mainly, by the phosphatase- and ATPase inhibitor, NaOV and to a lesser extent by H89 (Fig. 5C).

Table 2. Alterations in the body weight following adrenergic receptor agonist treatment

Treatment	1st day	4th day
Control	25.1 ± 1.0	26.3 ± 0.8
Phenylephrine	27.9 ± 0.4	28.6 ± 0.5
Isoprenaline	24.0 ± 0.6	25.2 ± 0.5

Body weight values are expressed in g. Phenylephrine, α_1-adrenergic receptor (AR) agonist; Isoprenaline, $\beta_{1/2}$-AR agonist.

In vivo assessment of the role of AR-linked pathways in PI3k/AKT/FoxO1 and cAMP/PKA activation

In order to further investigate the mechanism underlying the reduction in serum TG levels following PH or

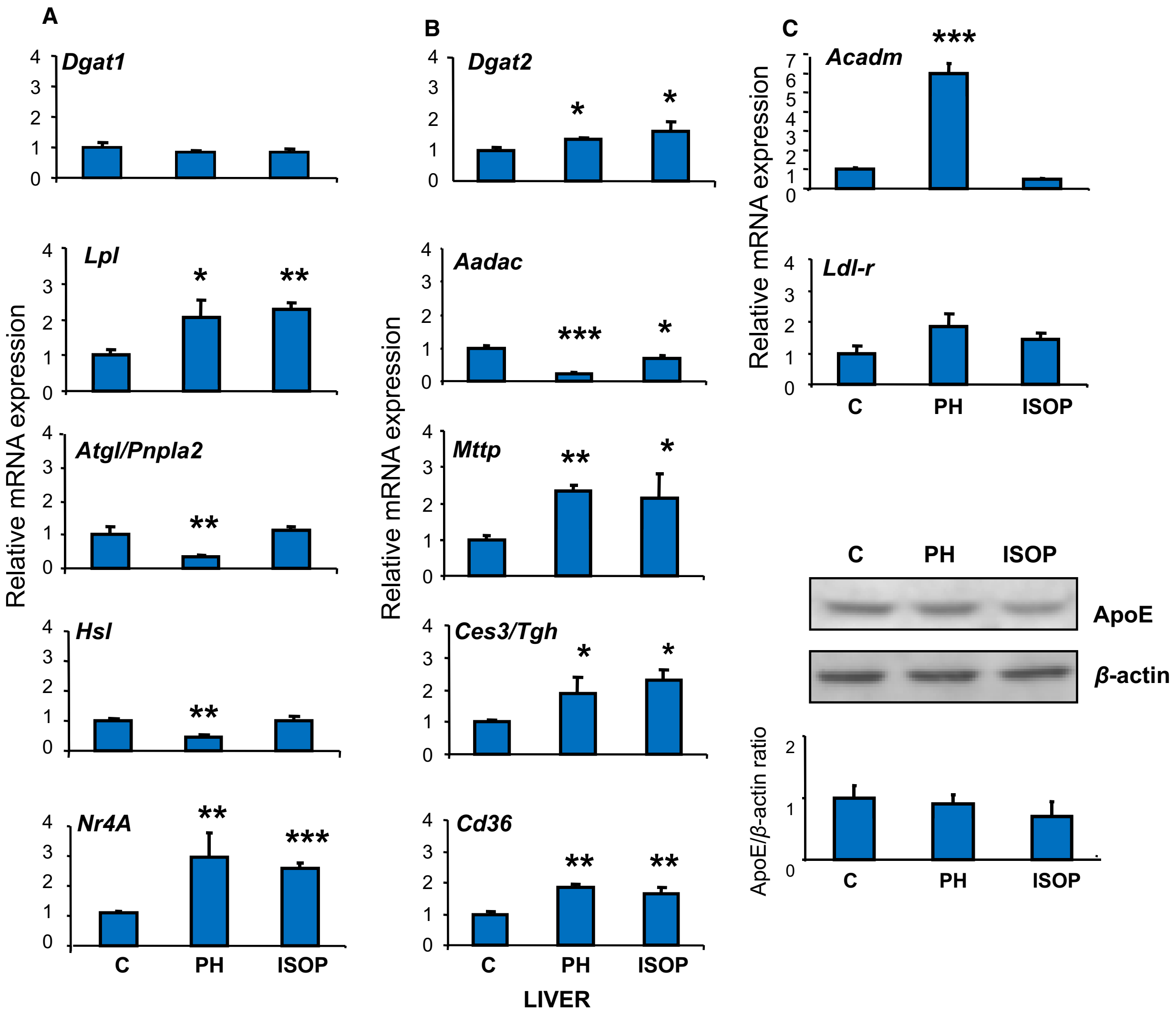

Fig. 3. Adrenergic receptor-mediated effect on hepatic factors regulating TG serum levels. (A) Effect of AR-agonists on genes involved in TG synthesis and lipolysis in the liver. (B) Effect of AR-agonists on genes involved in TG metabolism and clearance in the liver. (C) Effect of AR-agonists on factors important in lipid β-oxidation, the clearance of triglyceride-rich lipoproteins and the transport of free fatty acids. Comparisons were between controls and drug-treated mice. *Dgat1*: diacyl glycerol acyltransferase, *Dgat2*: diacyl glycerol acyltransferase 2, *Lpl*: lipoprotein lipase, *Hsl*: hormone-sensitive lipase, *Atgl/Pnpla2*: adipose triglyceride lipase/patatin-like phospholipase domain containing 2, *Nr4A*: orphan nuclear receptor NR4A, *Aadac*: arylacetamide deacetylase, Cd36: cluster of differentiation 36 or fatty acid transporter, *Ces3/tgh*: carboxylesterase 3, *Mttp*: microsomal triglyceride transfer, *Acadm*: acyl-CoA dehydrogenase, *Ldl-r*: low-density lipoprotein receptor, ApoE: apolipoprotein E. In the western blot, three samples per treatment were loaded in three different blots. C: Control, phenylephrine (α_1-AR-agonist, PH), isoprenaline ($\beta_{1/2}$-AR agonist, ISOP). Values are expressed as mean ± SEM, n:5-6 mice per treatment group. Group differences were calculated by one-way ANOVA, followed by Bonferroni's test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

ISOP treatment, total cellular proteins were analyzed by western blot. When compared to controls, both, PH and ISOP reduced AKT and consequently, FoxO1 phosphorylation in the liver (Fig. 6), whereas they increased CREB phosphorylation (Fig. 6), indicating inactivation of the PI3k/AKT/FoxO1 and activation of the AR/cAMP/PKA/CREB signaling pathway.

Discussion

Accumulated experience over the past many decades of basic and clinical research established unequivocally a major role of LDL-C in the development and progression of atherosclerosis. However, the aggressive LDL-C lowering in patients following the current

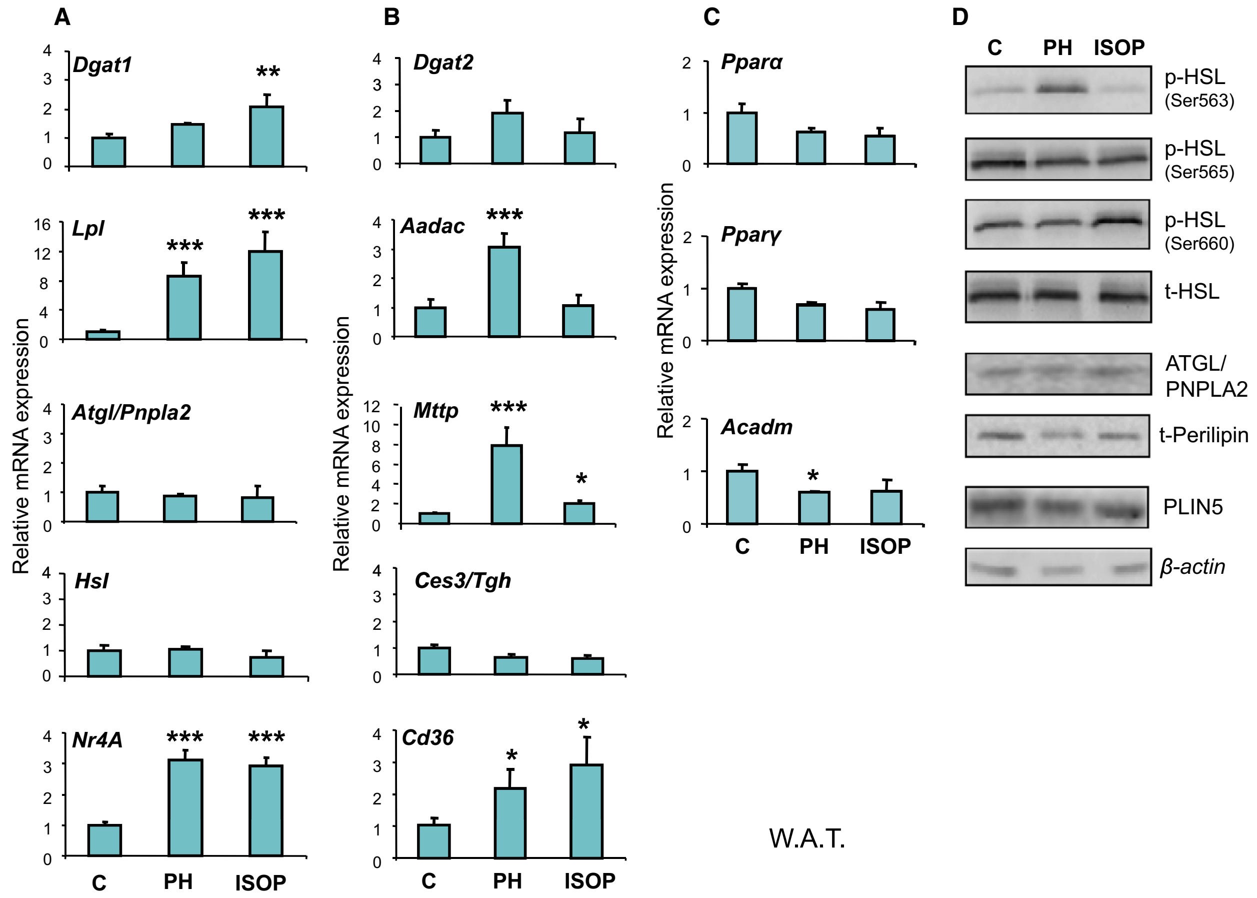

Fig. 4. Adrenergic receptor-mediated effect on various factors expressed in the W.A.T. regulating TG serum levels (A) Effect of phenylephrine (α_1-AR agonist, PH) and isoprenaline ($\beta_{1/2}$-AR agonist, ISOP) on the expression of genes involved in TG synthesis and lipolysis. (B) Effect of PH and ISOP on the expression of genes involved in TG metabolism and clearance. (C) Effect of PH and ISOP on factors important in lipid β-oxidation, the clearance of triglyceride-rich lipoproteins and the transport of free fatty acids. Comparisons were between controls and AR-agonist-exposed mice; *Dgat1*: diacyl glycerol acyltransferase 1 (acyl coenzyme A (CoA), *Dgat2*: diacyl glycerol acyltransferase 2, *Lpl*: lipoprotein lipase, *Hsl*: hormone-sensitive lipase, *Atgl/Pnpla2*: adipose triglyceride lipase/patatin-like phospholipase domain containing 2, *Nr4a*: orphan nuclear receptor, *Aadac*: arylacetamide deacetylase, *Cd36*: cluster of differentiation 36 or fatty acid transporter, *Ces3/tgh*: carboxylesterase 3, *Mttp*: microsomal triglyceride transfer, PLIN5: perilipin 5, AR: adrenergic receptor, C: Control, W.A.T.: white adipose tissue. Values are expressed as mean ± SEM, n:5-6 mice per treatment group; Group differences were calculated by one-way ANOVA, followed by Bonferroni's test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Lanes in western blots correspond to one sample per treatment and represent one sample of three separate samples tested in different blots.

medications is still associated with substantial residual cardiovascular risk, and strongly suggests that the benefit from LDL-C lowering strategies has reached a plateau [33]. Identifying and targeting alternative processes that are highly associated with atherogenesis may provide new ways to complement existing therapies and augment their benefit against the development of diseases, thus further reducing the residual cardiovascular risk, which is associated with the current pharmacotherapy [33].

The apparent causative relationship between high-TRL serum levels and atherosclerosis led to the development of several investigational drugs currently in clinical trials, that target TRL metabolism [33]. Volanesorsen, an apolipoprotein C3 (Apo C3) antisense oligonucleotide, targets selectively Apo C3 mRNA and blocks protein synthesis, due to the enhanced ribonuclease H1-mediated degradation of Apo C3 mRNA [10]. Another experimental drug is Evinacumab, an angiopoietin-like protein 3 (ANGPTL3) monoclonal antibody that blocks ANGPTL3, a protein known to increase plasma TRL and TG levels [11,34,35]. Another similar ANGPTL3-targeting drug is the IONIS-ANGPTL3-LRx, an ANGPTL3

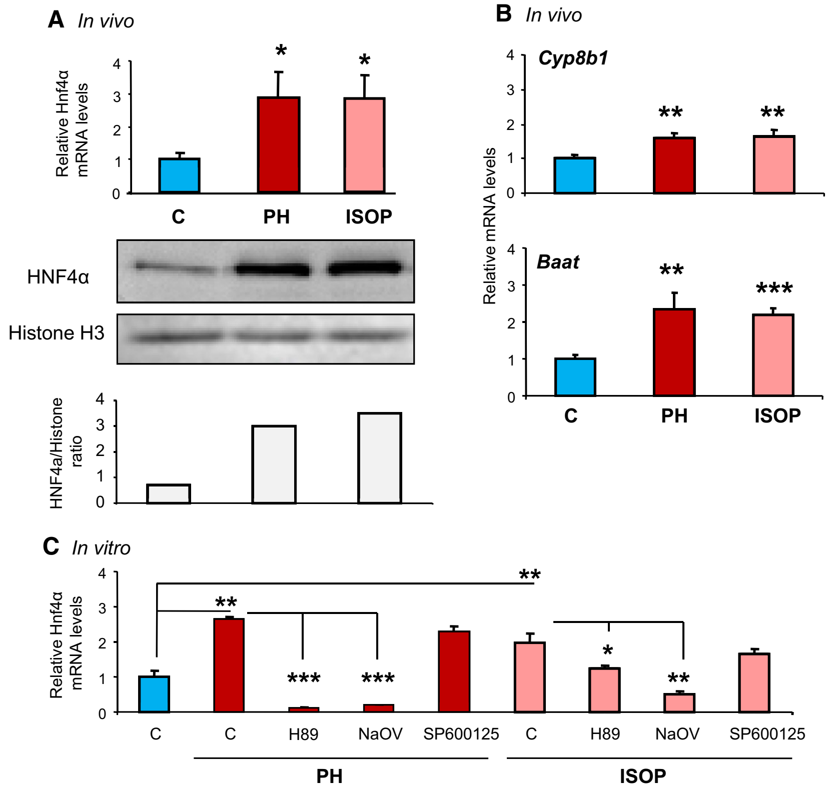

Fig. 5. The effect of adrenergic receptor-related pathways on *Hnf4a* expression. (A) Following treatment with AR-agonists, hepatic *Hnf4a* mRNA levels were analyzed in wild-type mice by qPCR. HNF4α protein was determined in liver nuclear fractions by western blot analysis. Histone H3 served as a loading control. In the bar graph, the quantified data from the western blot image are shown presented as the ratio of HNF4α/Histone H3. (B) *Cyp8b1* and *Baat* mRNA levels were analyzed in livers of wild-type mice by qPCR following treatment with AR-agonists. (C) *Hnf4a* mRNA levels were determined by qPCR following treatment of primary hepatocyte cultures with AR-agonists for 24 hours. Primary hepatocytes were also treated with AR-agonists in combination with either the JNK inhibitor, SP600125, the PKA inhibitor, H89, or the phosphatase- and ATPase inhibitor, NaOV (concentration of the inhibitors in the medium:10 μM and duration of incubation: 24 h). Values were normalized to β-actin and are expressed as mean ± SEM (*n* = 8–10). In the *in vivo* experiment, comparisons were between controls and drug-treated mice. In the *in vitro* experiment, comparisons were between DMSO and drug-treated hepatocytes, (*n* = 3–4). AR: adrenergic receptor, C: control (DMSO-treated primary hepatocytes), PH: Phenylephrine (α_1-AR agonist), ISOP: Isoprenaline ($\beta_{1/2}$-AR agonist). Group differences were calculated by one-way ANOVA, followed by Bonferroni's test. $^{*}P < 0.025$, $^{**}P < 0.01$, $^{***}P < 0.001$.

antisense oligonucleotide. Despite these developments, the molecular triggers that are associated with the disease development and eventually with the elevated plasma, TRL accumulation remain largely unexplored. There is a strong evidence that both, central and peripheral nervous systems, may be involved in this regulation [4,36–38].

Pharmacological stimulation of α_1- or $\beta_{1/2}$-AR–linked pathways markedly reduce the steady-state levels of serum TG in mice. These data are in line with studies reporting that subacute repeated restraint stress via mainly AR-related pathways reduces serum TG levels [4]. Investigation of the potential mechanisms involved in the AR agonist-induced serum TG reduction indicated that PPARα activation is not a part of this mechanism, because treatment of *Ppara*-null mice with either PH or ISOP triggered a reduction in serum TG levels that was comparable with that observed in PPARα expressing mice. Therefore, fibrates may not be an effective therapy for the stress-related hypertriglyceridemia. The contribution of other molecular factors, such as APO C3 and ANGPTL3, needs to be investigated.

The significant reduction in serum FFA observed following treatment with PH and ISOP may also suggest that AR agonists potentially increase energy requirements in the treated mice. This hypothesis is supported by previous studies reporting that agents, which stimulate adrenergic neurons increase energy expenditure, lipolysis, and fat oxidation [24]. Free fatty

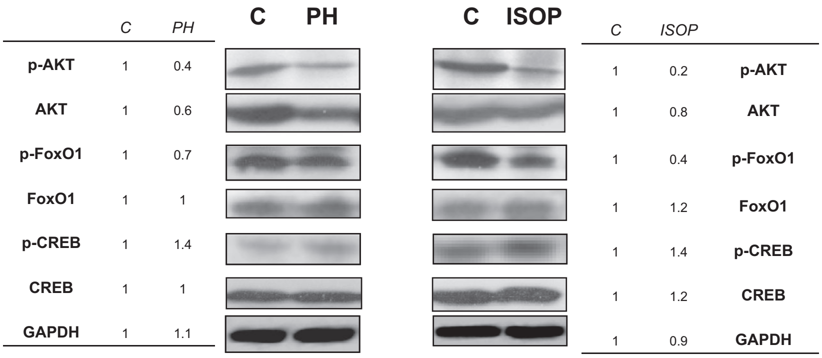

Fig. 6. The role of adrenergic receptors in the activation of insulin/PI3k/AKT/FoxO1 and AR/cAMP/PKA/CREB signaling pathways. Total and phosphorylated AKT and FoxO1 expression levels were examined in hepatic total cellular proteins using western blot analysis. CREB phosphorylation was assessed in hepatic total cellular proteins. C: control, PH: phenylephrine (α_1-AR agonist), ISOP: isoprenaline ($\beta_{1/2}$-AR agonist). The numbers next to the lanes represent the relative protein expression that is defined as the ratio between the drug-treated and control expression, which is set at 1.

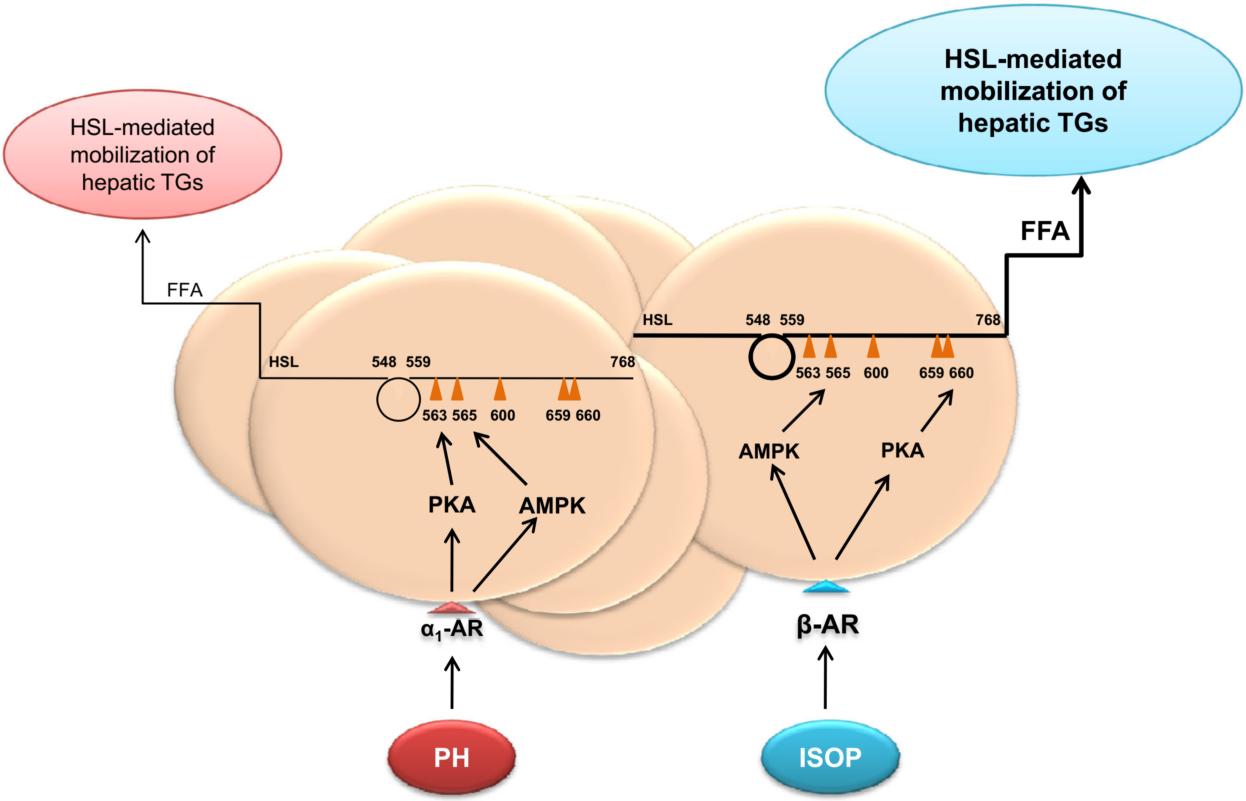

Fig. 7. Hypothetical model summarizing the impact of α_1- and β-AR agonists on HSL phosphorylation in the white adipose tissue and the subsequent hepatic TG mobilization. The present data indicated that exposure to phenylephrine (PH, α_1-AR agonist) activated the cAMP-PK (PKA) resulting in HSL phosphorylation at Ser563, whereas the activation of PKA, induced by isoprenaline (ISOP, $\beta_{1/2}$-AR agonist) led to phosphorylation of HSL at Ser660. Both, PH and ISOP, also activated the AMP-activated kinase (AMPK), which is considered to block the PKA-dependent activation of HSL in adipocytes, when HSL phosphorylation occurs at Ser563, while it is preserved when it occurs at Ser660. The current data confirm the lesser significance of α_1-ARs in the HSL-dependent lipolysis in adipocytes compared to that of β-ARs [42,44]. FFA: free fatty acids.

acids derived from TG β-oxidation are a major source of energy. It is plausible that the rapid reduction in serum TG levels observed in mice following treatment with AR agonists represents an immediate uptake of plasma TRLs by energy craving tissues in treated mice. Circulating TRLs serve as an immediate source of

FFA. However, since circulating TRLs represent a limited supply, HSL activity needs to be stimulated in order to mobilize additional intracellular deposits of TGs for sustained energy production in the W.A.T.

In addition, given the complex and multifactorial regulation of TG homeostasis, it is possible that AR stimulation by PH and ISOP influences numerous and diverse processes responsible for the observed reduction in serum TG levels. For example, PH and ISOP treatment could affect dietary lipid absorption, their packaging into chylomicrons, the processing of these chylomicrons in plasma via lipoprotein lipase and their subsequent clearance from the circulation by the LDL-r, the tissue deposition and mobilization of these TGs once they reach the respective tissues, their combustion via β-oxidation of fatty acids, and their shuttling between VLDL/LDL and HDL via CETP [25,26,30]. The precise effects of AR stimulation on these mechanisms need further investigation.

The present data indicated that stimulation of α_1- or $\beta_{1/2}$-ARs resulted in the up-regulation of several genes holding determinant roles in the fate of TGs [12,13,27,28,39,40]. In particular, AR agonists stimulated the hepatic expression of genes encoding factors involved in TG metabolism and clearance, including *Lpl*, *Nr4A*, *Mttp*, *Dgat2*, *Ces3*/*Tgh*, and *Cd36* [27,28]. These genes, with the exception of *Ces3*/*Tgh* and *Dgat2,* were also increased in the W.A.T. *Dgat1* was up-regulated only by ISOP and *Aadac* only by PH in the W.A.T. It is of interest also to note that both AR agonists activated HSL in the W.A.T. and reduced TG concentration in this tissue, indicating an increased TG hydrolysis rate [12,13,27,28,39–41]. Treatment of mice with either PH or ISOP promotes a considerable decrease in serum TG levels. Notably, ISOP results in a more significant effect. Real-time PCR analysis indicated that both agonists induce *Lpl* expression, indicating that the suppressive effect of ISOP on serum TG levels could be mainly due to increased *Lpl* expression; the nuclear receptor NR4A may have triggered the ISOP-induced Lpl up-regulation, while the effect of PH on TG is mediated by downstream to LPL events involved in the clearance of TG-rich lipoproteins, such as enhanced holoparticle uptake by the LDL-r [26]. ISOP treatment significantly increased hepatic VLDL-TG secretion, while PH had no effect, suggesting that mobilization and secretion of hepatic TGs are not a factor in the observed decrease in serum TG levels shown in PH-treated mice.

Although the above findings are strongly indicative for the role of AR agonists in the regulation of factors determining the fate of TGs in the body, future studies should be designed to pinpoint the AR agonist-induced alterations in the aforementioned proteins, focusing mainly in alterations at enzyme activity levels. It is well-established that HSL activity is highly regulated by adrenergic stimulation followed by PKA and AMPK activation [42]. In cases where catecholamines are physiologically elevated in humans (i.e,. during physical exercise), the level of HSL phosphorylation at Ser563 and Ser660 (PKA regulatory sites) is increased in both, skeletal muscles and adipose tissue. This induced phosphorylation results in HSL activation. FFA released from the enzymatic lipolysis of W.A.T triglycerides enters the hepatocytes where they are converted into triglycerides that will be eventually incorporated into nascent VLDL particles [43]. Epinephrine, an α/β-AR agonist, is known to induce phosphorylation of HSL at Ser563 and Ser660 to the same extent. Our present data indicate that PH and ISOP differ from epinephrine in that they selectively promote phosphorylation of either sites. Specifically, although PH induced HSL phosphorylation at Ser563 in the W.A.T. compared to controls (Fig. 4D), ISOP increased HSL phosphorylation at Ser660 in this tissue (Fig. 4D). Given that phosphorylation at both residues is required for a significant induction of the HSL activity, and based on the VLDL-TG secretion data (Fig. 2), we hypothesize that the PH-induced Ser563 phosphorylation may be a weaker inducer of HSL activity, thus resulting to less FFAs available for VLDL production compared to those following the ISOP-induced Ser660 phosphorylation (Fig. 7). In support of our hypothesis is the report that epinephrine (α/β-AR agonist), also activates the AMP-activated kinase (AMPK), which is considered to block the PKA-dependent activation of HSL in adipocytes, when HSL phosphorylation occurs at Ser563, while it is preserved when it occurs at Ser660. Our data are in line with previous findings indicating the lesser significance of α_1-ARs in the HSL-dependent lipolysis in adipocytes compared to that of β-ARs [42,44]. Clearly, additional enzymatic studies are essential to verify this hypothesis.

The increased fatty acid β-oxidation in the hepatic mitochondria also profoundly contributes to the α_1-AR–induced decline in serum TG steady-state levels, as PH led to an up-regulation of hepatic *Acadm* that encodes the rate-limiting enzyme in this metabolic pathway [12]. In the W.A.T., fatty acid β-oxidation does not appear to participate in PH- and ISOP-induced decline in serum TG levels as both AR agonists had no effect on *Ppar*α and *Ppar*γ expression, whereas PH repressed *Acadm* in this tissue (Fig. 4C).

Notably, PH and ISOP significantly up-regulated hepatic Hnf4α that holds determinant roles in a regulatory network required for the maintenance of the hepatocyte phenotype and the regulation of several metabolic genes involved in lipid homeostasis. It is of interest to also note that Hnf4α is acting in a coordinating fashion with the transcription regulators, Pparα and Pparγ, on their downstream target genes encoding factors important in fatty acid metabolism [9,12,45]. In both cases of AR agonists, the *Hnf4α* induced expression appears to be mediated by the activation of several phosphatase- and ATPase-linked signaling pathways, as pre-treatment of hepatocytes with the inhibitor of these enzymes, NaOV, drastically prevented the up-regulating effect of PH and ISOP on *Hnf4α*. The involvement of the α_1/β-AR/cAMP/PKA signaling pathway in this induction is also indicated by the fact that the PKA inhibitor, H89, restricted the drug-induced effect on *Hnf4α*.

It is well documented that the insulin/PI3k/AKT/FoxO1 signaling pathway regulates several lipogenic genes involved in TG synthesis [46,47]. Inactivation of this signaling pathway was detected following stimulation of α_1-ARs with PH or $\beta_{1/2}$-ARs with ISOP, suggesting that this effect may be responsible, at least in part, for the strong reduction in serum TG steady-state levels that are observed following treatment with these AR agonists.

Conclusion

The present data indicate that stimulation of α_1- or $\beta_{1/2}$-AR can efficiently reduce serum TRL levels via stimulation of TG hydrolysis, transport, metabolism and clearance, as well as inhibition of hepatic TG synthesis. Given that stress response includes adrenoceptor stimulation, our data further support that the stress-induced changes in serum TG levels are mediated by α_1- and $\beta_{1/2}$-ARs [4] in a PPARα-independent fashion, further supporting that PPARα activators, such as fibrates, may not be effective in the treatment of stress-related hypertriglyceridemia. Additional research may identify these PPARα-independent triggers providing alternative pharmacological targets for new pharmacological entities that may complement current therapies.

Materials and methods

Animals

Adult male *Ppara*-null mice [48,49], 7–8 weeks old, grown on the 129/SV background and strain-matched wild-type littermate controls, raised at NIH Animal Center, were used in this study. All mice followed a NIH-31 rodent chow-based diet (Zeigler, Gardners, PA) and had an *ad libitum* continuous access to drinking water. Five mice per cage were housed under a standard 12-h light, 12-h dark cycle, and all mice were monitored daily in order to detect outward signs of distress or adverse health effects. All studies involving experimental animals were carried out in accordance with the Institute of Laboratory Animal Resources guidelines and were approved by the National Cancer Institute Animal Care and Use Committee.

Drugs and treatment

Phenylephrine hydrochloride (Sigma-Aldrich; 2 mg·kg^{-1} i.p.; PH) and isoprenaline hydrochloride (Sigma-Aldrich; 2 mg·kg^{-1}, i.p.; ISOP) were dissolved in normal saline and administered two to three times a day and for four consecutive days (dosing regimen: Total 2-3-3-2 = 10 injections), in order to stimulate α_1-ARs and $\beta_{1/2}$-ARs, respectively. The selection of the dosing schedule of adrenergic receptor agonists was based on the literature to achieve sufficient stimulation of the adrenergic receptors [50]. The controls received normal saline and mice were not fasted during treatment. Two hours after the last drug treatment (3–4 p.m.), mice were killed by carbon dioxide asphyxiation and trunk blood was collected in BD Microtainer Serum Separator Tubes (Becton, Dickinson and Company, USA) for biochemical and hormonal analyses. Liver and white adipose tissue (W.A.T.) samples were dissected for total RNA, cellular and nuclear protein extraction and were kept along with serum samples at −80 °C until assayed. Each treatment group included five to six animals and the findings were confirmed by three different experiments.

Quantitative real-time PCR

Total RNA was isolated from the liver and W.A.T. using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The concentration of total RNA was determined spectrophotometrically. Quantitative real-time PCR (qPCR) was performed with cDNA generated from 1 μg total RNA using the SuperScript III reverse transcriptase kit (Invitrogen). The gene-specific primers were designed for qPCR using the PRIMER EXPRESS software (Applied Biosystems, Foster City, CA). The sequences for the forward and reverse primers used are shown in Table 3. For the real-time reactions, the SYBR Green PCR master mix (Applied Biosystems, Warrington, UK) was used. These reactions were carried out using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems). The relative mRNA expression levels were normalized to β-actin mRNA and the absolute levels were determined using the comparative threshold cycle method.

Western blot analysis

Nuclear extracts of liver samples were used for the immunoblot analysis of PPARα and hepatocyte nuclear factor

4α (HNF4α) protein expression. The NE-PER nuclear extraction kit (Pierce, Rockford, IL) was used for the preparation of these extracts. The phosphorylation of protein kinase B (Akt) and forkhead box protein O1 (FoxO1) was assessed in total cellular proteins, while the phosphorylation of cAMP-response element-binding protein (CREB) was analyzed in nuclear proteins. Drug-induced alterations at hepatic ApoE protein levels were assessed in total cellular proteins. Alterations in the phosphorylation of HSL, in total ATGL, perilipin 5 (PLIN5) and total perilipin apoprotein levels were assessed in total cellular proteins extracted from the W.A.T. The BCA protein assay (Pierce, Rockford, IL) was used for the determination of protein concentrations. Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using the following antibodies: goat polyclonal HNF4α IgG (Santa Cruz Biotechnology), rabbit polyclonal phospho-AKT IgG (Ser473; Santa Cruz Biotechnology), rabbit polyclonal phospho-FOXO1 (Ser256; Santa Cruz Biotechnology), rabbit polyclonal phospho-CREB-1 IgG (Ser133; Santa Cruz Biotechnology), and rabbit monoclonal anti-mouse ApoE IgG (Meridian USA). In addition, the rabbit polyclonal phospho-HSL (Ser563, Ser565, Ser660) and total HSL and Perilipin IgGs were also used (Lipolysis Activation Antibody Sampler kit, Cell Signaling). The goat polyclonal PLIN5 and ATGL IgGs, as well as the rabbit polyclonal AKT, FOXO1, and CREB IgGs (Santa Cruz Biotechnology) were also used. As loading control the immunoblotting with mouse β-actin, Histone-H3 and GAPDH antibodies (Santa Cruz Biotechnology) were used. The anti-rabbit, anti-goat, or anti-mouse IgG horseradish peroxidase-conjugated antibodies (Cell Signaling Technology) were used as secondary antibodies and the proteins were detected using an enhanced chemiluminescence detection kit (Thermo Scientific-Pierce, Rockford, IL). All western blot images were submitted to quantitation using the Image Processing and Analysis in Java soft ware (Image J).

Preparation of hepatocyte cultures

For the *in vitro* experiment, hepatocytes were prepared following a modified method based on a previous report [51]. In brief, for the isolation of parenchymal hepatocytes the *in situ* perfusion of the murine liver was used. The isolated hepatocytes were suspended in Williams' Medium E supplemented with L-glutamine, penicillin, and streptomycin and then, they were plated at a density of 0.80–1.0 $\times 10^6$ cells in 60 mm diameter collagen type I coated dish (BIOCOAT, Cell Environment, Becton Dickinson Labware, UK). The trypan blue dye exclusion was used to check the viability of isolated cells. Only primary hepatocytes with viability higher than 85% just before plating were cultured at 37 °C for 24 h under an atmosphere of humidified 5% CO_2 in order to allow the cells to adhere to the dish. Time and dose response experiments started 24 hours later. The cells were cultured in the presence of either AR agonists, PH or ISOP, at different doses (1–100 μM) and for a period of time raging from 4 to 36 hours. Here are presented only data from the incubation of primary hepatocytes with the AR agonists at a concentration of 25 μM for 24-h, as they clearly indicate the direct effect of PH and ISOP on *Hnf4α* expression.

Determination of post-prandial triglyceride kinetics following oral administration of olive oil

In order to compare the effect of AR-agonist treatment on the post-prandial triglyceride kinetics, groups of 6-8 mice were used. The determination of the post-prandial triglyceride kinetics was performed as previously described [52,53]. Values are expressed in mg·dL^{-1} ± standard error of the mean.

Table 3. The list of 5′ to 3′ oligonucleotide sequences used as forward and reverse primers

PPARα	CAGTGGGGAGAGAGGACAGA	AGTTCGGGAACAAGACGTTG
PPARγ	CACAAGAGCTGACCCAATGGT	AATAATAAGGTGGAGATGCAGGTTCT
HNF4α	CGGAGCCCCTGCAAAGT	ACTATCCAGTCTCACAGCCCATTC
Cyp8b1	ACGCTTCCTCTATCGCCTGAA	GTG CCTCAGACGCAGAGGAT
BAAT	ACAGGCCTGGCCCCCTTTCA	CCCATGGGGTGGACCCCCAT
ACADM	AGCTCTAGACGAAGCCACGA	GCGAGCAGAAATGAAACTCC
HSL	CCTCCAAGCAGGGCAAAGA	GCGTAAATCCATGCTGTGTGA
ATGL/PNPLA2	CCACTCACATCTACGGAGCC	TAATGTTGGCACCTGCTTCA
AADAC	ACCGCTTCCAGATGCTATTG	TGATTCCCAAAAGTTCACCA
MTTP	CGTGGTGAAAGGGCTTATTC	TCGCGATACCACAGAATGAA
DGAT1	GACGGCTACTGGGATCTGA	TCACCACACACCAATTCAGG
DGAT2	CGCAGCGAAAACAAGAATAA	GAAGATGTCTTGGAGGGCTG
LPL	TTTGGCTCCAGAGTTTGACC	TGTGTCTTCAGGGGTCCTTAG
CES3/TGH	TGGTATTTGGTGTCCCATCA	GCTTGGGCGATACTCAAACT
CD36	GCGACATGATTAATGGCACA	CCTGCAAATGTCAGAGGAAA
NR4A	ATTGAGCTTGAATACAGGGCA	GCTAGAAGGACTGCGGAGC
LDL-r	GGGAACATTTCGGGGTCTGT	AGTCTTCTGCTGCAACTCCG
β-actin	TATTGGCAACGAGCGGTTCC	GGCATAGAGGTCTTTACGGATGTC

Rate of hepatic very low-density triglyceride production in mice treated with phenylephrine or isoprenaline

In order to assess the effects of AR-agonist treatment on hepatic VLDL triglyceride secretion, 6–8 mice per treatment group were used. Briefly, treated mice were injected intraperitoneally with Triton-WR1339 at a dose of 500 $mg \cdot kg^{-1}$ b.w, using a 15% solution (w/v) in 0.9% NaCl. Triton-WR 1339 inhibits completely VLDL catabolism, as previously described [29,31]. Serum samples were collected 90 min following the injection with Triton WR 1339, in order to minimize the influence of handling stress on the tested mice. As a baseline control, serum samples were collected 1 min following the injection with the detergent. Then, serum TG levels were determined again at 90 min post-injection and linear graphs of TG concentration *vs* time were generated. The rate of VLDL triglyceride secretion (expressed in $mg \cdot dL^{-1} \cdot min^{-1}$) was calculated from the slope of the linear graphs for each individual mouse. The slopes were grouped together and plotted in a bar graph as mean ± standard error of the mean. Statistical analysis was performed using the Student *t*-test.

Determination of total hepatic cholesterol and triglyceride content

Tissue triglyceride determination was performed following the method previously described by Karavia *et al.* [53]. Results are expressed as milligram (mg) of triglycerides per gram of tissue ± standard error of the mean.

Hormonal and biochemical determinations

Serum total cholesterol levels were measured using the Cholesterol EIA kit (Wako Diagnostics, Richmond, VA) and the levels of serum non-esterified fatty acids were determined using the NEFA C, EIA kit (Wako Chemicals GmbH, Neuss, Germany).

Serum triglyceride levels were analyzed using the GPO-Trinder Kit (Sigma). In brief, the serum sample (10 μL) was diluted in 40 μL Phosphate buffered saline (PBS), and the dilute sample (7,5 μL) was analyzed for triglycerides, following the manufacturer's instructions. Triglyceride concentrations were determined spectrophotometrically at 540 nm as previously described [54].

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) [17] levels were determined using the Discrete Pak ALT and AST Reagents kits (Catachem Inc, Bridgeport, CT).

Statistical analysis

The data of the present study are presented as the mean ± SE and were analyzed using the one-way analysis of variance (ANOVA) that was followed by multiple comparisons with Bonferroni's and Tukey's least honest significant difference methods. The significance level for all analyses was set at probability of less than 0.05.

Acknowledgements

We would like to thank Dr Foteini Malliou for her valuable support and technical assistance. This research project has been co-financed by the European Union (European Regional Development Fund- ERDF) and Greek national funds through the Operational Program 'THESSALY-MAINLAND GREECE AND EPIRUS-2007–2013′ of the National Strategic Reference Framework (NSRF 2007–2013, Grant 346985/80753) and the National Cancer Institute Intramural Research Program.

Conflict of interest

The authors declare no conflict of interest.

Author contributions

MK conceived and coordinated the study. MK, KKy, TM, EX, YS, KKr, CA, AK, and FJG designed and performed the experiments, and analyzed the data. MK, KKy, and FJG wrote the paper.

References

1 Watts GF & Dimmitt SB (1999) Fibrates, dyslipoproteinaemia and cardiovascular disease. *CurrOpinLipidol* **10**, 561–574.

2 Balint BL & Nagy L (2006) Selective modulators of PPAR activity as new therapeutic tools in metabolic diseases. *EndocrMetab ImmuneDisordDrug Targets* **6**, 33–43.

3 Lemberger T, Saladin R, Vazquez M, Assimacopoulos F, Staels B, Desvergne B, Wahli W & Auwerx J (1996) Expression of the peroxisome proliferator-activated receptor alpha gene is stimulated by stress and follows a diurnal rhythm. *J Biol Chem* **271**, 1764–1769.

4 Konstandi M, Shah YM, Matsubara T & Gonzalez FJ (2013) Role of PPARα and HNF4α in stress-mediated alterations in lipid homeostasis. *PLoS ONE* **8**(8), e70675.

5 Michalik L, Auwerx J, Berger JP, Chatterjee VK, Glass CK, Gonzalez FJ, Grimaldi PA, Kadowaki T, Lazar MA, O'Rahilly S *et al.* (2006) International union of pharmacology. LXI. Peroxisome proliferator-activated receptors. *Pharmacol Rev* **58**, 726–741.

6 Rampler H, Weinhofer I, Netik A, Forss-Petter S, Brown PJ, Oplinger JA, Bugaut M & Berger J (2003) Evaluation of the therapeutic potential of PPARalpha agonists for X-linked adrenoleukodystrophy. *Mol Genet Metab* **80**, 398–407.

7 Qu S, Su D, Altomonte J, Kamagate A, He J, Perdomo G, Tse T, Jiang Y & Dong HH (2007) PPAR{alpha}

mediates the hypolipidemic action of fibrates by antagonizing FoxO1. *Am J Physiol Endocrinol Metab* **292**, E421–E434.

8 Kersten S, Seydoux J, Peters JM, Gonzalez FJ, Desvergne B & Wahli W (1999) Peroxisome proliferator-activated receptor alpha mediates the adaptive response to fasting. *J Clin Invest* **103**, 1489–1498.

9 Dongol B, Shah Y, Kim I, Gonzalez FJ & Hunt MC (2007) The acyl-CoA thioesterase I is regulated by PPARalpha and HNF4alpha via a distal response element in the promoter. *J Lipid Res* **48**, 1781–1791.

10 Graham MJ, Lee RG, Bell TA 3rd, Fu W, Mullick AE, Alexander VJ, Singleton W, Viney N, Geary R, Su J *et al.* (2013) Antisense oligonucleotide inhibition of apolipoprotein C-III reduces plasma triglycerides in rodents, nonhuman primates, and humans. *Circ Res* **112**, 1479–1490.

11 Shimamura M, Matsuda M, Yasumo H, Okazaki M, Fujimoto K, Kono K, Shimizugawa T, Ando Y, Koishi R, Kohama T *et al.* (2007) Angiopoietin-like protein3 regulates plasma HDL cholesterol through suppression of endothelial lipase. *Arterioscler Thromb Vasc Biol* **27**, 366–372.

12 Tolwani RJ, Farmer SC, Johnson KR, Davisson MT, Kurtz DM, Hinsdale ME, Cresci S, Kelly DP & Wood PA (1996) Structure and chromosomal location of the mouse medium-chain acyl-CoA dehydrogenase-encoding gene and its promoter. *Gene* **170**, 165–171.

13 Gauthier MS, Miyoshi H, Souza SC, Cacicedo JM, Saha AK, Greenberg AS & Ruderman NB (2008) AMP-activated protein kinase is activated as a consequence of lipolysis in the adipocyte: potential mechanism and physiological relevance. *J Biol Chem* **283**, 16514–16524.

14 Graham MJ, Lee RG, Brandt TA, Tai LJ, Fu W, Peralta R, Yu R, Hurh E, Paz E, McEvoy BW *et al.* (2017) Cardiovascular and metabolic effects of ANGPTL3 antisense oligonucleotides. *New Engl J Med* **377**, 222–232.

15 Johnson EO, Kamilaris TC, Chrousos GP & Gold PW (1992) Mechanisms of stress: a dynamic overview of hormonal and behavioral homeostasis. *Neurosci Biobehav Rev* **16**, 115–130.

16 Chrousos GP & Gold PW (1998) A healthy body in a healthy mind–and vice versa–the damaging power of "uncontrollable" stress. *J Clin Endocrinol Metab* **83**, 1842–1845.

17 Friedman TC, Mastorakos G, Newman TD, Mullen NM, Horton EG, Costello R, Papadopoulos NM & Chrousos GP (1996) Carbohydrate and lipid metabolism in endogenous hypercortisolism: shared features with metabolic syndrome X and NIDDM. *Endocr J* **43**, 645–655.

18 Rosmond R, Dallman MF & Bjorntorp P (1998) Stress-related cortisol secretion in men: relationships with abdominal obesity and endocrine, metabolic and hemodynamic abnormalities. *J Clin Endocrinol Metab* **83**, 1853–1859.

19 Karavanaki K, Tsoka E, Liacopoulou M, Karayianni C, Petrou V, Pippidou E, Brisimitzi M, Mavrikiou M, Kakleas K & Dacou-Voutetakis C (2008) Psychological stress as a factor potentially contributing to the pathogenesis of Type 1 diabetes mellitus. *J Endocrinol Invest* **31**, 406–415.

20 Golden SH (2007) A review of the evidence for a neuroendocrine link between stress, depression and diabetes mellitus. *Curr Diabetes Rev* **3**, 252–259.

21 Ware WR (2008) High cholesterol and coronary heart disease in younger men: the potential role of stress induced exaggerated blood pressure response. *Med Hypotheses* **70**, 543–547.

22 Depke M, Fusch G, Domanska G, Geffers R, Volker U, Schuett C & Kiank C (2008) Hypermetabolic syndrome as a consequence of repeated psychological stress in mice. *Endocrinology* **149**, 2714–2723.

23 Koch FS, Sepa A & Ludvigsson J (2008) Psychological stress and obesity. *J Pediatr* **153**, 839–844.

24 Astrup A & Lundsgaard C (1998) What do pharmacological approaches to obesity management offer? Linking pharmacological mechanisms of obesity management agents to clinical practice. *Exp Clin Endocrinol Diabetes* **106 Suppl 2**, 29–34.

25 Aalto-Setala K, Fisher EA, Chen X, Chajek-Shaul T, Hayek T, Zechner R, Walsh A, Ramakrishnan R, Ginsberg HN & Breslow JL (1992) Mechanism of hypertriglyceridemia in human apolipoprotein (apo) CIII transgenic mice. Diminished very low density lipoprotein fractional catabolic rate associated with increased apo CIII and reduced apo E on the particles. *J Clin Invest* **90**, 1889–1900.

26 Ai D, Chen C, Han S, Ganda A, Murphy AJ, Haeusler R, Thorp E, Accili D, Horton JD & Tall AR (2012) Regulation of hepatic LDL receptors by mTORC1 and PCSK9 in mice. *J Clin Invest* **122**, 1262–1270.

27 Brasaemle DL (2007) Thematic review series: adipocyte biology. The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. *J Lipid Res* **48**, 2547–2559.

28 Ducharme NA & Bickel PE (2008) Lipid droplets in lipogenesis and lipolysis. *Endocrinology* **149**, 942–949.

29 Kypreos KE, Teusink B, Van Dijk KW, Havekes LM & Zannis VI (2001) Analysis of the structure and function relationship of the human apolipoprotein E in vivo, using adenovirus-mediated gene transfer. *FASEB J* **15**, 1598–1600.

30 Zannis VI, Chroni A, Kypreos KE, Kan HY, Cesar TB, Zanni EE & Kardassis D (2004) Probing the pathways of chylomicron and HDL metabolism using adenovirus-mediated gene transfer. *Curr Opin Lipidol* **15**, 151–166.

31 Kypreos KE, van Dijk KW, Havekes LM & Zannis VI (2005) Generation of a recombinant apolipoprotein E variant with improved biological functions: hydrophobic residues (LEU-261, TRP-264, PHE-265, LEU-268, VAL-269) of apoE can account for the apoE-induced hypertriglyceridemia. *J Biol Chem* **280**, 6276–6284.

32 Kypreos KE (2008) ABCA1 promotes the de novo biogenesis of apolipoprotein CIII-containing HDL particles in vivo and modulates the severity of apolipoprotein CIII-induced hypertriglyceridemia. *Biochemistry* **47**, 10491–10502.

33 Kypreos KE, Bitzur R, Karavia EA, Xepapadaki E, Panayiotakopoulos G & Constantinou C (2019) Pharmacological management of dyslipidemia in atherosclerosis: limitations, challenges, and new therapeutic opportunities. *Angiology* **70**(3), 197–209.

34 Ono M, Shimizugawa T, Shimamura M, Yoshida K, Noji-Sakikawa C, Ando Y, Koishi R & Furukawa H (2003) Protein region important for regulation of lipid metabolism in angiopoietin-like 3 (ANGPTL3): ANGPTL3 is cleaved and activated in vivo. *J Biol Chem* **278**, 41804–41809.

35 Stitziel NO, Khera AV, Wang X, Bierhals AJ, Vourakis AC, Sperry AE, Natarajan P, Klarin D, Emdin CA, Zekavat SM *et al*. (2017) ANGPTL3 deficiency and protection against coronary artery disease. *J Am Coll Cardiol* **69**, 2054–2063.

36 Lichtor T, Davis HR, Johns L, Vesselinovitch D, Wissler RW & Mullan S (1987) The sympathetic nervous system and atherosclerosis. *J Neurosurg* **67**, 906–914.

37 Guan L, Collet JP, Mazowita G & Claydon VE (2018) Autonomic nervous system and stress to predict secondary ischemic events after transient ischemic attack or minor stroke: possible implications of heart rate variability. *Front Neurol* **9**, 90.

38 Chrousos GP (2009) Stress and disorders of the stress system. *Nat Rev Endocrinol* **5**, 374–381.

39 Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ & Londos C (1991) Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. *J Biol Chem* **266**, 11341–11346.

40 Zimmermann R, Lass A, Haemmerle G & Zechner R (2009) Fate of fat: the role of adipose triglyceride lipase in lipolysis. *Biochem Biophys Acta* **1791**, 494–500.

41 Schweiger M, Schreiber R, Haemmerle G, Lass A, Fledelius C, Jacobsen P, Tornqvist H, Zechner R & Zimmermann R (2006) Adipose triglyceride lipase and hormone-sensitive lipase are the major enzymes in adipose tissue triacylglycerol catabolism. *J Biol Chem* **281**, 40236–40241.

42 Watt MJ, Holmes AG, Pinnamaneni SK, Garnham AP, Steinberg GR, Kemp BE & Febbraio MA (2006) Regulation of HSL serine phosphorylation in skeletal muscle and adipose tissue. *Am J Physiol Endocrinol Metab* **290**, E500–E508.

43 Mensenkamp AR, Havekes LM, Romijn JA & Kuipers F (2001) Hepatic steatosis and very low density lipoprotein secretion: the involvement of apolipoprotein E. *J Hepatol* **35**, 816–822.

44 Lafontan M, Barbe P, Galitzky J, Tavernier G, Langin D, Carpene C, Bousquet-Melou A & Berlan M (1997) Adrenergic regulation of adipocyte metabolism. *Hum Reprod* **12** (Suppl 1), 6–20.

45 Martinez-Jimenez CP, Kyrmizi I, Cardot P, Gonzalez FJ & Talianidis I (2010) Hepatocyte nuclear factor 4alpha coordinates a transcription factor network regulating hepatic fatty acid metabolism. *Mol Cell Biol* **30**, 565–577.

46 Altomonte J, Cong L, Harbaran S, Richter A, Xu J, Meseck M & Dong HH (2004) Foxo1 mediates insulin action on apoC-III and triglyceride metabolism. *J Clin Invest* **114**, 1493–1503.

47 Cheng Z & White MF (2011) Targeting Forkhead box O1 from the concept to metabolic diseases: lessons from mouse models. *Antioxid Redox Signal* **14**, 649–661.

48 Lee SS, Pineau T, Drago J, Lee EJ, Owens JW, Kroetz DL, Fernandez-Salguero PM, Westphal H & Gonzalez FJ (1995) Targeted disruption of the alpha isoform of the peroxisome proliferator-activated receptor gene in mice results in abolishment of the pleiotropic effects of peroxisome proliferators. *Mol Cell Biol* **15**, 3012–3022.

49 Akiyama TE, Nicol CJ, Fievet C, Staels B, Ward JM, Auwerx J, Lee SS, Gonzalez FJ & Peters JM (2001) Peroxisome proliferator-activated receptor-alpha regulates lipid homeostasis, but is not associated with obesity: studies with congenic mouse lines. *J Biol Chem* **276**, 39088–39093.

50 Konstandi M, Johnson EO, Marselos M, Kostakis D, Fotopoulos A & Lang MA (2004) Stress-mediated modulation of B(alpha)P-induced hepatic CYP1A1: role of catecholamines. *Chem Biol Interact* **147**, 65–77.

51 Seglen PO (1976) Preparation of isolated rat liver cells. *Methods Cell Biol* **13**, 29–83.

52 Karavia EA, Papachristou DJ, Liopeta K, Triantaphyllidou IE, Dimitrakopoulos O & Kypreos KE (2012) Apolipoprotein A-I modulates processes associated with diet-induced nonalcoholic fatty liver disease in mice. *Mol Med* **18**, 901–912.

53 Karavia EA, Papachristou DJ, Kotsikogianni I, Giopanou I & Kypreos KE (2011) Deficiency in apolipoprotein E has a protective effect on diet-induced nonalcoholic fatty liver disease in mice. *FEBS J* **278**, 3119–3129.

54 Kypreos KE, van Dijk KW, van Der Zee A, Havekes LM & Zannis VI (2001) Domains of apolipoprotein E contributing to triglyceride and cholesterol homeostasis in vivo. Carboxyl-terminal region 203–299 promotes hepatic very low density lipoprotein-triglyceride secretion. *J Biol Chem* **276**, 19778–19786.