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

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Abstract

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.

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Author contribution

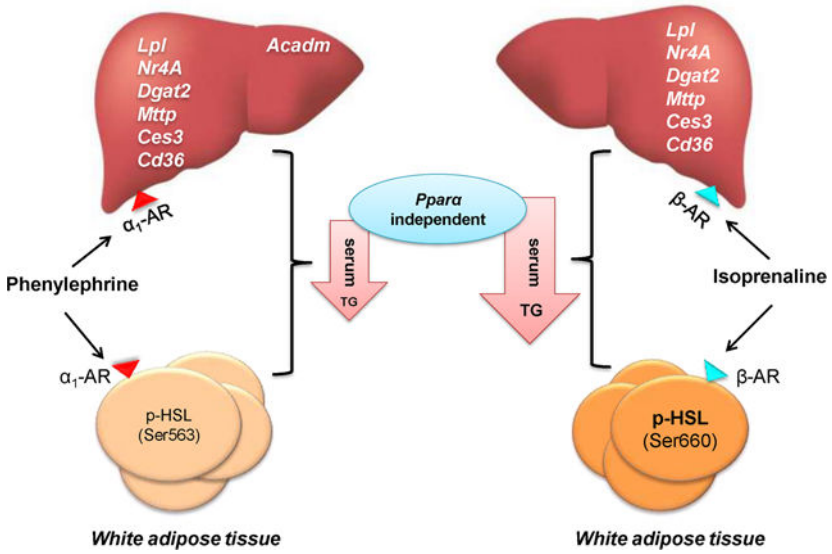
MK conceived and coordinated the study. MK, KKy, TM, EX, YS, KKr, CA, AK and FJG designed and performed the experiments, and analyzed the data. MK, KKy and FJG wrote the paper.

Conflict of interest

The authors declare no conflict of interest.

Graphical Abstract

Drugs stimulating α1- or β1/2-adrenergic receptors (AR) can efficiently reduce serum triglycerides (TG) by inducing various genes in the liver and white adipose tissue (WAT) that regulate TG hydrolysis, transport, metabolism and clearance in a PPARα-independent mechanism. β1/2-AR induced hormone-sensitive lipase (HSL) phosphorylation at Ser660 in the WAT appears to play a central role in the isoprenaline-mediated mobilization of TGs from the liver and their reduction in serum.



Keywords

Triglycerides; PPARα; Adrenergic receptors; TRLs; hypertriglyceridemia; LDL

Introduction

Hypertriglyceridemia is a major pathological feature of metabolic syndrome, which is associated with accumulation of triglyceride-rich lipoproteins (TRLs) in circulation. Patients with elevated serum TRLs are at high risk for cardiovascular and renal disease, as well as for steatohepatitis and other disorders. To this 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 for 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 rage 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 has been also 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–1C). Beta-AR stimulation decreased only serum TG and FFA concentration, whereas it had no effect on total cholesterol levels (Fig. 1A–1C). Interestingly, stimulation of ARs also suppressed serum TG steady state levels in *Ppara*-null mice thus indicating a PPARα-independent 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 *Pparα*-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 (Fig. 1A & 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. 3A). In contrast, the expression of *Atgl/Pnpla2, Hsl* and *Aadac* mRNAs were suppressed by PH in this tissue (Fig. 3A and 3B). *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. 4A). *Hsl* and *Atgl/Pnpla2* mRNA and protein were not affected by either PH or ISOP in this tissue (Fig. 4A and 4D). 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).

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).

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 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 signalling 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 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 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 of 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 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 is not a factor in the observed decrease of 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 afore-mentioned 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 coming from the enzymatic lipolysis of W.A.T triglycerides enter to 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 of serum TG levels as both AR-agonists had no effect on *Ppara* and *Pparγ* 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 also to 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 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 of 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 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; 2mg/kg i.p.; PH) and isoprenaline hydrochloride (Sigma-Aldrich; 2mg/kg, i.p.; ISOP), were dissolved in normal saline and administered twice 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 hrs after the last drug treatment (3–4p.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 ^{0}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) was 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 heoatocytes with viability higher than 85% just before plating were cultured at 37^0C 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 4–36 hours. Here are presented only data from the incubation of primary hepatocytes with the AR-agonists at a concentration of 25μM 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 ± standard error of the mean.

Rate of hepatic very low density (VLDL) 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/kg 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/dl/min) 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 analysed using the GPO-Trinder Kit (Sigma). In brief, the serum sample (10μl) was diluted in 40 μl Phosphate buffered saline (PBS), and the dilute sample (7,5μl) 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 analysed using the one-way analysis of variance (ANOVA) that was followed by multiple comparisons with Bonferonni's and Tuckey's least honest significant difference methods. The significance level for all analyses was set at probability of less than 0.05.

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Abbreviations:

AADAC	arylacetamide deacetylase
ACADM	acyl-CoA dehydrogenase
ACOX	acyl-CoA oxidase
ACOT	acyl-CoA thioesterase

AKT	protein kinase B
ALT	alanine aminotransferase
AST	aspartate aminotransferase
ApoE	apolipoprotein E
AR	adrenergic receptor
ATGL	adipose triglyceride lipase
BBAT	bile acid CoA
CREB	cAMP-response element-binding protein
cAMP	cyclic AMP
CD36	cluster of differentiation 36
CES3/TGH	carboxylesterase 3
DGAT	diacylglycerol O-acyltransferase
EIA	Elisa
FFA	free fatty acids
FoxO1	forkhead box protein O1
HNF4α	hepatocyte nuclear factor 4α
HDL	high density lipoprotein
HSL	hormone sensitive lipase
ISOP	isoprenaline
MTTP	microsomal triglyceride transfer protein
NEFA	non-esterified fatty acids
Nr4A	nuclear receptor 4a
LPL	lipoprotein lipase
LDL-r	low density lipoprotein receptor
VLDL	very low density lipoprotein
PCSK9	proprotein convertase subtilisin/kexin type 9
PH	phenylephrine
PI3k	phosphatidylinositol 3-kinase
PKA	protein kinase A

PPARα	peroxisome proliferator-activated receptor-α
PCR	polymerase chain reaction
RIA	radioimmunoassay
TG	triglycerides
TRLs	triglyceride-rich lipoproteins
W.A.T.	white adipose tissue

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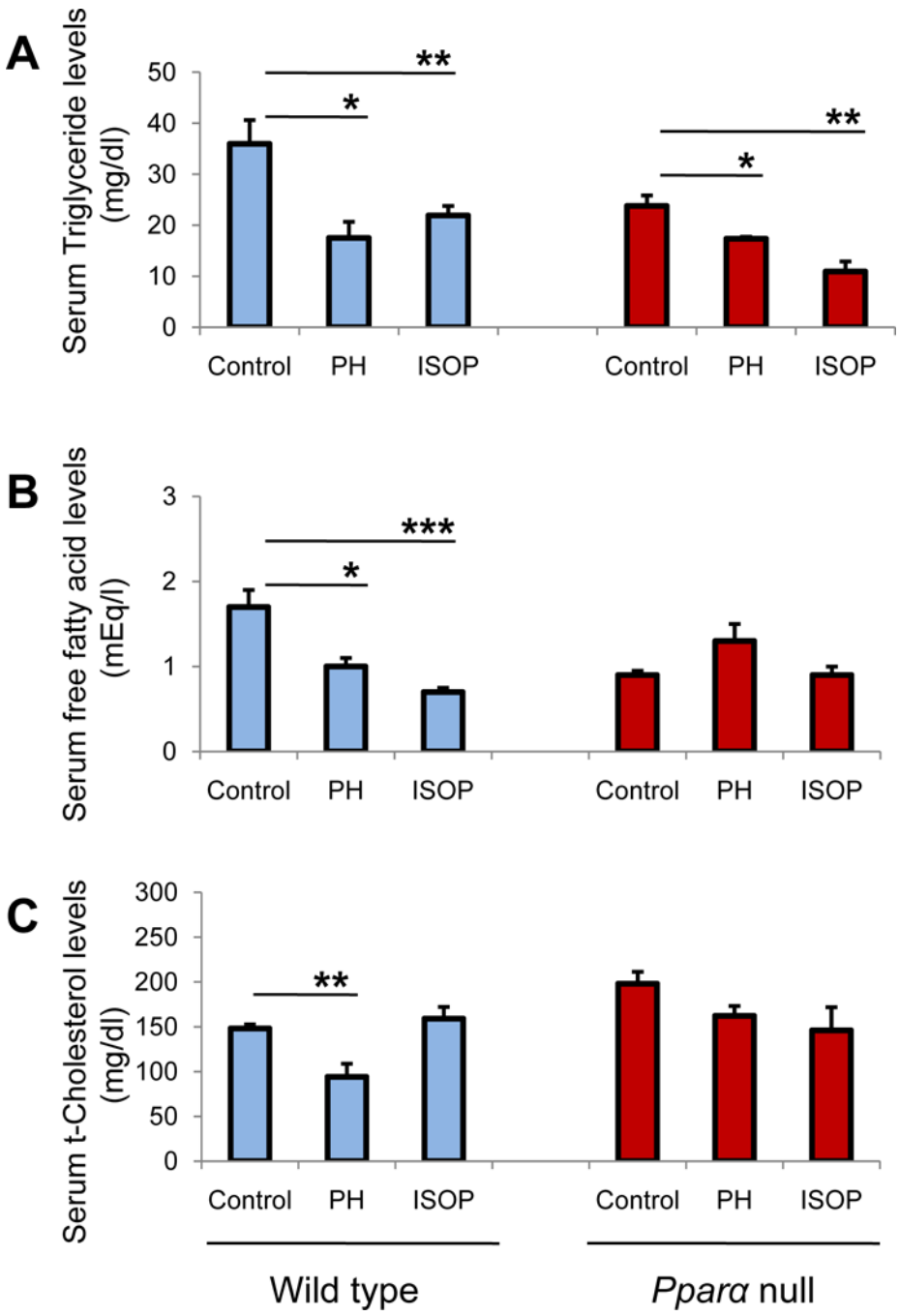

Fig. 1.

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.

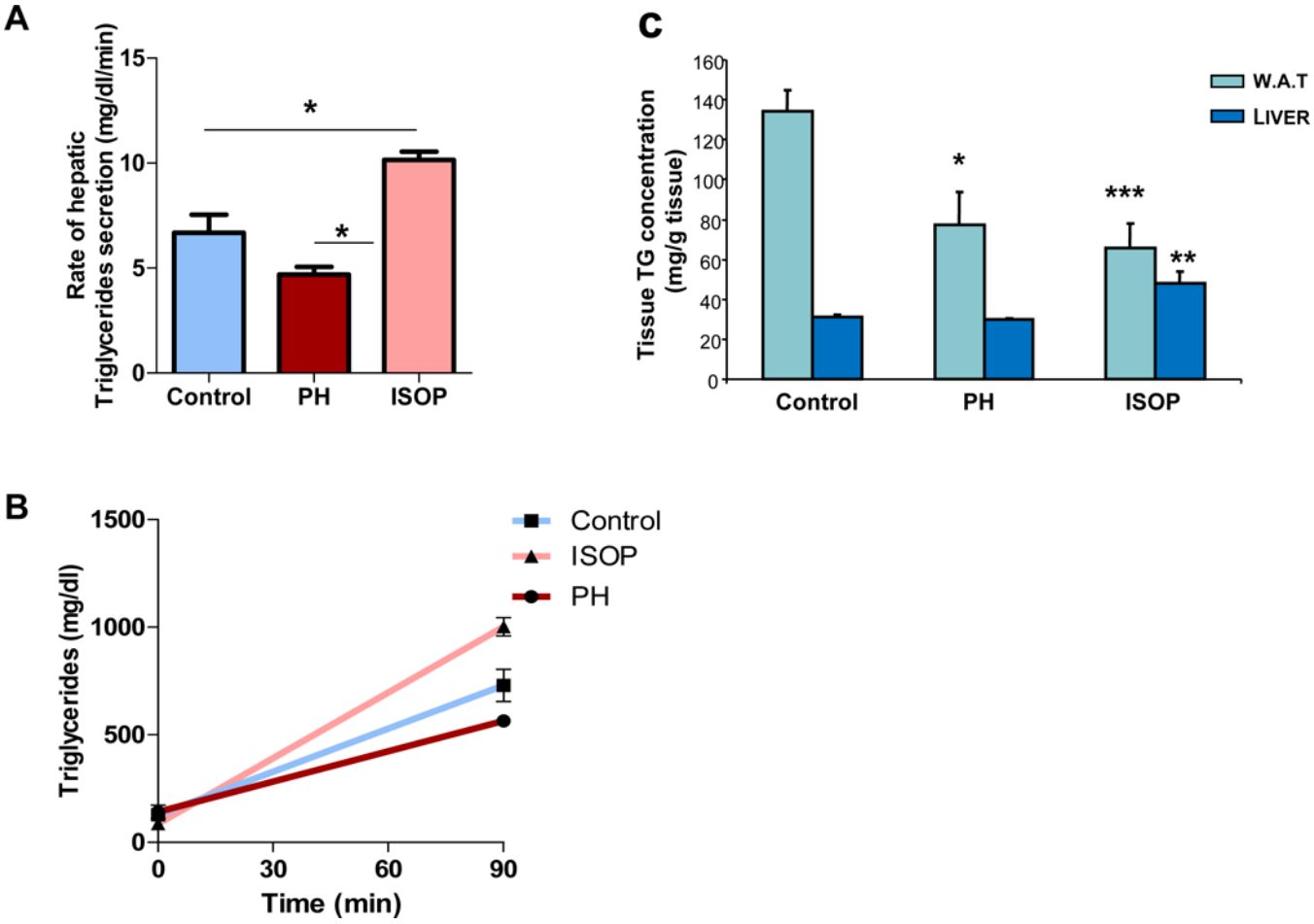

Fig. 2.

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 Bonferonni's test. All experiments were performed as described in Materials and Methods. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

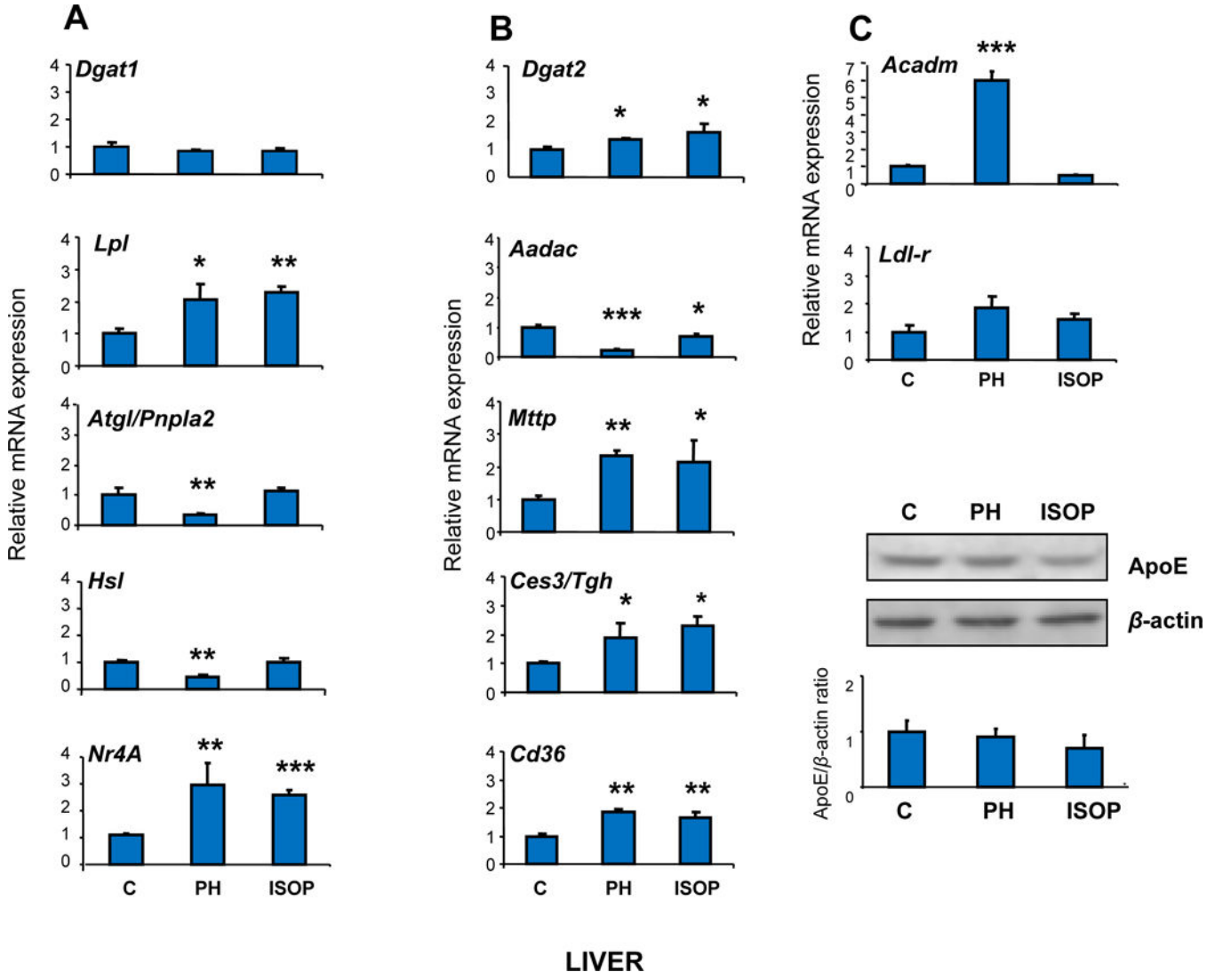

Fig. 3. Adrenergic receptor-mediated effect on hepatic factors regulating TG serum levels.

(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 Bonferonni's test. *P < 0.05, **P < 0.01, ***P < 0.001.

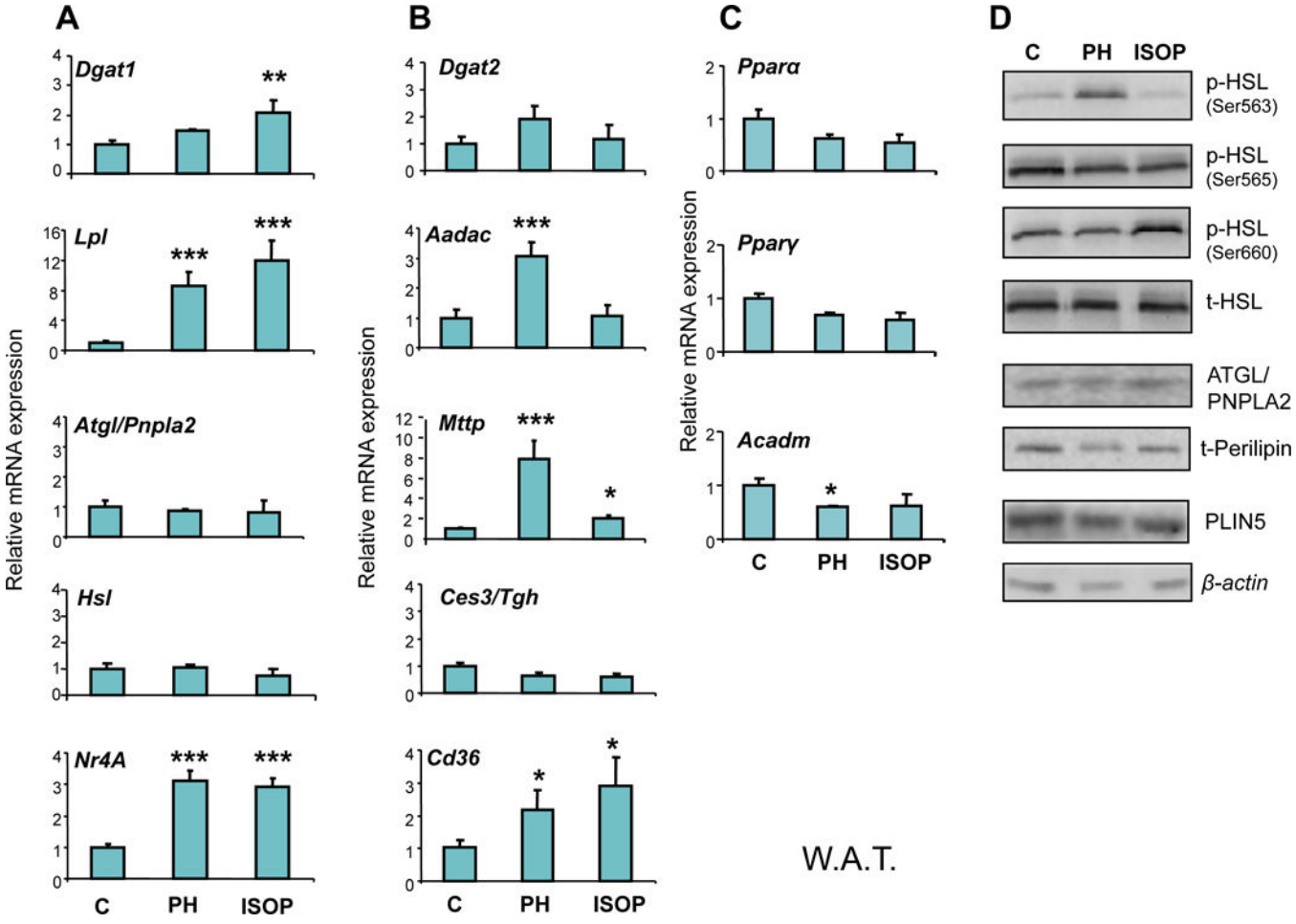

Fig. 4.

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 Bonferonni'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.

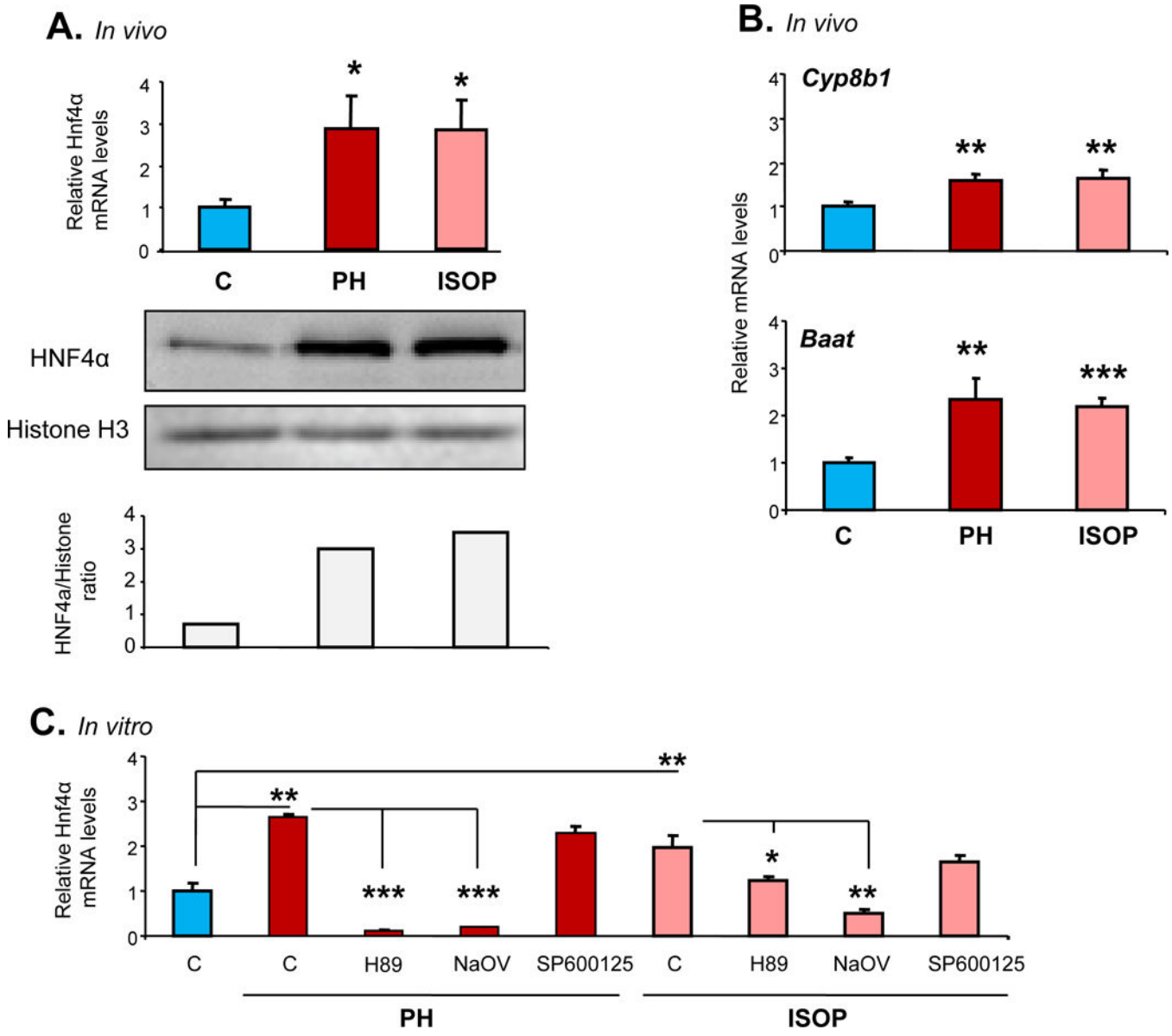

Fig. 5.

The effect of adrenergic receptor-related pathways on *Hnf4a* expression. (A) Following treatment with AR-agonists, hepatic *Hnf4a* mRNA levels were analysed 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:10μM and duration of incubation: 24 hrs). 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 Bonferonni's test. $^{*}P < 0.025$, $^{**}P < 0.01$, $^{***}P < 0.001$.

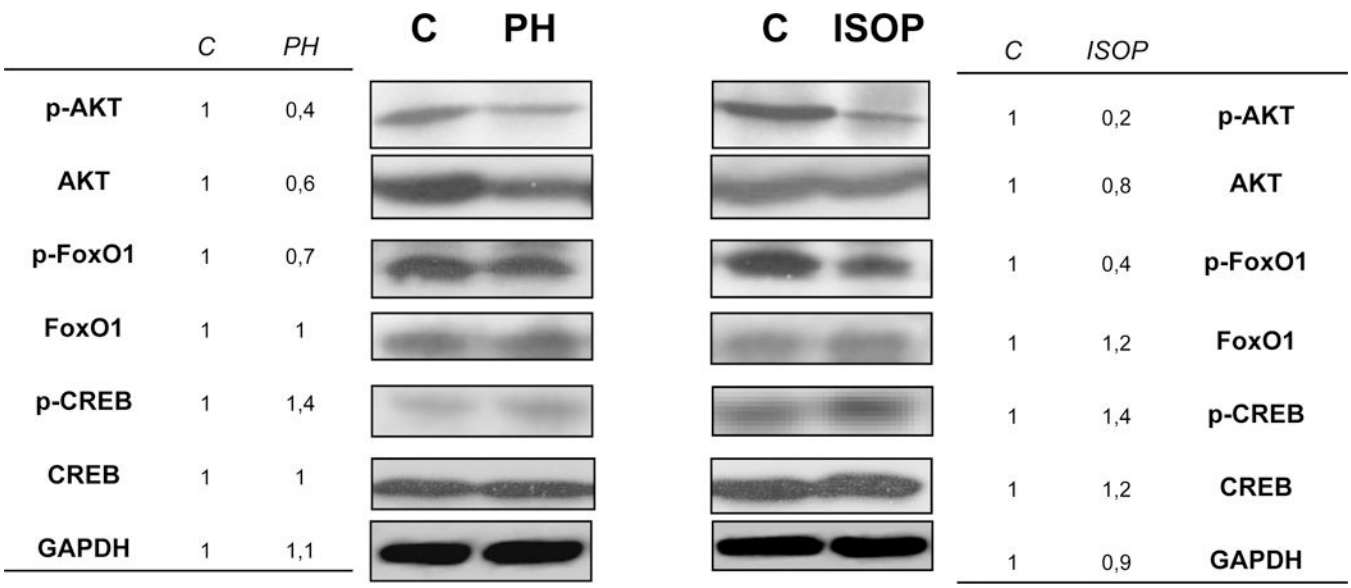

Fig. 6.

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.

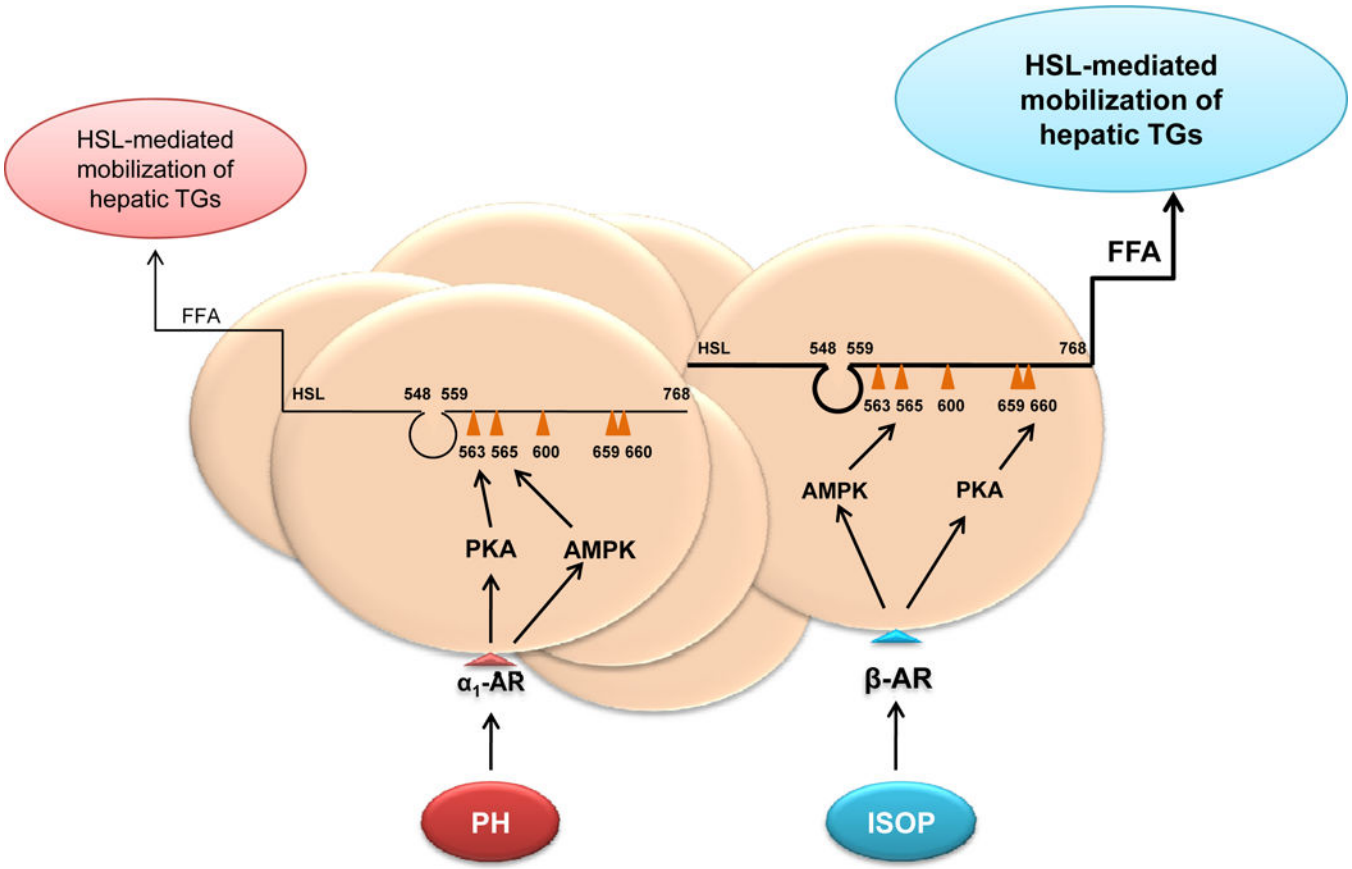

Fig. 7.

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 activation of PKA, the 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.

Table 1.

AR-induced alterations in serum ALT and AST concentration

	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

Adrenergic receptor (AR); Alanine aminotransferase (ALT), aspartate aminotransferase (AST), phenylephrine (PH), isoprenaline (ISOP), (wild type, n=20; *Ppara*-null, n=15).

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.

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