Role of PPARα and HNF4α in Stress-Mediated Alterations in Lipid Homeostasis

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Abstract

Stress is a risk factor for several cardiovascular pathologies. PPARα holds a fundamental role in control of lipid homeostasis by directly regulating genes involved in fatty acid transport and oxidation. Importantly, PPARα agonists are effective in raising HDL-cholesterol and lowering triglycerides, properties that reduce the risk for cardiovascular diseases. This study investigated the role of stress and adrenergic receptor (AR)-related pathways in PPARα and HNF4α regulation and signaling in mice following repeated restraint stress or treatment with AR-agonists administered prior to stress to block AR-linked pathways. Repeated restraint stress up-regulated Pparα and its target genes in the liver, including Acox, Acot1, Acot4, Cyp4a10, Cyp4a14 and Lipin2, an effect that was highly correlated with Hnf4α. In vitro studies using primary hepatocyte cultures treated with epinephrine or AR-agonists confirmed that hepatic AR/cAMP/PKA/CREB- and JNK-linked pathways are involved in PPARα and HNF4α regulation. Notably, restraint stress, independent of PPARα, suppressed plasma triglyceride levels. This stress-induced effect could be attributed in part to hormone sensitive lipase activation in the white adipose tissue, which was not prevented by the increased levels of perilipin. Overall, this study identifies a mechanistic basis for the modification of lipid homeostasis following stress and potentially indicates novel roles for PPARα and HNF4α in stress-induced lipid metabolism.

Introduction

The frequency of obesity related health risks, including hypertension, insulin resistance, type 2 diabetes, dyslipidemia, atherosclerosis, and cardiovascular disorders are increasing in Western societies [1]. Chronic stress is considered a risk factor for atherosclerosis, coronary artery disease and the entire spectrum of metabolic syndrome X, including visceral obesity, insulin resistance, dyslipidemia, dyscoagulation, and hypertension. The role of stress in the development of these pathologies is mainly attributed to disturbances in lipid and carbohydrate metabolism [2–9]. In particular, the chronic stress-induced hyperlipidemia, which is characterised by elevated plasma levels of cholesterol, low-density lipoproteins, triglycerides and low levels of high-density lipoprotein, has been connected with increased incidence of atherosclerosis, myocardial infarct and congestive heart failure [2,3,5,10].

Circulating epinephrine and norepinephrine released from the adrenal medulla predominantly sets the tone of sympathetic system. Peripheral organs, receive sympathetic innervation with post-ganglionic neurons releasing norepinephrine [5]. These biogenic amines, along with glucocorticoids, major effectors of the stress system, inhibit glucose uptake, fatty acid storage, protein synthesis at storage sites, and stimulate the release of energy substrates, including glucose, amino acids and free fatty acids from muscle, adipose tissue and liver [2,11,12].

Peroxisome proliferator-activated receptors (PPARs), members of the nuclear receptor superfamily, directly regulate lipid, transport, storage and metabolism, glucose metabolism, adipogenesis, and inflammatory responses; they also modulate carcinogenesis [13,14] Most notably, PPARs act as lipid sensors that translate changes in lipid/fatty acid levels into metabolic activity, leading to either fatty acid catabolism or lipid storage [15–17]. PPARs have been associated with obesity and type 2 diabetes [18]. In particular, PPARα, which is mainly expressed in the liver, kidney, and heart, is critical in lipid homeostasis by directly regulating genes involved in fatty acid uptake, ω-oxidation and β-oxidation [13,18]. PPARα agonists, such as the fibrate class of drugs, are effective in the treatment of dyslipidemia, where they raise HDL and reduce serum triglycerides, properties that decrease incidence of atherosclerosis and reduce risk for the development of cardiovascular disorders [19–21]. It is worth noting that the FIELD trial [22] and ACCORD Lipid study [23] revealed that fibrates reduced nonfatal coronary events in patients at risk for cardiovascular disease, including those with type 2 diabetes. The significant benefit of the fenofibrate-simvastatin combination therapy over statins alone regarding the incidence of major cardiovascular events in patients with atherogenic ‘mixed’ dyslipidemia (moderate hypertriglyceridemia and low HDL-cholesterol) was especially important [23]. Moreover, there is accumulating evidence supporting a direct protective role for
PPARα agonists in cardiomyocytes via the PPARα/IGF-1 pathway, an effect though, needing further investigation in humans [19–21,24].

It was previously reported that exposure to acute restraint stress and glucocorticoids up-regulated PPARα in rats [25]. The present study investigated the role of repeated restraint stress and the major components of the stress system, glucocorticoids and adrenergic receptors on PPARα regulation. Emphasis was given on the role of adrenergic receptor-linked pathways, employing wild type and Ppara-null mice. The data showed that exposure to psychophysiological stress modulated PPARα activity followed by alterations in serum lipid markers.

**Materials and Methods**

**Animals**

Adult male SV129 and Ppara-null mice [26,27] were used in this study. Mice were fed NIH-31 rodent chow (Zeigler, Gardners, PA) ad libitum with continuous access to fresh drinking water and were housed up to five per cage under a standard 12-h light, 12-h dark cycle. Mice were monitored daily for outward signs of distress or adverse health effects. All animal studies were carried out in accordance with Institute of Laboratory Animal Resources (ILAR) guidelines and approved by the National Cancer Institute Animal Care and Use Committee.

**Restraint stress paradigm**

Experimental animals were exposed to restraint stress two h daily (10:00–12:00) for four consecutive days in an isolated environment [28]. Throughout the stress task, animals were restricted to adequately ventilated individual 30 ml conical plastic tubes (3×10 cm Falcon tubes). They could rotate from supine to prone position but they could not turn head to tail. Non-stressed controls were left undisturbed, but food and water were removed for 2 h to match the drinking and feeding condition of the stress group.
Drugs and treatment
Prazosin hydrochloride (Sigma-Aldrich, 20 mg/kg b.w., i.p.; Prazosin, Orion Pharma, Pfizer, 200 μg/kg b.w., s.c.; Atipamezole), an α2-AR blocker were administered 30 min prior to the stress task. Propranolol hydrochloride (Sigma-Aldrich, 10 mg/kg, i.p.; propranolol), a beta-AR blocker, was administered 15 min before the stress. Immediately after the last stress task and 2 h after the last drug treatment, mice were killed by CO2 asphyxiation and blood was collected in BD Microtainer Serum Separator Tubes (Becton, Dickinson and Company, USA) for hormonal and biochemical analyses. Liver and white adipose tissue samples were dissected for total RNA, total cellular protein, cytosolic and nuclear protein extraction. All tissue and serum samples were kept at −80°C until assayed.

Hepatocyte α2-adrenergic receptor pathways were stimulated using the α2-AR agonist, phenylephrine hydrochloride (Sigma-Aldrich; PH). Isoprenaline hydrochloride (Sigma-Aldrich; ISOP) was used to activate beta-AR-linked pathways.

Quantitative real-time PCR (qPCR)
Total RNA from livers and white adipose tissue was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The concentration of total RNA was determined spectrophotometrically. Quantitative real-time reverse transcriptase PCR (qPCR) was performed with cDNA generated from 1 μg total RNA with a SuperScript III reverse transcriptase kit (Invitrogen). 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 S1. SYBR Green PCR master mix (Applied Biosystems, Warrington, UK) was used to perform the real-time reactions, which were carried out using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems). Relative mRNA expression levels were normalized to β-actin mRNA and absolute levels determined using the comparative threshold cycle method.

Western blot analysis
Immunoblot analysis of PPARγ and HNF4α was carried out using nuclear extracts of liver samples. For the preparation of the nuclear extracts, the NE-PER nuclear extraction kit (Pierce, Rockford, IL) was used. Akt and FOXO1 phosphorylation was assessed in nuclear and cytosolic proteins, while CReB and STAT5b phosphorylation was analyzed only in nuclear proteins and that of p-p70S6K was assessed only in cytosolic proteins. Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL). Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting using the following antibodies: Rabbit monoclonal anti-mouse PPARγ (Santa Cruz Biotechnology, Santa Cruz, CA), HNF4α goat polyclonal IgG (Santa Cruz), rabbit monoclonal phospho-AKT IgG (Ser473; Santa Cruz), rabbit polyclonal phospho-FOXO1 (Ser256; Santa Cruz Biotechnology), rabbit polyclonal phospho-STAT5b IgG (Tyr694; Cell Signaling Technology) and rabbit polyclonal p-p70S6K IgG (Thr389; Cell Signaling Technology) were used. Immunoblotting with mouse β-actin, histone-H1 and -H3 antibodies (Santa Cruz Biotechnology) was used as loading control. As secondary antibodies, the anti-rabbit, anti-goat or anti-mouse IgG horseradish peroxidase conjugated antibodies (Cell Signaling Technology) were used and the proteins were detected using an enhanced chemiluminescence detection kit (Thermo Scientific-Pierce, Rockford, IL).

Preparation of hepatocyte cultures
Hepatocytes were prepared using a modified method based on a previous report [29] In brief, parenchymal hepatocytes were isolated from mice weighing 20–25 g using in situ perfusion. The isolated hepatocytes were suspended in Williams’ Medium E supplemented with L-glutamine, penicillin and streptomycin and were plated at a density of 0.80–1.0 x 10⁶ cells in 60 mm diameter collagen type I coated dish [BioCoat, Cell Environment, Becton Dickinson Labware, UK]. The viability of isolated cells was checked with trypan blue dye exclusion and those with viability higher than 85% just before plating were used. Hepatocytes were cultured at 37°C for 24 h under an atmosphere of humidified 5% CO₂ in order to allow the cells to adhere to the dish. Time and dose response experiments started 24 hours later. Primary hepatocyte cultures were treated with either corticosterone or...
the AR-agonists, epinephrine (EPIN), PH and ISOP, at different doses (1–100 μM for EPIN, PH and ISOP) and for a period of time ranging from 4–36 hours.

Hormonal and biochemical determinations

Serum corticosterone concentrations were measured using the Corticosterone EIA kit (ACE, Cayman Chemical Company, USA). The detection limit was about 40 pg/ml and the intra-assay coefficient of variation (CV) was 4.1%. Serum triglycerides were determined using the ThermoTrace kit (Melbourne, Australia). Serum total cholesterol concentration was measured with the Cholesterol EIA kit (Wako Diagnostics, Richmond, VA). The levels of non-esterified fatty acids in serum were determined using the NEFA C, EIA kit (Wako Chemicals GmbH, Neuss, Germany). Alanine Aminotransferase (ALT) and aspartate aminotransferase (AST) serum levels were measured with the DiscretePak ALT and AST Reagents kits (Catachem Inc, Bridgeport, CT). Serum catecholamine levels, norepinephrine (NE) and EPIN, were determined using the IBL-International EIA kit (Cayman Chemicals, USA).

Figure 2. In vitro evaluation of the role of glucocorticoids and hepatic adrenergic receptor-linked pathways in PPARα and target gene expression. α. Following treatment of primary hepatocyte cultures with adrenergic receptor agonists for 24 hours, Acox, Cyp4a10, Cyp4a14, Acot1, Acot4, Lipin1 and Lipin2 mRNA levels were analyzed by qPCR. β. Ppara mRNA levels were also determined by qPCR in primary hepatocytes treated with either corticosterone, epinephrine or AR-agonists for 24 hours. Primary hepatocytes were also treated with AR-agonists in combination with the JNK inhibitor, SP600125 (SP), or the PKA inhibitors, H89 or sodium orthovanadate (NaOV). Values were quantified using the comparative CT methods normalized to β-actin and are expressed as mean ± SE (n = 3–4). Experiments were repeated three times. Comparisons were between control (DMSO) and drug-treated hepatocytes. C: control, CORT: corticosterone, PH: phenylephrine (alpha-AR agonist), ISOP: isoprenaline (beta-AR agonist), EPIN: epinephrine (alpha- and beta-AR agonist), cAMP: 8-Br-cAMP. Group differences were calculated by one-way ANOVA, followed by Bonferonni’s test. * P<0.025, **P<0.01, ***P<0.001. doi:10.1371/journal.pone.0070675.g002
Normal saline + + - - - - + + + + + + Normal saline
Restraint stress - + + + + + + - - + + + + Restraint stress
All inhibitors - - - - - - + + - - + + - All inhibitors
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Figure 3. Stress-induced effect on PI3K/AKT, cAMP/PKA/CREB and GH/STAT5b signaling pathways. The evaluation of the stress effect on these pathways was conducted in nuclear and cytosolic proteins with Western blot. Histone H3 served as a loading control for nuclear proteins and β-actin for cytosolic proteins. AR: adrenergic receptor; AR-antagonists given prior to stress, blocked the up-regulating effect of stress (Fig. 1B). Since none of the AR-blockers used prevented the stress-induced Acoth up-regulation, it was likely not mediated by AR-related pathways, (Fig. 1B). Interestingly, exposure to restraint stress up-regulated in the liver the co-activator Lipin1 and retinoid X receptor alpha (RXRα) that forms heterodimers with PPARα. These effects were prevented by all AR-antagonists (Fig. 1B). In the liver of Ppara null mice, Ppara, Cyp4a10 and Acox mRNA levels were detected at markedly lower levels compared to those detected in wild type mice and restraint stress did not affect them (P<0.01, Fig. S1).

Statistical analysis
The data are presented as the mean ± SE and were analysed using one-way analysis of variance (ANOVA) followed by multiple comparisons with Bonferroni’s and Tuckey’s list honest significant difference methods. The significance level for all analyses was set at probability of less than 0.05. Moreover, correlation statistical analysis was performed using the Pearson’s coefficient correlations in order to investigate possible correlations between alterations in the relative hepatic PPARα mRNA expression and those observed in relative HNF4α mRNA levels.

Results
Up-regulating effect of stress on PPARα expression: Role of AR-linked pathways
Serum corticosterone and epinephrine levels were detected at higher levels in all stress-exposed animals compared to non-stressed controls (Table 1), indicating that all mice responded to stress [2]. The effect of stress on the expression of PPARα was determined by qPCR and western blot in the livers of wild-type mice. Restraint stress increased Ppara mRNA and PPARα protein levels (Fig. 1A) and this increase was prevented by the α1-AR antagonist, prazosin, the α2-AR antagonist, atipamezole and the beta-AR antagonist, propranolol (Fig. 1A). To test if the stress-induced Ppara expression could affect the expression of PPARα target genes, qPCR analysis of PPARα target gene mRNAs encoding enzymes critical in fatty acid homeostasis, were assessed. Stress markedly increased expression of hepatic acyl-coenzyme A oxidase (Acox), Cyp4a10, Cyp4a14, Lipin 2, cytosolic acyl-coenzyme A thioesterases (Acox)1 and Acox4 (Fig. 1B). Alpha- and beta-AR antagonists, given prior to stress, blocked the up-regulating effect of stress on Acox, Cyp4a10 and Cyp4a14 mRNAs (Fig. 1B). In the case of the stress-induced Acoth up-regulation, α2-ARs were found to have a major role as only prazosin blocked its induction (Fig. 1B). The stress-induced Lipin2 up-regulation is mediated mainly by α2- and beta-ARs as atipamezole and propranolol blocked the up-regulating effect of stress (Fig. 1B). Since none of the AR-blockers used prevented the stress-induced Acoth up-regulation, it was likely not mediated by AR-related pathways, (Fig. 1B). Interestingly, exposure to restraint stress up-regulated in the liver the co-activator Lipin1 and retinoid X receptor alpha (RXRα) that forms heterodimers with PPARα. These effects were prevented by all AR-antagonists (Fig. 1B). In the liver of Ppara null mice, Ppara, Cyp4a10 and Acox mRNA levels were detected at markedly lower levels compared to those detected in wild type mice and restraint stress did not affect them (P<0.01, Fig. S1).

Altering serum lipid indices indicative of PPARα activation
PPARα activation by stress resulted in suppression of plasma triglycerides (TG), free fatty acids (FFA) and total cholesterol levels, whereas blockade of ARs during stress, diminished the suppressive effect of stress on these lipids (Table 1). No significant changes in plasma FFA and total cholesterol concentrations were detected in Ppara-null mice following stress, again indicating a role for PPARα (Table 1). Nonetheless, it is of interest to note that the stress-mediated decrease in plasma TG levels was also detected in the plasma of Ppara-null mice (Table 1), indicating that PPARα is potentially not involved in this effect. No significant changes in serum AST, ALT, and body weights were observed following stress or drug treatment, thus revealing that the alterations observed in this study are not associated with any toxic effect or starvation (Table 1 and Table S2).

In vitro assessment of hepatocyte adrenoceptor involvement in PPARα regulation
To investigate whether the EPIN-stimulated hepatic PPARα up-regulation is due to a direct effect on hepatocyte ARs, primary hepatocytes were cultured in the presence of EPIN. The involvement of the specific AR-linked pathways in PPARα and
HNF4α regulation was assessed by treating primary hepatocytes with the AR-agonists, PH and ISOP. Stimulation of alpha1- and beta-ARs, induced Acox, Cyp4a10, Cyp4a14, Acot1 and Lipin2 mRNA levels in treated hepatocytes (Fig. 2A). Acot4 mRNA was up-regulated by only PH, and Lipin1 by only ISOP (Fig. 2A). Both AR-agonists used markedly induced Ppara expression in primary hepatocytes (Fig. 2B). Pretreatment of primary hepatocytes with the PKA inhibitor, sodium orthovanadate (NaOV, Sigma-Aldrich), prevented the PH-induced Ppara up-regulation (Fig. 2B). The ISOP-induced effect on Ppara expression was blocked by the JNK inhibitor, SP600125 (Enzo) and the PKA inhibitors, H89 (Sigma-Aldrich) and NaOV, (Fig. 2B). EPIN also markedly increased Ppara expression in hepatocytes and this effect was blocked by pretreatment with NaOV (Fig. 2B). The up-regulating effect of the cAMP/PKA pathway on Ppara expression was confirmed by treatment of primary hepatocytes with 8-Br-cAMP (Sigma-Aldrich) (Fig. 2B). Corticosterone also induced Ppara mRNA in primary hepatocyte cultures (Fig. 2B) in accordance with earlier studies [25].

**In vivo assessment of the stress-induced effect on several major signal transduction pathways related to lipid homeostasis and Pparα regulation**

The insulin/PI3k/Akt signaling pathway has a critical role in the regulation of several genes encoding critical factors in TG synthesis [30,31]. Exposure to restraint stress increased Akt phosphorylation in the liver (Fig. 3). However, this effect was not blocked by α1-AR inhibition, but only by α2- and beta-AR blockade (Fig. 3). Interestingly, the forkhead box protein O1 beta (FOXO1b) phosphorylation and that of p70S6K was not altered by stress, thus indicating that other downstream elements in the PI3k/Akt signaling pathway are involved in the stress-induced PPARα activation and synthesis of factors with essential roles in lipid homeostasis (Fig. 3). Interestingly, inhibition of α2-ARs prior to stress increased p70S6K activation, but this effect was not followed by any alteration in PPARα activation and lipid-related gene regulation (Fig. 3).

Stress also increased the cAMP response element-binding protein (CREB) phosphorylation in the liver (Fig. 3), although...
the increase was not prevented by the AR-inhibitors used in this study (Fig. 3). It is of interest also to note that restraint stress decreased the growth hormone (GH) pulse activated signal transducer and activator of transcription 5b (STAT5b) phosphorylation in the liver, which was not inhibited by the AR-antagonists used (Fig. 3).

**In vivo** and **in vitro** assessment of the role of HNF4α in the stress-induced alterations in lipid homeostasis

HNF4α and PPARα coordinate regulation of several common target genes [32]. In order to assess the involvement of HNF4α in the stress-induced up-regulation of PPARα target genes, hepatic RNA was analyzed by qPCR, and nuclear proteins quantified by Western blotting. HNF4α mRNA and protein levels were increased in the liver of restrained mice compared to non-stressed animals (Fig. 4A). The stress-induced up-regulation of Hnf4a was blocked only by the alpha1-AR antagonist, prazosin, whereas atipamezole and propranolol had no effect thus indicating a prevalent role for alpha1-AR-related pathways in the stress-induced Hnf4a up-regulation (Fig. 4A). The stress-induced Hnf4a expression triggered up-regulation of the HNF4α target genes, bile acid CoA (Baat) and Cyp8b1 (Fig. 4B). Further investigation revealed that the stress-released EPIN up-regulated Hnf4a by directly stimulating hepatocyte alpha1- and beta-ARs, as treatment of primary hepatocytes with either EPIN, PH or ISOP markedly induced hepatocyte Hnf4a expression (Fig. 4C). The PH- and ISOP-induced up-regulating effect on Hnf4a mRNA was blocked by pre-treatment with the PKA inhibitors, H89 and NaOV (Fig. 4C). The JNK inhibitor, SP600125, also prevented the ISOP-induced Hnf4a up-regulation (Fig. 4C). The EPIN-induced effect on Hnf4a was blocked by pre-treatment of the hepatocytes with either H89 or NaOV (Fig. 4C). The involvement of the cAMP/PKA signaling pathway in the regulation of Hnf4a was also apparent when the hepatocytes were treated with 8-Br-cAMP (Fig. 4C). Interestingly, corticosterone markedly increased Hnf4a mRNA in primary hepatocytes (Fig. 4C), similar to what was noted with Ppara mRNA. Pearson’s coefficient correlation revealed that the in vivo stress-induced alteration in Ppara expression is highly correlated to that observed in Hnf4a expression (Fig. S2). On the other hand, basal Hnf4a mRNA expression raged at equivalent levels in wild type and Ppara null mice, and exposure to restraint stress up-regulated Hnf4a in both animal models, indicating that this effect was not mediated by Ppara (Fig. S1 and Fig. 4A).

**In vivo** assessment of the stress-mediated effects on lipid beta-oxidation, cholesterol synthesis and LDLr expression

Exposure to stress up-regulated acyl-coenzyme A dehydrogenase (Acadm) mRNA in the mouse liver (Fig. 5A) and this effect was completely blocked only by the alpha1-AR antagonist, prazosin (Fig. 5A). In contrast, stress did not alter Acadm expression in the white adipose tissue. Stress also markedly increased the protein convertase subtilisin/kexin type 9 (Pcsk9) mRNA transcripts in the liver, an effect that was completely inhibited by all AR-antagonists used (Fig. 5B). In contrast, low-density lipoprotein receptor (Ldlr) expression was not affected by stress (Fig. 5B).

**In vivo** assessment of the role of stress in TG homeostasis

To further elucidate the mechanism underlying the suppressive effect of stress on TG serum concentration, the expression of various genes encoding factors involved in TG synthesis, metabolism and clearance were determined by qPCR and western blot analysis. The data revealed that stress increased Dgat1, Atgl/Pnpla2 and Hsl mRNA expression in hepatic tissue (Fig. 6A). The stress-induced effect on Dgat1 and Hsl, was blocked by prazosin and propranolol, and that on Atgl/Pnpla2 was blocked only by prazosin (Fig. 6A). In contrast, stress suppressed Lpl mRNA levels in the liver (Fig. 6A).

It should be noted that compared to non-stressed controls, stress increased the Dgat1, Lpl, Atgl/Pnpla2, Nr4a1, Dgat2, Aadac and Mttn mRNA transcripts in the white adipose tissue (Fig. 7A and 7B). Further investigation revealed that stress increased the HSL phosphorylation at Ser563 and Ser660, as well as perilipin apoprotein levels in the white adipose tissue (Fig. 7C), clearly confirming the involvement of stress in TG homeostasis. The
involvement of AR-related pathways in the stress-mediated alterations in TG homeostasis, was also investigated in animals treated with AR-antagonists prior to their exposure to restraint stress. The data revealed that alpha1-AR blockade by prazosin prevented the stress-induced \textit{Dgat1}, \textit{Lpl}, \textit{Atgl/Pnpla2}, \textit{Dgat2} and \textit{Aadac} up-regulation in the white adipose tissue (Fig. 7A and 7B). Both, alpha2- and beta-AR-antagonists, atipamezole and propranolol, respectively, prevented the \textit{Atgl/Pnpla2}, \textit{Dgat2} and \textit{Aadac} up-regulation in the white adipose tissue (Fig. 7A and 7B). In the case of the stress-induced \textit{Nr4a} up-regulation in the white adipose tissue the involvement of alpha2-AR-related pathways appears to be prevalent (Fig. 7A). The stress-induced HSL phosphorylation at Ser563 was blocked by propranolol (Fig. 7C), whereas that at Ser660 was blocked by prazosin (Fig. 7C). Notably, both prazosin and propranolol, prevented the stress-induced increase in perilipin apoprotein levels in the white adipose tissue (Fig. 7C).

**Discussion**

Genomic and biochemical data in the present study confirmed that stress modifies lipid homeostasis [2–10]. Serum lipid markers,
such as free fatty acids and total cholesterol, were detected at lower concentrations in stress-exposed wild-type mice compared to non-stressed controls, whereas, no significant change was observed in Pparα-null mice. These observations clearly indicate a significant role for PPARα in the stress-induced alterations in lipid homeostasis, further elucidating the regulatory mechanisms involved in the maintenance of cellular lipid balance under normal physiological conditions, psychological stress, or in the context of disease states. The role of PPARα as a cellular “lipostat”, transducing changes in cellular lipid levels to the transcriptional regulation of target genes involved in fatty acid utilization [16,33] was confirmed by the present study.

A previous investigation on the role of stress in PPARα regulation, revealed that glucocorticoids can induce expression of Ppara [25]. The present data confirmed the up-regulating effect of glucocorticoids on Ppara and further indicated the determinant role of EPIN in the stress-induced effect. Nonetheless, the role of the AR-linked pathways and, in particular, the role of alpha-ARs in the stress-induced regulation of PPARα signal transduction was not clear. Therefore, within the scope of the present study was investigation of the role of AR-related pathways, major components of the sympathoadrenal response to stress and targets of drugs used for the treatment of hypertension, asthma, angina pectoris, congestive heart failure, cardiac arrhythmias, prostatic hypertrophy, glaucoma, and depression [34,35]. In this context, it is also of interest to note that ARs hold major physiological roles, such as the regulation of carbohydrate, lipid and amino acid metabolism.

Figure 7. Stress-induced effect on TG homeostasis-related gene expression in the white adipose tissue. (A) Effect of restraint stress on the expression of genes involved in TG synthesis and lipolysis. (B) Effect of restraint stress on the expression of genes involved in TG metabolism and clearance. (C) Effect of restraint stress on the expression and activation of genes involved in TG hydrolysis. Comparisons were between controls and stress-exposed mice (alone or treated simultaneously with the AR-antagonists, prazosin, propranolol or atipamezole; black bars). 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, orphan nuclear receptor NR4A, AADAC: arylacetamide deacetylase, CD36: cluster of differentiation 36 or fatty acid transporter, CES3/TGH: carboxylesterase 3, MTTP: microsomal triglyceride transfer, PLINS: perilipin S. AR: adrenergic receptor, C: Control, Prazosin (alpha1-AR antagonist), Atipamezole (alpha2-AR antagonist), Propranolol (beta-AR antagonist), white adipose tissue (W.A.T.). Values are expressed as mean ± SE, n=5–6 per treatment group. Lanes in western blots correspond to one sample per treatment and represent one sample of three separate samples tested in different blots. *P<0.005, **P<0.01, ***P<0.001.

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metabolism in the liver, among others, but the mechanisms remain still unclear [36–39].

In vivo studies employing pharmacological manipulations of AR-related pathways using AR-antagonists given prior to stress, revealed a critical role for ARs in Pparα regulation by stress. In particular, blockade of alpha-1, alpha-2 or beta-ARs during the stress manipulation, prevented the stress-induced effect on Pparα and PPARα target gene expression. The up-regulating effect of stress on PPARα and its target genes is potentially, in part, associated with the up-regulation of RXRαβ, which forms heterodimers with PPARα. This complex binds to peroxisome proliferator response element (PPRE) in the promoter of target genes and stimulates their transcription [40]. Stress also induced Lipin1 expression, which functions as a nuclear transcriptional coactivator with peroxisome proliferator-activated receptor α thus modulating the expression of several genes involved in lipid metabolism [41].

At the signal transduction level, the insulin/PI3k/Akt signalling pathway appears to have a role in the stress-induced regulation of PPARα and genes encoding factors involved in lipid homeostasis [30,31]. Stress activated Akt in the liver via pathways not related to α1-ARs but to α2- and beta-ARs, as only atipamezole and propranolol blocked the stimulating effect of stress on Akt phosphorylation. Interestingly, activation of Akt appears to stimulate downstream elements in the PI3k/Akt pathway, other than FOXO1β and p70S6K, which mediate the up-regulating effects of stress.

In vitro stimulation of hepatectomy alpha2-AR/cAMP/PKA signalling pathway with PH, the beta-AR/cAMP/PKA and beta-AR/JNK-linked pathways with ISOP, was followed by up-regulation of PPARα and its target genes. To our knowledge, this is the report of a role for alpha2-ARs in the regulation of PPARα. In vivo, the stress-induced release of EPIN activated hepatic α1- and beta-AR/cAMP/PKA/CREB signalling pathway thus activating PPARα. It should be noted though that the AR-agonists at the doses given, did not prevent the stress-induced effect. Potentially, other central and peripheral AR-linked pathways have overridden the effect of hepatic AR-linked pathways.

Stress also decreased phosphorylation of the GH-pulse activated transcription factor, STAT3β, in the liver, an effect though that was not blocked by the AR-inhibitors used, indicating that the stress-induced inactivation of STAT3β is mediated by glucocorticoids [42] and not by central or peripheral AR-linked pathways. The suppressive effect of stress on the GH/STAT3β pathway potentially mediates the stress-induced Pparα up-regulation. The bidirectional inhibitory cross-talk between PPAR and STAT3β is well documented [43].

The stress-induced Pparα and target gene up-regulation was followed by reduced plasma T-cholesterol, TG and FFA levels. The present data are consistent with previous reports indicating that stress reduced plasma TG levels [44–47]. The effect of stress on the above mentioned lipid markers appears to be mediated mainly by stimulation of AR-linked pathways with epinephrine as it was completely blocked by AR-antagonists. It is of interest to note that stress also suppressed plasma TG levels in Ppara-null mice without affecting plasma FFA and T-cholesterol levels, thus indicating that the stress-induced suppressive effect on TG is not exclusively mediated by PPARα. Several other transcription factors can contribute to the TG lowering effects of stress, including SREBP-1c, NF-kappaB, RXRs, LXRα, FXR and HNF4α [48]. The mechanism mediating the stress- and AR-related effect on lipid homeostasis remains unclear and currently a subject of thorough investigation. TG are stored in lipid droplets mainly in adipocytes, and during lipolysis they are hydrolyzed into free fatty acids and glycerol, a sequential process involving different lipases, mainly the HSL and ATGL, which accounts for most of the HSL-independent TG hydrolase activity in white adipose tissue [49–51]. In this process, perilipin holds a key role; it is localized at the periphery of lipid droplets serving as a protective coating against lipases [49,50,52,53]. It was reported that stimulation of the cAMP/PKA signalling pathway may activate perilipin, thus leading to conformational alterations, which facilitate the exposure of lipid droplets to endogenous lipases [52].

In the cascade of events involved in the stress-induced significant decline in plasma TG levels, increased mitochondrial lipid beta-oxidation may be partly involved, as stress up-regulated Acadin, an enzyme that catalyzes fatty acid beta-oxidation [54,55]. It is of interest to note that repeated restraint stress also induced expression of the nuclear receptor HepaR α, which is central to the maintenance of hepatic transactivation and the regulation of genes involved in lipid metabolism [56,57]. Importantly, as in the case of Ppara, the hepatic alpha2- and beta-AR/cAMP/PKA signalling pathway along with that of beta-AR/JNK-linked pathway appear to mediate HepaRα up-regulation [58]. The in vivo and in vitro data indicated that the alpha2- and beta2-AR-induced HepaRα up-regulation, should be attributed to a direct stimulation of hepatic alpha2- or beta2-AR signalling pathways by EPIN. The critical role of the hepatic ARs in HepaRα regulation by stress is strengthened by the fact that stimulation of hypothalamic α1- or beta-ARs leads to suppressed plasma GH levels [59], a hormonal state connected with down-regulation of HNF4α target genes. A bidirectional cross-talk between the GH/STAT5b signalling and HNF4α is well documented. In particular, STAT5b activation has been connected with augmentation of HNF4α-dependent gene transcription, while HNF4α inhibits STAT5b transcriptional activity via inhibition of JAK2 phosphorylation [60]. In contrast, α2-ARs, expressed on pancreatic beta-cells, possess a positive control on insulin secretion [61], which in turn, via activation of hepatic mTOR signaling, has a negative control on HNF4α [62]. In addition, cytokines, which are released from immune cells following stimulation of all types of ARs have a suppressive effect on HepaRα regulation [63–65]. Based on these reports and the present findings, it is apparent that the hepatic alpha1- and beta-AR-related effects on HepaRα regulation override that of central and other peripheral AR-mediated effects. The findings of this study confirm the positive feedback regulatory mechanism involving PPARα and HNF4α that is activated when there is an increased demand for these two transcription factors to activate target genes [57].

Current evidence suggests that stress up-regulated several genes in the white adipose tissue, which are critical in the synthesis and metabolism of TG, such as the Dgat1, Dgat2, Lpl, Aci, Aduc, Mitpa and Nr4a2. The stress-induced effect on Lpl, which has dual functions of TG hydrolysis and lipid-bridging factor of receptor mediated lipoprotein uptake [52,53], is apparently mediated via alpha2-ARs. Interestingly, the stress-induced up-regulating effect on Dgat1, Dgat2, Aduc and Aci is mediated by alpha- and beta-ARs. It should be also noted that stress induced the Nr4a2 expression only via alpha2-ARs. Importantly, the stress activated HSL, one of the major enzymes contributing to TG breakdown in the white adipose tissue [66] and this effect was mediated by α1- and beta-ARs. Apparently, the stress-induced increase in perilipin concentration in the white adipose tissue, which was mediated by α1- and beta-AR-related pathways, did not prevent the effect of lipases and hydrolyses on TG. Notably stress markedly up-regulated Ppck9 that plays a major role in cholesterol synthesis, but
this increase apparently was not strong enough to affect LDLr and in turn, plasma cholesterol levels [62].

Taken together these studies revealed that short-term mild stress may be beneficial in lipid homeostasis, as it can stimulate an array of genes involved in lipid metabolism thus resulting in suppression of total cholesterol, triglycerides and free fatty acid plasma levels. This effect could be attributed to the overall adaptive mechanisms that are triggered by mild stress in order to enable the organism to maintain homeostasis [67]. In support of this notion are the findings of several previous studies indicating the beneficial impact of short-term mild stress in various disease states including cancer, immune diseases [68], Alzheimer’s disease [69] and in situations that trigger oxidative damage [70]. Nonetheless, the long term detrimental effects of chronic stress should not be underestimated as it has long been considered as a crucial causative factor in the development of a myriad of diseases including those related to lipid and carbohydrate metabolism, with cardiovascular disorders and obesity holding significant portions of the stress-induced morbidity [2,3,5,10]. From a clinical point of view, the challenge is the management of stress and not to let the sympathetic nervous system stay chronically aroused. A critical role for beta-ARs in PPARα activation was also well defined by previous [71] and the present data, which revealed a role for alpha1-ARs in Pparg regulation. These findings could be the basis for the design of new molecules targeting these receptors for PPARα activation. It should be also underscored the alpha- and beta-AR mediated stress-induced suppression in plasma TG levels, an effect that could be mainly attributed to increased TG lipolysis, metabolism and clearance. It is conceivable though that a thorough investigation of the mechanisms involved should be the centre of a new study. Overall, this study sheds more light on the complex pathophysiological states related to the stress-induced lipid disturbances and potentially suggests innovative therapeutic approaches indicating new targets in the regulation of PPARα and lipid homeostasis.

Supporting Information

Table S1 List of 5′ to 3′ oligonucleotide sequences used as forward and reverse primers.

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Table S2 Alterations in the body weight after stress. Body weight values are expressed in g.

**Figure S1** Stress-induced effect on PPARα expression in Pparα null mice. A. PPARα mRNA and protein levels were examined in the liver of Pparα null mice followed restraint stress or treatment with AR agonists. B. Hepatic Acox and Cyp4a10 mRNA levels were also analyzed by qPCR in these mice. C. HNF4α mRNA levels were examined in the liver of Pparα-null mice followed restraint stress or treatment with AR agonists. C: controls; PH: phenylephrine (α1-agonist), DEXT: dexmedetomidine (α2-agonist), ISOP: isoprenaline (β-agonist). Values were quantified using the comparative CT methods normalized to β-actin and are expressed as mean ± SE (n = 8–10). Comparisons took place between controls and stress-exposed or drug-treated mice. Group differences were calculated by one-way ANOVA, followed by Bonferroni’s test. * P<0.025, **P<0.001.

**Figure S2** Correlation between stress- and AR-induced alterations in PPARα and HNF4α expression. A. Correlation between stress-induced alterations in PPARα and HNF4α relative mRNA expression. B. Correlation between β1-AR-induced alterations in PPARα and HNF4α relative mRNA expression. C. Correlation between β-AR induced alterations in PPARα and HNF4α relative mRNA expression. D. Correlation of α2-AR induced alterations in PPARα and HNF4α mRNA relative expression.

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Author Contributions

Conceived and designed the experiments: MK YMS TM JFG. Performed the experiments: MK TM YMS. Analyzed the data: MK. Contributed reagents/materials/analysis tools: JFG. Wrote the paper: MK JFG.
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