

Role of PPAR α and HNF4 α in Stress-Mediated Alterations in Lipid Homeostasis

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Abstract

Stress is a risk factor for several cardiovascular pathologies. PPAR α holds a fundamental role in control of lipid homeostasis by directly regulating genes involved in fatty acid transport and oxidation. Importantly, PPAR α 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 PPAR α and HNF4 α regulation and signaling in mice following repeated restraint stress or treatment with AR-antagonists administered prior to stress to block AR-linked pathways. Repeated restraint stress up-regulated *Ppar α* and its target genes in the liver, including *Acox*, *Acot1*, *Acot4*, *Cyp4a10*, *Cyp4a14* and *Lipin2*, an effect that was highly correlated with *Hnf4 α* . *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 PPAR α and HNF4 α regulation. Notably, restraint stress, independent of PPAR α , 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 PPAR α and HNF4 α in stress-induced lipid metabolism.

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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, PPAR α , 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]. PPAR α 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

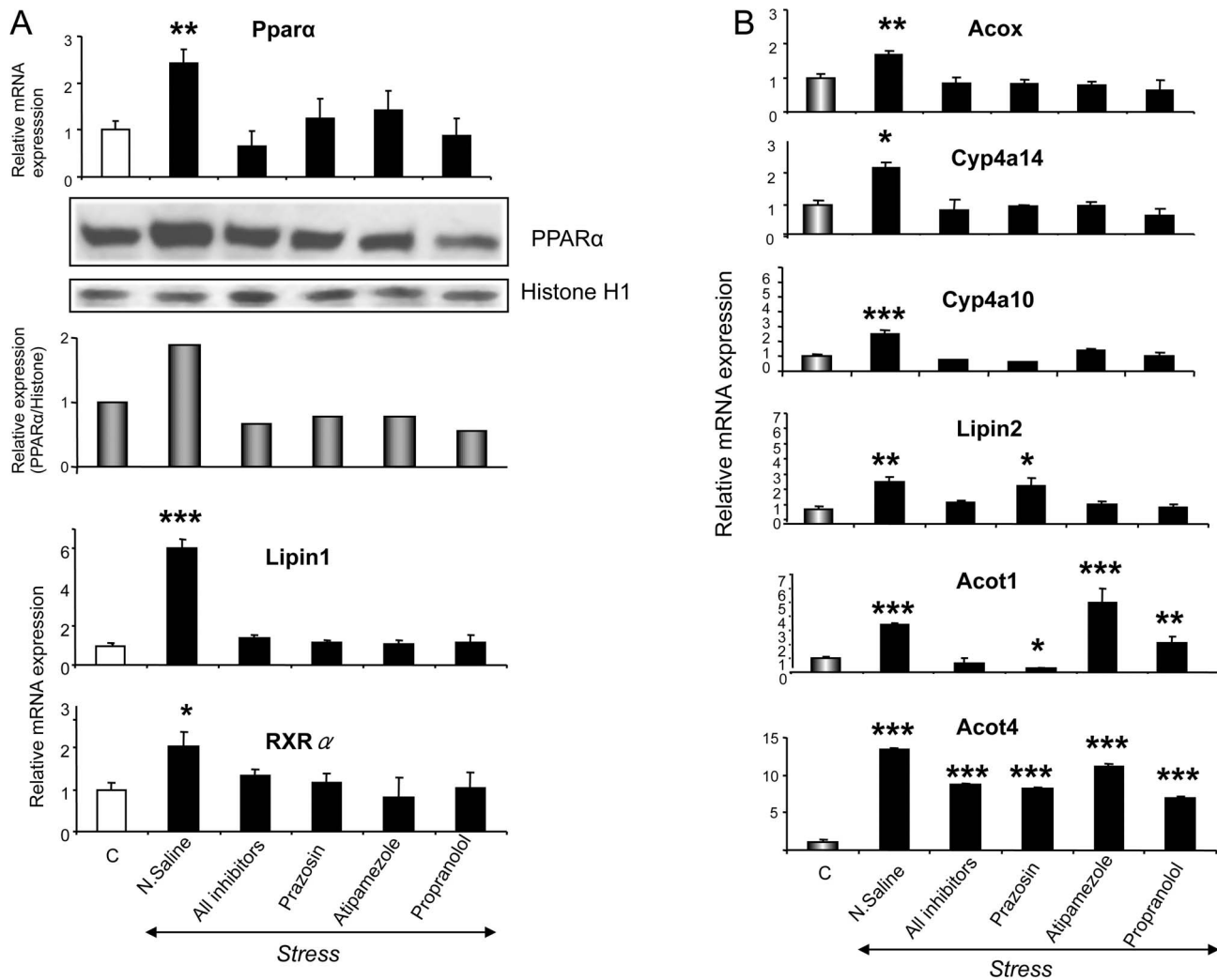


Figure 1. Stress-induced effect on *PPARα* and target gene expression. A. Following exposure of wild-type mice to restraint stress, *Ppara* mRNA levels were examined by qPCR analysis in their liver and *PPARα* protein was measured in the nuclear fraction by Western Blot analysis. Histone H1 served as a loading control. B. *Acox*, *Cyp4a10*, *Cyp4a14*, *Lipin2*, *Acot1*, *Acot4*, *Lipin1* and *RXRα* mRNA levels were also analyzed by qPCR in wild-type mice. Values were quantified using the comparative CT methods normalized to β -actin and are expressed as mean \pm SE (n = 8–10). Comparisons were between controls and stress-exposed mice (alone or previously treated with the AR-antagonists, prazosin, propranolol or atipamezole). 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.g001

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 50 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.

Table 1. Role of PPAR α in the stress-induced changes in serum lipid markers.

	Wild-type						<i>Ppara</i> -null	
	Control	Stress	Stress+ all AR-antagonists	Stress+ Prazosin	Stress+ Atipamezole	Stress+ Propranolol	Control	Stress
TG	141 \pm 13	83.2 \pm 8.0**	122 \pm 3.1	141 \pm 7.2	144 \pm 10	129 \pm 6.4	154 \pm 17	100 \pm 22
FFA	1.3 \pm 0.1	0.9 \pm 0.1**	1.5 \pm 0.3	1.8 \pm 0.4	1.4 \pm 0.2	1.3 \pm 0.1	2.0 \pm 0.3	1.9 \pm 0.3
T-Cholesterol	261 \pm 55	181 \pm 5.3**	265 \pm 78	194 \pm 20	255 \pm 46	305 \pm 14	314 \pm 61	291 \pm 6.5
Glucose	147 \pm 4.6	189 \pm 8.2**	151 \pm 10	101 \pm 11	154 \pm 11	157 \pm 17	155 \pm 5.7	192 \pm 7.8**
ALT	14.8 \pm 3.4	22.0 \pm 2.6	15.2 \pm 3.2	16.7 \pm 4.1	14.9 \pm 4.7	16.3 \pm 7.2	15.0 \pm 2.9	24.1 \pm 2.6
AST	46.8 \pm 7.2	62.0 \pm 9.5	57.3 \pm 13	64.3 \pm 8.9	48.8 \pm 15	54.2 \pm 14	56.5 \pm 8.6	79.4 \pm 14
CORT	68.4 \pm 7.3	110.4 \pm 11**	94.8 \pm 13*	115 \pm 28*	121 \pm 18**	95.6 \pm 17*	70.7 \pm 13	129 \pm 26*
EPIN	0.3 \pm 0.08	1.1 \pm 0.3***	0.2 \pm 0.01	0.2 \pm 0.01	0.2 \pm 0.0	0.2 \pm 0.01	0.4 \pm 0.1	1.4 \pm 0.2***
NE	2.9 \pm 0.8	3.3 \pm 0.4	2.5 \pm 0.3	2.5 \pm 0.2	2.6 \pm 0.1	2.4 \pm 0.3	3.1 \pm 0.9	3.7 \pm 1.2

Triglycerides (TG), free fatty acids (FFA) and total cholesterol (T-cholesterol). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), corticosterone (CORT), epinephrine (EPIN), norepinephrine (NE), (WT, n=20; *Ppara*-null, n=12).

*P<0.05,

**P<0.01.

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Drugs and treatment

Prazosin hydrochloride (Sigma-Aldrich, 20 mg/kg b.w., i.p.; Prazosin), an α_1 -AR blocker, or atipamezole hydrochloride (Antisedan, Orion Pharma, Pfizer, 200 μ g/kg b.w., s.c.; Atipamezole), an α_2 -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 trunk 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 α_1 -adrenergic receptor pathways were stimulated using the α_1 -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) following 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 μ g 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 for 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 PPAR α and HNF4 α 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 PPAR α (Santa Cruz Biotechnology, Santa Cruz, CA), HNF4 α goat polyclonal IgG (Santa Cruz), rabbit monoclonal phospho-AKT IgG (Ser473; Santa Cruz), rabbit polyclonal phospho-FOXO1 (Ser256; Santa Cruz Biotechnology), rabbit polyclonal phospho-CREB-1 IgG (Ser133; Santa Cruz Biotechnology), monoclonal anti-mouse phosphorylated STAT5b IgG (Tyr694, Cell Signaling Technology) and rabbit polyclonal p-p70S6K IgG (Thr 389; 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 $0.80\text{--}1.0 \times 10^6$ 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

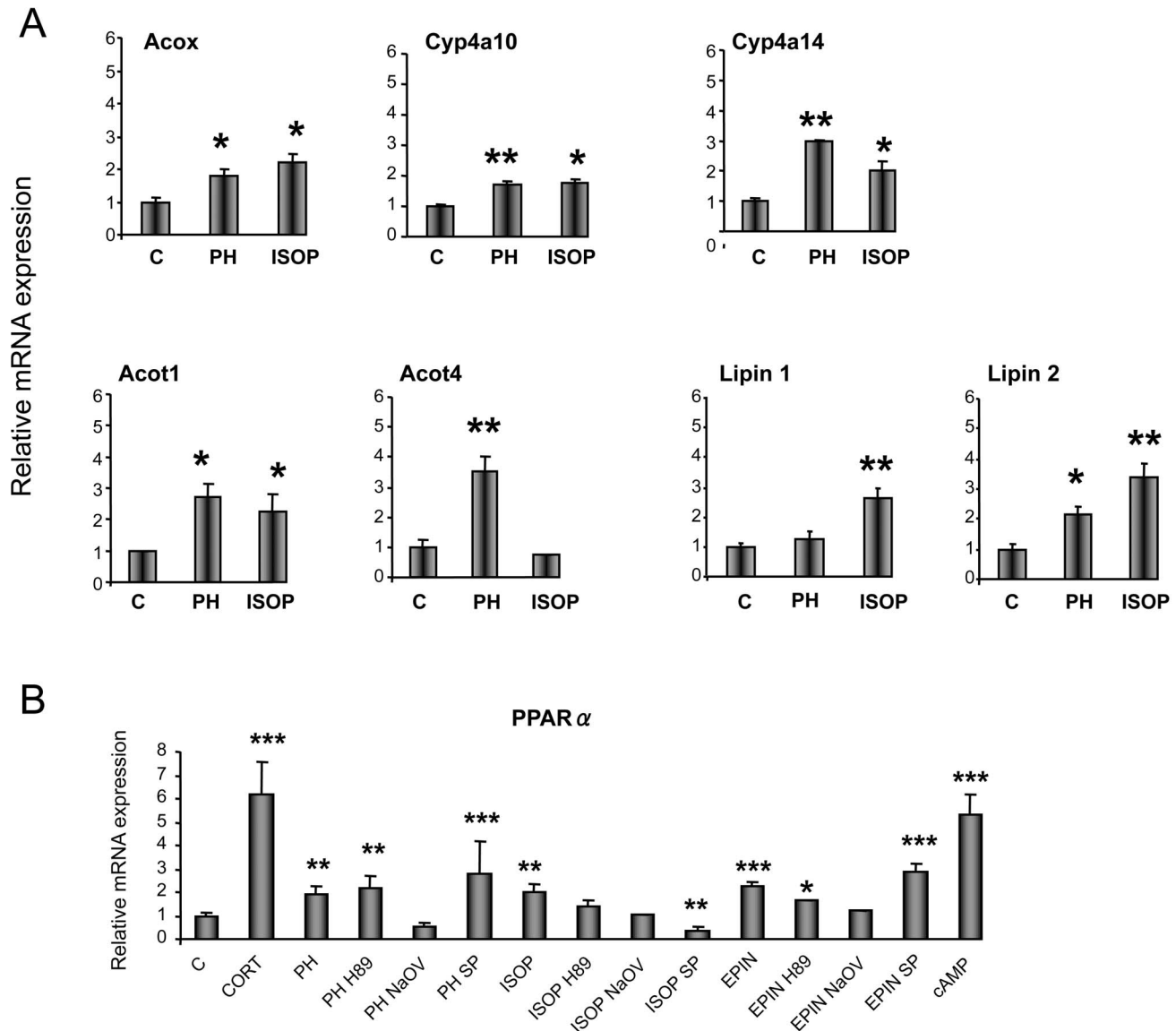


Figure 2. *In vitro* evaluation of the role of glucocorticoids and hepatic adrenergic receptor-linked pathways in *PPARα* and target gene expression. *α*. Following treatment of primary hepatocyte cultures with adrenergic receptor agonists for 24 hours, *Acox*, *Cyp4a10*, *Cyp4a14*, *Acot1*, *Acot4*, *Lipin1* and *Lipin2* mRNA levels were analyzed by qPCR. *B*. *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 \pm SE ($n=3-4$). Experiments were repeated three times. Comparisons were between control (DMSO) and drug-treated hepatocytes. C: control, CORT: corticosterone, PH: phenylephrine (α_1 -AR agonist), ISOP: isoprenaline (beta-AR agonist), EPIN: epinephrine (α - and beta-AR agonist), cAMP: 8-Br-cAMP. Group differences were calculated by one-way ANOVA, followed by Bonferroni's test. * $P<0.025$, ** $P<0.01$, *** $P<0.001$. doi:10.1371/journal.pone.0070675.g002

the AR-agonists, epinephrine (EPIN), PH and ISOP, at different doses (1–100 μ M 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 (Melburne, 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).

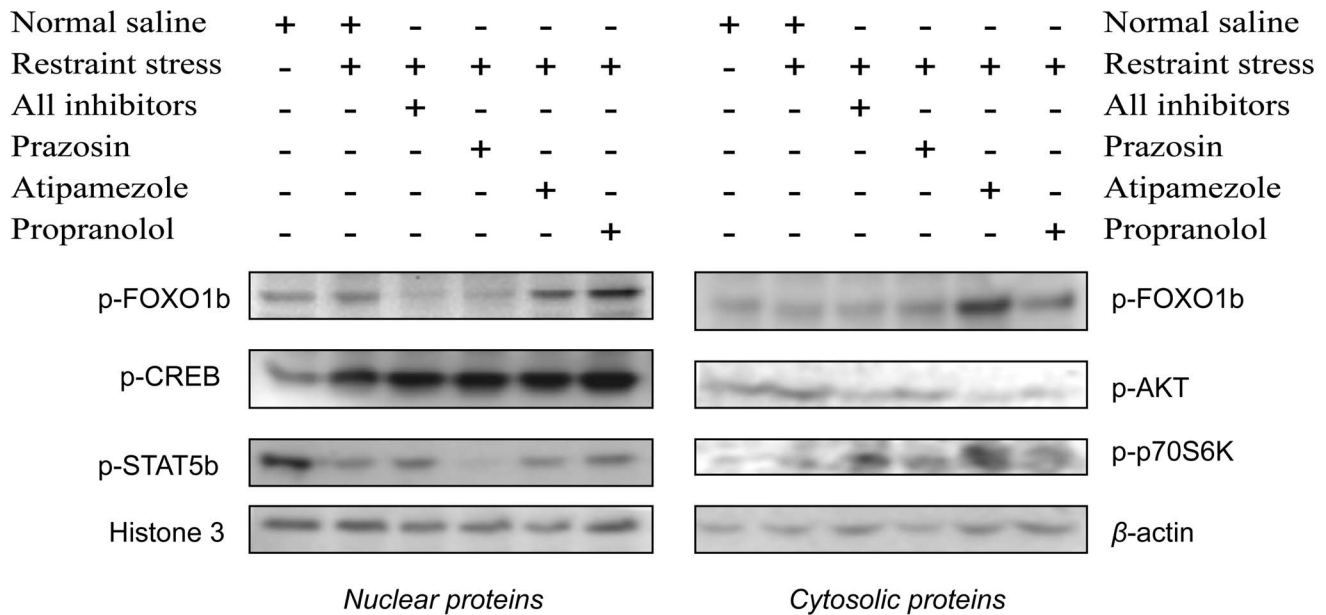


Figure 3. Stress-induced effect on PI3K/AKT, cAMP/PKA/CREB and GH/STAT5b signaling pathways. The evaluation of the stress effect on these pathways was conducted in nuclear and cytosolic proteins with Western blot. Histone H3 served as a loading control for nuclear proteins and β -actin for cytosolic proteins. AR: adrenergic receptor; AR-antagonists given prior to stress inhibited α_1 -, α_2 - and beta-AR signaling; prazosin (α_1 -AR inhibitor), atipamezole (α_2 -AR inhibitor), propranolol (β -AR inhibitor). doi:10.1371/journal.pone.0070675.g003

Statistical analysis

The data are presented as the mean \pm SE and were analysed using one-way analysis of variance (ANOVA) followed by multiple comparisons with Bonferonni'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 PPAR α mRNA expression and those observed in relative HNF4 α mRNA levels.

Results

Up-regulating effect of stress on PPAR α 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 PPAR α was determined by qPCR and western blot in the livers of wild-type mice. Restraint stress increased *Ppar α* mRNA and PPAR α protein levels (Fig. 1A) and this increase was prevented by the α_1 -AR antagonist, prazosin, the α_2 -AR antagonist, atipamezole and the beta-AR antagonist, propranolol (Fig. 1A). To test if the stress-induced *Ppar α* expression could affect the expression of PPAR α target genes, qPCR analysis of PPAR α 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 (*Acot1* and *Acot4*) (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 *Acot1* up-regulation, α_1 -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 α_2 - 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 *Acot4* 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 (RXR α) that forms heterodimers with PPAR α . These effects were prevented by all AR-antagonists (Fig. 1B). In the liver of *Ppar α* null mice, *Ppar α* , *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).

Alterations in serum lipid indices indicative of PPAR α activation

PPAR α 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 PPAR α (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 PPAR α 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 PPAR α regulation

To investigate whether the EPIN-stimulated hepatic PPAR α 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 PPAR α and

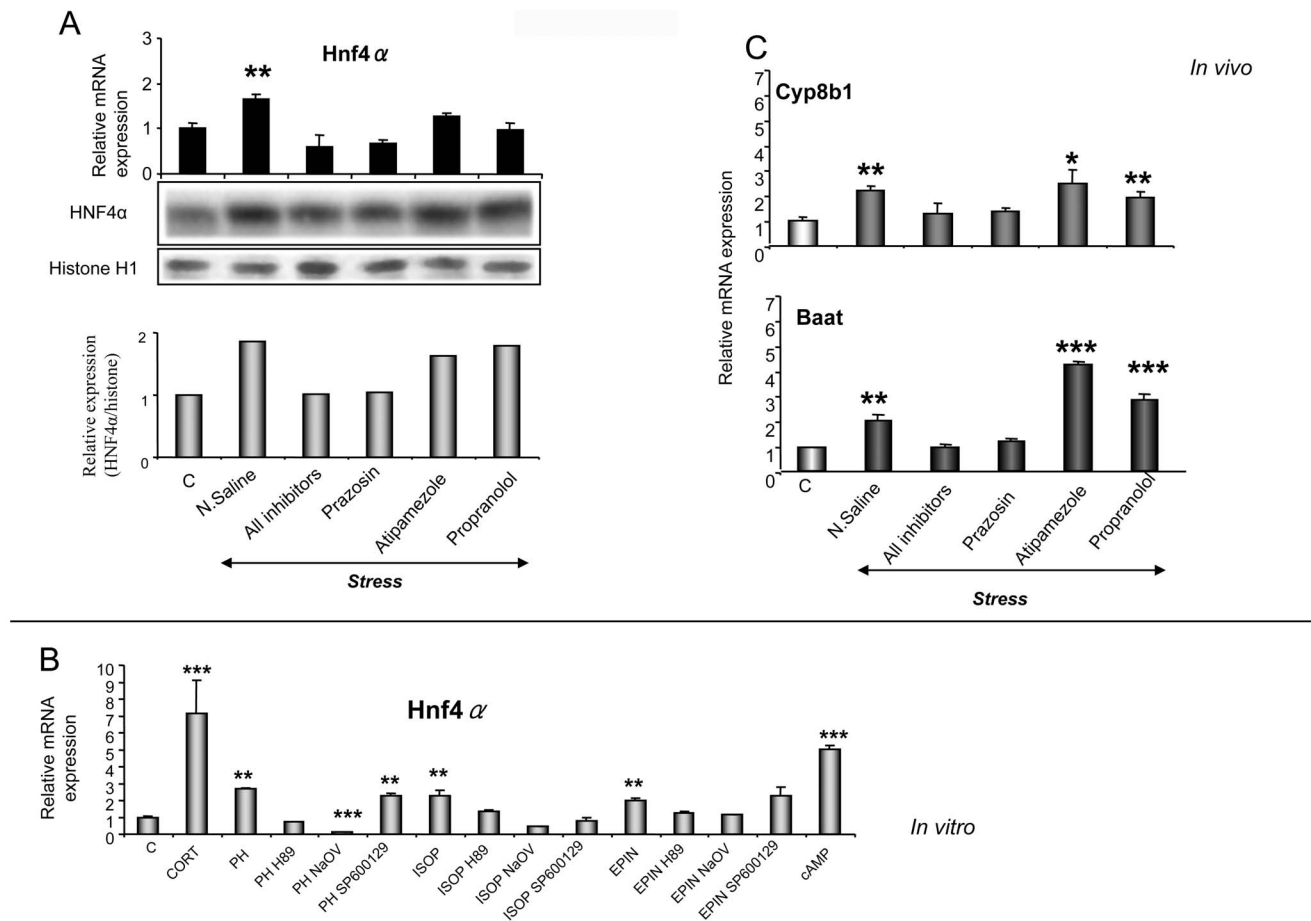


Figure 4. Stress-induced effect on *Hnf4α* expression. A. Following restraint stress alone or coupled with AR-antagonists hepatic *Hnf4α* mRNA levels were analysed in wild-type mice by qPCR. HNF4α protein was measured in liver nuclear fractions by Western blot analysis. Histone H1 served as a loading control. B. *Cyp8b1* and *Baat* mRNA levels were analyzed in the livers of wild-type mice by qPCR following treatment with either restraint stress alone or coupled with AR-antagonists (dark bars). C. *Hnf4α* mRNA levels were determined by qPCR following treatment of primary hepatocyte cultures with either corticosterone (CORT), epinephrine (EPIN) 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 normalized to β -actin and are expressed as mean \pm SE (n = 8–10). Comparisons were between controls and stress- or drug-treated mice. AR: adrenergic receptor, C: control, N. Saline: normal saline, All inhibitors: mice were treated with all AR-antagonists prior to stress, Prazosin (α_1 -AR antagonist), Atipamezole (α_2 -AR antagonist), Propranolol (β -AR antagonist), PH: Phenylephrine (α_1 -AR agonist), ISOP: Isoprenaline (β -AR agonist). 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.g004

HNF4 α regulation was assessed by treating primary hepatocytes with the AR-agonists, PH and ISOP. Stimulation of α_1 - and β -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 Ppara 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 α_1 -AR inhibition, but only by α_2 - and β -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 PPAR α activation and synthesis of factors with essential roles in lipid homeostasis (Fig. 3). Interestingly, inhibition of α_2 -ARs prior to stress increased p70S6K activation, but this effect was not followed by any alteration in PPAR α 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

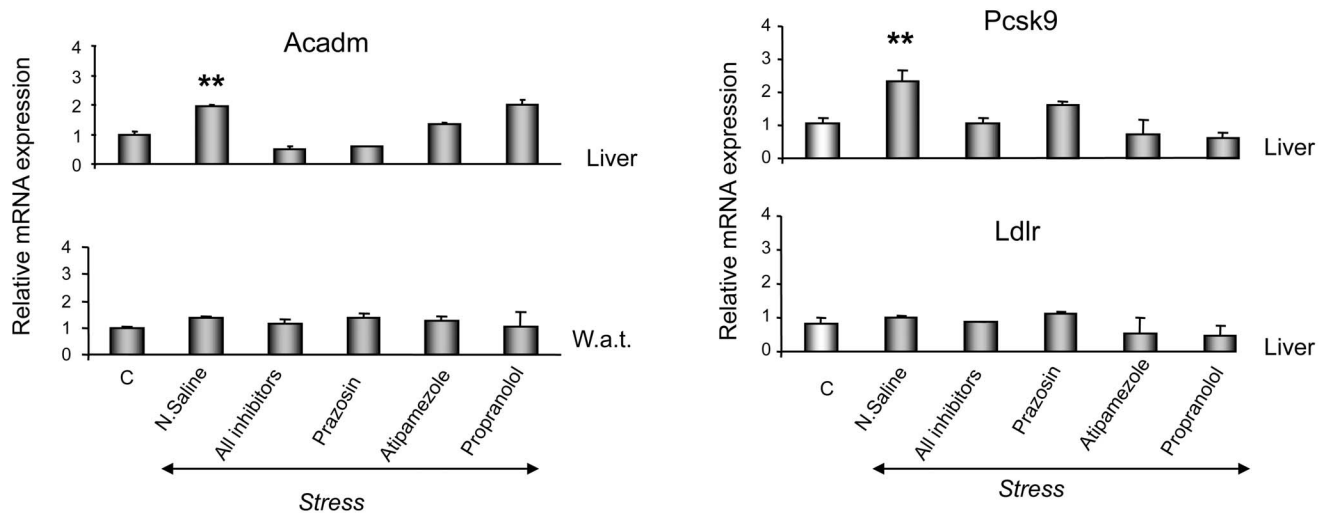


Figure 5. Stress-induced effect on *Acadm* expression. Effect of restraint stress and AR-related pathways on *Acadm* mRNA expression in the liver and in white adipose tissue (A), on *Pcsk9* and *Ldlr* mRNA expression in the liver (B). Values were normalized to β -actin and are expressed as mean \pm SE (n = 8–10). Comparisons were between controls and stress alone or coupled with AR-antagonists. AR: adrenergic receptor, C: control, N. Saline: normal saline, All inhibitors: mice were treated with all AR-antagonists prior to stress, prazosin (α_1 -AR antagonist), atipamezole (α_2 -AR antagonist), propranolol (β -AR antagonist). **P<0.01, ***P<0.001. doi:10.1371/journal.pone.0070675.g005

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 HNF4 α in the stress-induced alterations in lipid homeostasis

HNF4 α and PPAR α coordinate regulation of several common target genes [32]. In order to assess the involvement of HNF4 α in the stress-induced up-regulation of PPAR α target genes, hepatic RNA was analyzed by qPCR, and nuclear proteins quantified by Western blotting. HNF4 α 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 *Hnf4 α* was blocked only by the α_1 -AR antagonist, prazosin, whereas atipamezole and propranolol had no effect thus indicating a prevalent role for α_1 -AR-related pathways in the stress-induced *Hnf4 α* up-regulation (Fig. 4A). The stress-induced *Hnf4 α* expression triggered up-regulation of the HNF4 α target genes, bile acid CoA (*Baat*) and *Cyp8b1* (Fig. 4B). Further investigation revealed that the stress-released EPIN up-regulated *Hnf4 α* by directly stimulating hepatocyte α_1 - and β -ARs, as treatment of primary hepatocytes with either EPIN, PH or ISOP markedly induced hepatocyte *Hnf4 α* expression (Fig. 4C). The PH- and ISOP- induced up-regulating effect on *Hnf4 α* mRNA was blocked by pre-treatment with the PKA inhibitors, H89 and NaOV (Fig. 4C). The JNK inhibitor, SP600125, also prevented the ISOP-induced *Hnf4 α* up-regulation (Fig. 4C). The EPIN-induced effect on *Hnf4 α* 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 *Hnf4 α* was also apparent when the hepatocytes were treated with 8-Br-cAMP (Fig. 4C). Interestingly, corticosterone markedly increased *Hnf4 α* 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 *Hnf4 α* expression (Fig. S2). On the other hand, basal *Hnf4 α* mRNA expression ranged at equivalent levels in wild type and *Ppara* null mice, and exposure to restraint stress up-regulated *Hnf4 α* 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 α_1 -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 *Dcat1* 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*, *Nr4a*, *Dgat2*, *Aadac* and *Mtth* 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

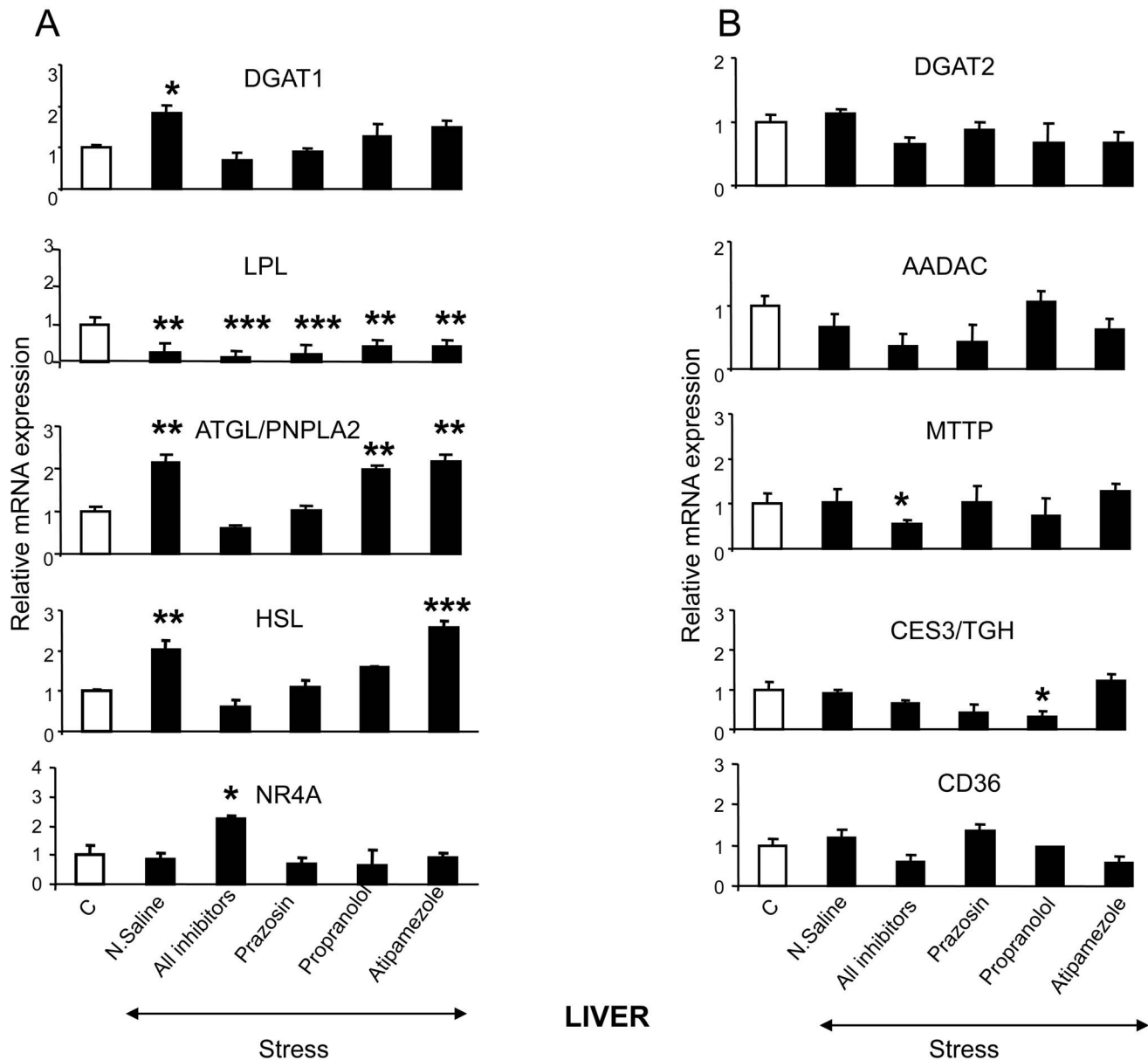


Figure 6. Stress-induced effect on hepatic TG homeostasis. (A) Effect of restraint stress on genes involved in TG synthesis and lipolysis in the liver. (B) Effect of restraint stress on genes involved in TG metabolism and clearance in the liver. Comparisons were between controls and stress-exposed mice (alone or simultaneously treated with the AR-antagonists, prazosin, propranolol or atipamezole; black bars). DGAT1: Diacylglycerol acyltransferase 1 (acyl coenzyme A (CoA)), DGAT2: Diacylglycerol 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, MTP: microsomal triglyceride transfer, PLIN5: perilipin 5. C: Control, N.Saline: normal saline, Prazosin (α_1 -AR antagonist), Atipamezole (α_2 -AR antagonist), Propranolol (β -AR antagonist). Values are expressed as mean \pm SE, n:5–6 per treatment group. * $P < 0.005$, ** $P < 0.01$, *** $P < 0.001$. doi:10.1371/journal.pone.0070675.g006

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 α_1 -AR blockade by prazosin prevented the stress-induced *Dgat1*, *Lpl*, *Atgl/Pnpla2*, *Dgat2* and *Aadac* up-regulation in the white adipose tissue (Fig. 7A and 7B). Both, α_2 - and β -AR-antagonists, atipamezole and propranolol, respectively, prevented the *Atgl/Pnpla2*, *Dgat2* and *Aadac* up-regulation in the white adipose tissue (Fig. 7A and 7B). In the case of the stress-induced *Nr4a* up-regulation in the white adipose tissue

the involvement of α_2 -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,

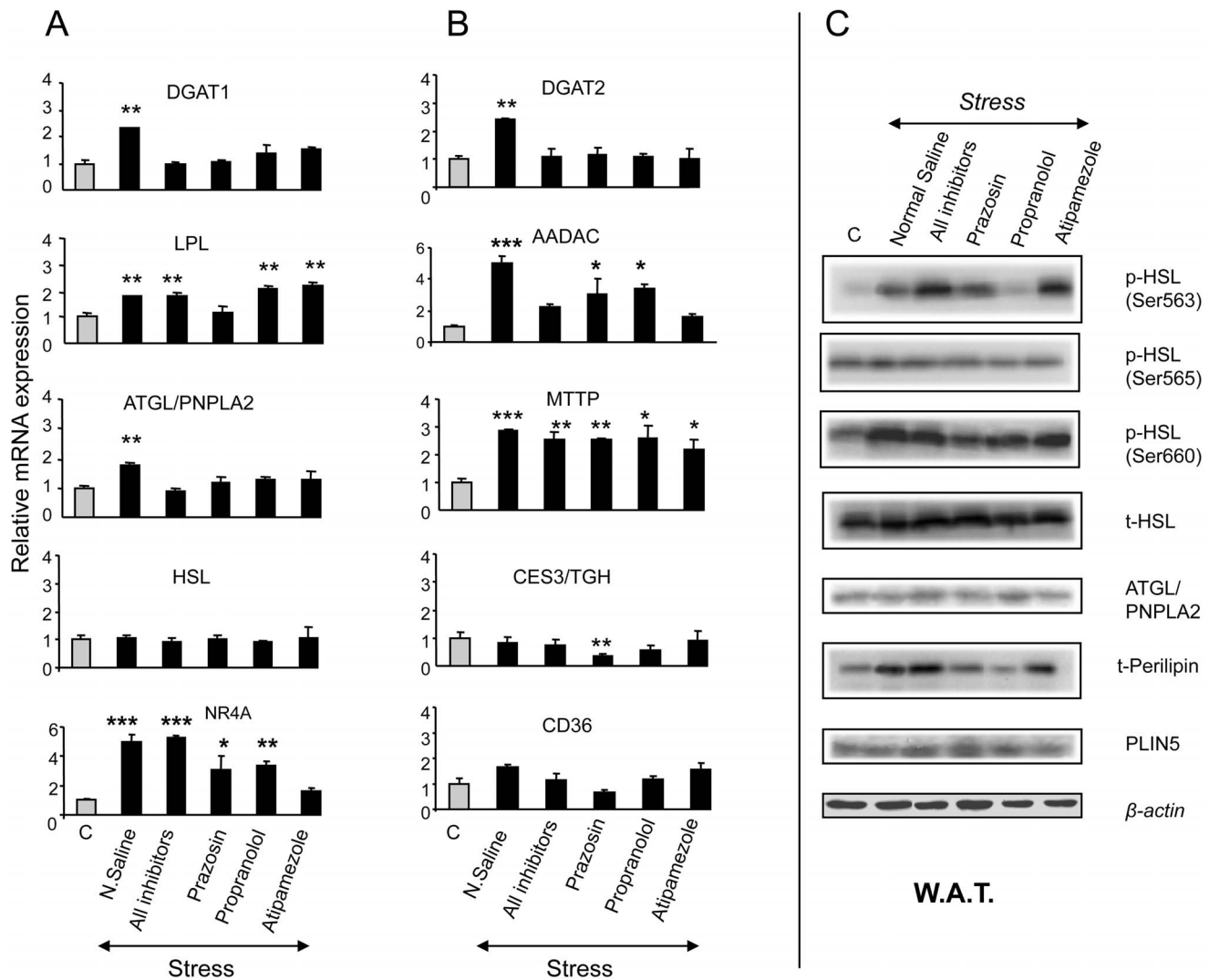


Figure 7. Stress-induced effect on TG homeostasis-related gene expression in the white adipose tissue. (A) Effect of restraint stress on the expression of genes involved in TG synthesis and lipolysis. (B) Effect of restraint stress on the expression of genes involved in TG metabolism and clearance. (C) 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, PLIN5: perilipin 5. AR: adrenergic receptor, C: Control, Prazosin (α_1 -AR antagonist), Atipamezole (α_2 -AR antagonist), Propranolol (β -AR antagonist), white adipose tissue (W.A.T.). Values are expressed as mean \pm 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. * $P < 0.005$, ** $P < 0.01$, *** $P < 0.001$. doi:10.1371/journal.pone.0070675.g007

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 *Ppara*-null mice. These observations clearly indicate a significant role for PPAR α 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 PPAR α 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 PPAR α 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 α -ARs in the stress-induced regulation of PPAR α 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 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 *Ppara* regulation by stress. In particular, blockade of α_1 -, α_2 - or beta-ARs during the stress manipulation, prevented the stress-induced effect on *Ppara* and PPAR α target gene expression. The up-regulating effect of stress on PPAR α and its target genes is potentially, in part, associated with the up-regulation of RXR α , which forms heterodimers with PPAR α . 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 PPAR α and genes encoding factors involved in lipid homeostasis [30,31]. Stress activated Akt in the liver via pathways not related to α_1 -ARs but to α_2 - 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 FOXO1b and p70S6K, which mediate the up-regulating effects of stress.

In vitro stimulation of hepatocyte α_1 -AR/cAMP/PKA signaling pathway with PH, the beta-AR/cAMP/PKA and beta-AR/JNK-linked pathways with ISOP, was followed by up-regulation of PPAR α and its target genes. To our knowledge, this is the report of a role for α_1 -ARs in the regulation of PPAR α . *In vivo*, the stress-induced release of EPIN activated hepatic α_1 - and beta-AR/cAMP/PKA/CREB signaling pathway thus activating PPAR α . It should be noted though that the AR-antagonists 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, STAT5b, in the liver, an effect though that was not blocked by the AR-inhibitors used, indicating that the stress-induced inactivation of STAT5b is mediated by glucocorticoids [42] and not by central or peripheral AR-linked pathways. The suppressive effect of stress on the GH/STAT5b pathway potentially mediates the stress-induced *Ppara* up-regulation. The bidirectional inhibitory cross-talk between PPAR and STAT5b is well documented [43].

The stress-induced *Ppara* 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 PPAR α . Several other transcription factors can contribute to the TG lowering effects of stress, including SREBP-1c, NF-kappaB, RXRs, LXRs, FXR and HNF4 α [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 *Aadm*, 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 *Hnf4 α* , which is central to the maintenance of hepatocyte differentiation and the regulation of genes involved in lipid metabolism [56,57]. Importantly, as in the case of *Ppara*, the hepatocyte α_1 - and beta-AR/cAMP/PKA signaling pathway along with that of beta-AR/JNK-linked pathway appear to mediate *Hnf4 α* up-regulation [58]. The *in vivo* and *in vitro* data indicated that the α_1 - and beta- α_2 -AR-induced *Hnf4 α* up-regulation, should be attributed to a direct stimulation of hepatic α_1 - or beta- α_2 -AR signaling pathways by EPIN. The critical role of the hepatic ARs in *Hnf4 α* regulation by stress is strengthened by the fact that stimulation of hypothalamic α_1 - or beta-ARs leads to suppressed plasma GH levels [59], a hormonal state connected with down-regulation of HNF4 α target genes. A bidirectional cross-talk between the GH/STAT5b signalling and HNF4 α is well documented. In particular, STAT5b activation has been connected with augmentation of HNF4 α -dependent gene transcription, while HNF4 α inhibits STAT5b transcriptional activity via inhibition of JAK2 phosphorylation [60]. In contrast, α_2 -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 HNF4 α [62]. In addition, cytokines, which are released from immune cells following stimulation of all types of ARs have a suppressive effect on *Hnf4 α* regulation [63–65]. Based on these reports and the present findings, it is apparent that the hepatic α_1 - and beta-AR-related effects on *Hnf4 α* regulation override that of central and other peripheral AR-mediated effects. The findings of this study confirm the positive feedback regulatory mechanism involving PPAR α and HNF4 α 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*, *Atgl*, *Aadac*, *Mitp* and *Nr4a*. The stress-induced effect on *Lpl*, which has dual functions of TG hydrolase and ligand/bridging factor of receptor mediated lipoprotein up-take [52,53], is apparently mediated via α_1 -ARs. Interestingly, the stress-induced up-regulating effect on *Dgat1*, *Dgat2*, *Aadac* and *Atgl* is mediated by α - and beta-ARs. It should be also noted that stress induced the *Nr4a* expression only via α_2 -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 α_1 - and beta-ARs. Apparently, the stress-induced increase in perilipin concentration in the white adipose tissue, which was mediated by α_1 - and beta-AR-related pathways, did not prevent the effect of lipases and hydrolases on TG. Notably stress markedly up-regulated *Pcsk9* 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 PPAR α activation was also well defined by previous [71] and the present data, which revealed a role for alpha₁-ARs in PPAR α regulation. These findings could be the basis for the design of new molecules targeting these receptors for PPAR α 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 PPAR α and lipid homeostasis.

Supporting Information

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

(DOC)

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Table S2 Alterations in the body weight after stress.

Body weight values are expressed in g.

(DOC)

Figure S1 Stress-induced effect on PPAR α expression in Ppara null mice.

A. PPAR α mRNA and protein levels were examined in the liver of Ppara 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. HNF4 α mRNA levels were examined in the liver of Ppara-null mice followed restraint stress or treatment with AR agonists. C: controls, PH: phenylephrine (α_1 -agonist), DEXT: dexmedetomidine (α_2 -agonist), ISOP: isoprenaline (β -agonist). Values were quantified using the comparative CT methods normalized to β -actin and are expressed as mean \pm 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 Bonferonni's test. * P<0.025, **P<0.01. (DOC)

Figure S2 Correlation between stress- and AR-induced alterations in PPAR α and HNF4 α expression.

A. Correlation between stress-induced alterations in PPAR α and HNF4 α relative mRNA expression. B. Correlation between α_1 -AR-induced alterations in PPAR α and HNF4 α relative mRNA expression. C. Correlation between β -AR induced alterations in PPAR α and HNF4 α relative mRNA expression. D. Correlation of α_2 -AR induced alterations in PPAR α and HNF4 α mRNA relative expression. (DOC)

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Author Contributions

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

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