Peroxisome proliferator activated receptors-α and -γ, and cAMP-mediated pathways, control retinol-binding protein-4 gene expression in brown adipose tissue

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Precis :

Thermogenic activation causes enhanced expression of the RBP4 gene and enhanced release of RBP4 protein by brown adipose tissue.

Abstract

Retinol binding protein-4 (RBP4) is a serum protein involved in the transport of vitamin A. It is known to be produced by the liver and white adipose tissue. RBP4 release by white fat has been proposed to induce insulin resistance. We analyzed the regulation and production of RBP4 in brown adipose tissue. RBP4 gene expression is induced in brown fat from mice exposed to cold or treated with PPAR agonists. In brown adipocytes in culture, norepinephrine, cAMP and activators of PPARγ and PPARα induced RBP4 gene expression and RBP4 protein release. The induction of RBP4 gene expression by norepinephrine required intact PPAR-dependent pathways, as evidenced by impaired response of the RBP4 gene expression to norepinephrine in PPARα-null brown adipocytes or in the presence of inhibitors of PPARγ and PPARα. PPARγ and norepinephrine can also induce the RBP4 gene in white adipocytes, and over-expression of PPARα confers regulation by this PPAR subtype to white adipocytes. The RBP4 gene promoter transcription is activated by cAMP, PPARα and PPARγ. This is mediated by a PPAR-responsive element capable of binding PPARα and PPARγ and required also for activation by cAMP. The induction of the RBP4 gene expression by norepinephrine in brown adipocytes is protein synthesis-dependent and requires PPARγ-co-activator-1-α (PGC-1α), that acts as a norepinephine-induced co-activator of PPARs on the RBP4 gener. We conclude that PPARγ- and PPARα-mediated signalling control RBP4 gene expression and release in brown adipose tissue, and thermogenic activation induces RBP4 gene expression in brown fat through mechanisms involving PGC-1α co-activation of PPAR signalling..

Introduction

Retinol binding protein-4 (RBP4) is a serum protein whose primary recognized function is the transport of vitamin A (1). The liver is the main site of RBP4 production while other tissues, particularly white adipose tissue (WAT), have been reported to contribute also to RBP4 synthesis and release (2). RBP4 has been proposed to act as an adipokine, i.e. a protein released by adipose tissue with regulatory functions distinct from its role in vitamin A homeostasis (3). A negative correlation between RBP4 levels (and, specifically, synthesis in WAT) and overall insulin sensitivity has been reported in several rodent models of obesity, and it has been proposed that RBP4 released by WAT acts on liver as a systemic signal promoting insulin resistance. In humans, obesity and the associated insulin resistance have been described as being associated with abnormally high RBP4 protein levels in serum whereas insulin sensitization achieved via weight loss or exercise appears to reduce serum RBP4 levels (4). However, not all studies in humans have confirmed the relationship of obesity and insulin resistance with RBP4 levels (5-9), and the role of RBP4 as a putative adipokine inducing insulin resistance remains controversial. Moreover, it has been recently reported that the RBP4-vitamin A complex inhibits cellular insulin responses through the interaction with the membrane protein STRA6 ("stimulated by retinoic acid") present in the cell surface (10).

The two main types of adipose tissue in mammals, WAT and BAT (brown adipose tissue) play opposing roles in energy balance. WAT is an energy-storing tissue, and BAT constitutes a site of energy expenditure due to its thermogenic capacity. Both types of adipose tissue are present in substantial amounts in adult rodents. In adult humans, WAT is predominant but BAT has been recognized recently to have also a relevant presence (11, 12). Both types of adipose tissue store vitamin A derivatives and have functions in metabolism of retinoids, including release of retinol (13). All-trans retinoic acid acts usually as an anti-adipogenic agent in brown and white adipocyte differentiation (14, 15). However, retinoids participate in the differential development of both types of adipose tissues. Thus, all-trans retinoic acid, acting through retinoic acid receptors (RAR), is a powerful inducer uncoupling protein-1 (UCP1) expression, the specific marker of brown-versus-white fat and responsible for thermogenic function (16). On the other hand, activation of the 9-cis retinoic acid receptor RXR favors brown and white adipogenesis through activation of the RXR moiety of the adipogenic peroxisome proliferators activated receptor-γ (PPARγ)/RXR heterodimers (17). PPARα is highly expressed in BAT, whereas expression in WAT is very low (18). PPARα controls the transcription of genes for lipid catabolism in brown fat as well as UCP1 gene transcription, and it has been proposed that it establishes a link between lipid oxidation and thermogenesis in BAT (19).

The major pathway of control of brown fat-specific thermogenic activity involves the action of norepinephrine, released by the sympathetic nervous system (20). The activation of β-

adrenergic receptors leads to a rise in intracellular cAMP, which mediates most of the thermogenic responses of the brown adipocyte, i.e.: enhanced lipolysis and induction of UCP1 gene expression.

The molecular signals controlling RBP4 gene expression and synthesis in adipose tissues are poorly understood. Treatment of rodents with rosiglitazone, an activator of PPARγ, has been reported to reduce RBP4 gene expression in WAT, an effect contributing to the insulin sensitizing effects of this drug (3). However, other reports in humans do not support such an effect and treatment of human patients with the thiazolidinedione (TZD) pioglitazone, another activator of PPARγ, has been reported to increase RBP4 gene expression in WAT (8, 21).

In the present study we show that RBP4 gene expression is under the control of PPARα and PPARγ-mediated regulation as well as by noradrenergic, cAMP-mediated, stimulus involving PGC-1α in brown adipocytes These pathways of control may contribute to the differential regulation of RBP4 gene expression and protein release by BAT and WAT.

Materials and Methods

Wy14,643, bezafibrate, GW7647, GW501516, GW6471, GW9662, all-*trans* and 9-cis retinoic acid, norepinephrine, dibutyryl-cAMP and cycloheximide were from Sigma (Saint Louis, MO). Rosiglitazone was from Cayman (Ann Arbor, MC). GW7647 and TTNPB were from Tocris (Ellisville, USA).

The care and use of mice were in accordance with the European Community Council Directive 86/609/EEC. For studies in lactating dams, Swiss mice 15 days after parturition were injected with a single dose of Wy14,643 (50 μg/g bw) or bezafibrate (100 μg/g bw) in 20% dimethylsulfoxide (DMSO)/saline, or equivalent volumes of vehicle solution, and sacrificed 6h later. To determine the effects of cold exposure, two-month-old PPARα-null mice and strain-matched wild-type mice were acclimated to a thermoneutral temperature (28ºC) for one week and then exposed to 4ºC for 24 h. Interscapular BAT and periovarian WAT were dissected for further analysis.

RNA was extracted from BAT, WAT and cultured cells (RNAeasy kit, Qiagen). Reverse transcription was performed in 20 μl, using random hexamer primers (Applied Biosystems) and 0.5 μg RNA. PCR reactions were conducted in the ABI/Prism-7700 Sequence-Detector System using 25 μl of reaction mixture containing 1 μl of cDNA, 12.5 μl of TaqMan Universal PCR Master Mix, 250 nM probes and 900 nM primers from the following Assays-on-Demand (Applied Biosystems) kits: Mm00803266, mouse RBP4; Hs00198830, human RBP4; Mm00447183, PGC-1α; and Hs99999901, 18S rRNA. Each sample was run in duplicate and

the mean value of the duplicate was normalized to that of the 18S rRNA gene using the comparative ($2^{-\Delta CT}$) method following the manufacturer's instructions.

Primary brown adipocytes were differentiated in culture as described previously (16). Brown adipocytes were exposed to treatments, and cells and medium were harvested 24 h later, unless otherwise indicated. The HIB-1B brown adipocyte cell line was cultured as reported elsewhere (22). Primary cultures of mouse white adipocytes were performed using pre-adipocytes isolated from mouse subcutaneous WAT, as described (23). Experiments were performed on day 12 of differentiation and cells and medium were harvested 24 h after treatment. SGBS human adipocyte cells were cultured and differentiated as already reported (24) and experiments were performed on day 12 after induction of differentiation, when more than 70% cells were differentiated.

Mouse embryonic fibroblasts (MEFs) were isolated from 13-day-old (embryonic age) wild-type and PGC-1α-null mice (kindly provided by B.Spiegelman) according to standard procedures, and adipogenic differentiation of MEFs was achieved as already described, with slight modifications (25, 26). Experiments were performed when 80-90% of the cells were differentiated, on the basis of acquisition of adipocyte morphology

The plasmids -1192-RBP4-Luc, -535-RBP4-Luc and -285-RBP4-Luc, in which the corresponding human RBP4 promoter fragments drive Firefly luciferase, have been described previously (27). The plasmid -1192-Mut-RBP4-Luc is a version of -1192-RBP4-Luc in which point mutations to disrupt the putative PPAR-responsive element at position –765/-745 had been included. The point mutant construct was generated using a QuickChange site-directed mutagenesis kit (Stratagene), the forward primer 5′-GAA ACT AAA GAA CAA ATA TTG Aagc GAG GGa cCC ACA ACG CTC CTG AAA GAG AG-3′ (forward), 5′-CTC TCT TTC AGG AGC GTT GTG GGT CCC TCG CTT CAA TAT TTGTTCTTTAGTTTC 3′ (lower case indicates the mutated base pairs) and the complementary reverse primer. The plasmids were transfected into HIB-1B cells using FuGENE6 (Roche). Each point was assayed in triplicate and contained 0.3 μg RBP4-Luc reporter plasmid, 0.5 ng of the expression vector pRL-CMV (Promega, Madison, WI), and, when indicated, 0.06 μg pSG5-PPARα, pSG5-PPARγ or pSV-PGC-1α. Cells were incubated for 48 h after transfection and, when indicated, treated for 24h with 1μM GW7647, 10 μM rosiglitazone or 1 mM dibutyryl-cAMP. Luciferase activities were measured using the Dual-Luciferase-Reporter assay (Promega, Madison, WI). Firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase as an internal control.

Differentiated SGBS adipocytes were transduced with adenoviral vectors driving human PPARα (AdCMV-hPPARα) (28), murine PGC-1α (AdCMV-PGC-1α, provided by Dr B. Spiegelman) or GFP (AdCMV-GFP, control) at a multiplicity of infection of 400, for 4h.

Experiments were performed after further incubation in differentiation medium for 48h. Efficiency of transduction was approximately 80%, on the basis of GFP fluorescence. Cells were treated for 24h with 10μM GW7647, 10μM rosiglitazone or vehicle (DMSO).

Immunoblot analysis of RBP4 protein in cell culture medium (35 μl) was performed using 13% SDS-PAGE, RBP4 antiserum reacting against human or mouse RBP4 (1:2000, Alexis), and the ECL detection kit (Amersham). Quantitative analysis was performed by densitometry (Phoretics ID Software, Newcastle, UK).

Chromatin immunoprecipitation experiments were performed as described elsewhere (29). HIB-1B cells were transfected with the RBP4 promoter constructs, pSG5-PPARαor pSG5-PPARγ and exposed to 1μM GW7647 or 10 μM rosiglitazone respectively, as described above. Immunoprecipitation was carried out using anti-PPARα (sc-900, Santa Cruz Biotechnology) or anti-PPARγ (sc-7196, Santa Cruz Biotechnology) antibodies, or an equal amount of an unrelated immunoglobulin (Sigma). Input DNA and immunoprecipitated DNA were analyzed by quantitative PCR using SYBR green fluorescent dye and the following primers encompassing the putative PPAR-responsive region in the RBP4 gene: forward, 5'-ACC AGG GTT GCG TTT CTG GAG AAT-3´; reverse, 5´-TCT GAG GTC CAC TTG TGC AGG AAT-3 (250-bp fragment). Primers amplifying a 237-bp fragment of the cyclophilin-A gene, used as control, were: forward, 5'-CCA TCC AGC CAT TCA GTC TT-3' and reverse, 5'-TTA CAG GAC ATT GCG AGC AG-3'. The protein-bound DNA was calculated as a ratio to input DNA. The specificity of amplification using the primers above was assessed by by PCR amplification (30 cycles of 30 seconds at 95ºC, 30 seconds at 60ºC, and 30 seconds at 72ºC), electrophoreses on 1.5% agarose gel and staining with ethidium bromide and visualization of the expected bands.

Where appropriate, statistical analysis was performed using the Student's t test; significance is indicated in the text.

Results

RBP4 gene expression and induction by thermogenic activation in brown adipose tissue.

Basal expression of RBP4 mRNA was not significantly different between BAT and WAT in mice reared at thermoneutral temperature. Exposure of mice to cold (4ºC) caused a significant increase in RBP4 mRNA levels in BAT whereas no effects were observed in WAT (Fig 1A). In fact, mice placed at the usual room temperature (21ºC), in which a mild thermogenic stimulus occurs, showed significantly higher levels of RBP4 mRNA in BAT than in WAT (3±0.25 fold, $p < 0.05$), thus confirming the sensitivity of the RBP4 gene to the thermogenic stimulus in BAT.

The effects of acute treatment of mice with drugs distinct from a noradrenergic stimulus but known to influence brown fat activity, i.e. PPAR activators, were studied. Previous studies have indicated that the action on BAT of acute treatment of mice with PPAR activators was optimally visualized when controls were mice in a physiological state involving low levels of circulating free fatty acids, i.e. lactating dams (19). Thus, when lactating mice were treated with a single dose of bezafibrate, a PPAR activator with poor selectivity for PPAR subtypes, a significant rise in RBP4 mRNA was observed (Fig 1B). The PPARα-specific activator Wy14,643 also significantly induced RBP4 mRNA levels. In order to further establish the role of PPARα in the control of RBP4 gene expression in BAT, PPARα-null mice were studied. No differences in RBP4 mRNA levels were observed in mice in basal, thermoneutral conditions (Fig 1C). However, cold exposure caused a minor induction of RBP4 mRNA levels in PPARα-null mice relative to controls (Fig 1C), thus suggesting an interaction between noradrenergic and PPAR-dependent pathways of control of the RBP4 gene.

Effects of noradrenergic activation, PPAR agonists and retinoids on RBP4 gene expression in brown adipocytes .

Norepinephrine as well as cAMP caused a dramatic induction of RBP4 mRNA levels in brown adipocytes in primary culture. Bezafibrate, PPARα (GW7647) and PPARγ (rosiglitazone) specific activators also significantly induced RBP4 mRNA levels whereas a PPARβ/δ activator (GW501516) did not (Fig 2A). Both 9-cis retinoic acid and a synthetic activator of RXR, AGN194204 (a gift from Dr. R.Chandraratna), also increased RBP4 mRNA abundance. In contrast neither all-trans retinoic acid nor TTNPB, a specific activator of RARs, induced RBP4 mRNA expression. In order to establish the consequences of the increase in RBP4 mRNA levels, RBP4 protein levels were determined in brown adipocyte culture medium. Results indicated that norepinephrine, cAMP, bezafibrate and the PPARγ-specific (rosiglitazone) and PPARα-specific (GW7647) activators caused a significant rise in the abundance of RBP4 protein in the medium (Fig 2B).

In order to further analyze the role of PPARα in the control of the RBP4 gene and its interaction with the cAMP-dependent, noradrenergic pathway of regulation, primary cultures of brown adipocytes were established from PPARα-null mice (Fig 4). Morphological differentiation of adipocytes from PPARα-null mice was unaltered respect to cultures from wild-type mice, as described elsewhere (30). Basal levels of RBP4 mRNA expression were also unaltered in PPARα-null brown adipocytes, whereas the increase in RBP4 mRNA levels due to the PPARα agonist GW7647 was completely suppressed (Fig 3). The effects of the RXR activator AGN194204 were essentially unchanged, and the action of 9-cis retinoic acid and of rosiglitazone were slightly reduced. The effects of norepinephrine, although not completely

impaired, were significantly reduced in adipocytes lacking PPARα, thus further indicating a cross-talk between PPARα-dependent and noradrenergic pathways of regulation of RBP4 gene expression (Fig 3).

In a second approach, the PPARα antagonist GW6471 was used. This drug not only suppressed the action of the PPARα activator GW7647 but it also significantly reduced the action of norepinephrine (Fig 4). The PPARγ antagonist GW9662 also significantly decreased the effects of norepinephrine on RBP4 mRNA expression, while treatment with both PPAR subtype-specific drugs completely suppressed noradrenergic induction. This indicated that an active PPAR-dependent pathway of regulation, either through PPARα or PPARγ, is required for effective noradrenergic induction of the RBP4 gene in brown adipocytes.

Transcriptional control of the RBP4 gene by PPARs and cAMP in brown adipocytes

In order to determine the action of cAMP and PPARα-dependent pathways in transcriptional control of the RBP4 gene in brown adipocytes, transfection assays on HIB-1B brown fat-derived cells were performed using a luciferase construct driven by the 1192-bp promoter region of the human RBP4 gene. Exposure of the transfected construct to PPAR activators indicated significant induction by PPARγ activation (rosiglitazone), a weaker but significant induction by PPARα activation (GW7647), and a significant induction due to cAMP (Fig 6A). Co-transfection with PPARα, and particularly further addition of GW7647, caused a dramatic induction of the RBP4 promoter activity. Similar observations were obtained when PPARγ was co-transfected and cells were exposed to rosiglitazone. No effects were observed with PPARβ/δ co-transfection, further indicating the lack of sensitivity of the RBP4 gene to this PPAR subtype (data not shown).

Through a deletion mutant analysis, it was determined that the RBP4 promoter construct lacking the fragment from -1192 to -535 retained most of the basal activity but became completely non-sensitive to PPAR-dependent regulation (Fig 6A). Most of cAMP-mediated responsiveness was lost in this short –535-RBP4-Luc construct. Further deletion of the reporter construct between –535 to –285 dramatically reduced basal promoter activity (11% of the full-length construct levels), and –285-RBP4-Luc was completely insensitive to PPAR- or cAMP-dependent regulation (data not shown). The -1192 to -535 region of the RBP4 gene was previously reported to contain a putative PPAR-responsive sequence at –765 to –745 (27). When we analyzed a construct of the RBP4 promoter in which point mutations had been introduced to disrupt PPAR binding at this site (–1192-Mut-RBP4-Luc) (Fig 5C), the responsiveness to PPARs and to cAMP was essentially lost (Fig 5A). Chromatin immunoprecipitation in conditions of PPAR-dependent induction of the RBP4 promoter in HIB-1B cells using antibodies against PPARα and PPARγ indicated a specific enrichment in the PCR amplification of the RBP4 promoter region (Fig 5B). When –1192-Mut-RBP4-Luc mutated

version of the RBP4 promoter construct was analyzed in comparison with the wild-type promoter construct, a significant impairment in the enrichment of amplified DNA after chromatin immunoprecipitation using PPARα and PPARγ antibodies. Was observed (Fig 5D). Collectively, this data indicate that PPARα and PPARγ regulate the RBP4 gene promoter mainly through the –765 to –745 region of the RBP4 gene.

Regulation of RBP4 gene expression in white adipocytes

We determined whether the observed effects were specific to brown adipocytes or were shared by white adipose cells. Differentiated 3T3-L1 and 3T3-F442A adipocytes express extremely low levels of RBP4 mRNA, hardly detectable even using highly sensitive quantitative real-time PCR. In contrast, mouse white adipocytes differentiated in primary culture expressed substantial levels of RBP4 mRNA. Norepinephrine and the PPARγ agonist rosiglitazone significantly induced RBP4 mRNA levels in white adipocytes whereas the PPARα activator GW7647 had no effect (Fig 6A). Parallel observations were made for RBP4 protein levels in the adipocyte culture medium in response to these agents (Fig 6B). The study of SGBS human white adipocytes led essentially to similar results (Fig 6C). This indicated that the main features of regulation of RBP4 gene expression in white adipocytes were shared by human and murine cells.

The lack of effect observed for the PPARα agonist on RBP4 gene expression in white adipocytes was likely to be due to the much lower expression of PPARα in white, in comparison to brown, adipocytes (18). We therefore investigated whether increasing PPARα expression in white adipocytes could confer to these cells sensitivity to activation of RBP4 gene expression. Adenoviral transduction of human SGBS white adipocytes led to highly PPARα expression, with levels around thirty-fold those in basal conditions. PPARα expression led to a significant induction of RBP4 mRNA levels in SGBS white adipocytes after exposure to GW7647 (Fig.6D). This confirmed the positive action of PPARα on the RBP4 gene in the context of adipose cells, either brown or white.

Effects of PGC-1a on RBP4 gene regulation

The requirement of intact PPAR signaling pathway for cAMP responsiveness of RBP4 gene expression, led us to study whether the effects of noradrenergic, cAMP-dependent, stimulus of the RBP4 gene in brown adipocytes could occur through indirect processes. Treatment of brown adipocytes with cycloheximide, a suppressor of protein synthesis, blunted RBP4 mRNA induction in response to norepinephrine (Fig 7A) indicating that, effectively, RBP4 mRNA induction was protein-synthesis dependent. We examined the role of PGC-1α in RBP4 gene regulation in brown adipocytes, considering that PGC-1α expression is induced by

norepinephrine in brown adipocytes via cAMP-dependent signaling and it is a powerful co-activator of PPARα and PPARγ (31). The time-course of norepinephrine effects on gene expression in brown adipocytes showed a much earlier peak of induction of PGC-1α mRNA than RBP4 mRNA (Fig 7B). Cycloheximide did not suppress PGC-1α mRNA induction (17-fold induction of PGC-1α mRNA in the presence of cycloheximide versus 15-fold induction in the absence, 2h exposure to norepinephrine). These results were compatible with the hypothesis that PGC-1α may play a role in stimulus of the RBP4 gene in response to noradrenergic cAMP-mediated signaling, via co-activation of PPARs. This possibility was assessed by studying the effects of cAMP on adipocytes derived from MEFs obtained from wild-type or PGC-1α-null mice. Adipocytes from wild-type MEFs exhibit an intermediate brown-to-white adipocyte phenotype, including the expression of substantial levels of PGC-1α (26, 32 and present results). After adipogenic differentiation MEFs from wild-type and from PGC-1α-null mice acquired adipose morphology and expressed marker genes of adipogenesis, such as ap2 mRNA or PPARγ (data not shown) similarly, as expected from a previous report using RNAi-mediated PGC-1α invalidation systems (33). Basal expression of RBP4 mRNA was slightly impaired in PGC-1α-null adipocytes (71 $\pm$ 28 percent respect to wild-type adipocytes). Adipocytes derived from PGC-1α-null embryos showed a significant impairment in cAMP-mediated induction of RBP4 mRNA expression (Fig 7C), thus indicating that PGC-1α is required for cAMP-mediated, regulation of the RBP4 gene. A further analysis using transfection of HIB-1B cells was undertaken. PGC-1α co-transfection induced RBP4 gene promoter activity as well as enhanced the activation elicited by rosiglitazone and PPARγ co-transfection (Fig 7D). Similar results were observed for PPARα and GW7647 effects (data not shown). The effects of PGC-1α co-transfection were lost for the –1192 hRBP4-Mut-Luc construct, in which the PPAR responsive region is mutated. Finally, the effects of PGC-1α on RBP4 gene expression were analyzed in SGBS white adipocytes, which lack a substantial expression of PGC-1α in contrast with brown adipocytes, but express PPARγ. Adenoviral-mediated over-expression of PGC-1α was enough to case a significant induction of RBP4 mRNA expression (Fig 7E).

Discussion

The present study demonstrates that RBP4 gene expression is tightly regulated in BAT by cAMP-mediated pathways, PPARγ and PPARα .Moreover, the present findings supports the notion that, in contrast to other adipokines that are preferentially released by white (rather than brown) adipocytes, substantial RBP4 expression and release occurs BAT, specially after thermogenic activation.

The cAMP-dependent regulation observed in brown adipocytes may be related to the induction of RBP4 gene expression by the thermogenic stimulus in BAT. A likely scenario after thermogenic stimulus would be that enhanced lipolysis results in increased hydrolysis of retinyl-esters inside the fat depots of BAT, with the subsequent release of retinol and the potential need for coordinate synthesis of the RBP4 protein to bind it. The capacity of BAT to become an enhanced source of retinol and/or RBP4 protein in response to thermogenic stress has not been analyzed to date, but it might be expected to occur on the basis of the present findings. However, we observed that serum RBP4 levels were reduced in mice exposed to cold, in association with reduced RBP4 gene expression in liver (M.Rosell, F.Villarroya and R.Iglesias, unpublished observations). It is likely that lower hepatic expression compensate for enhanced RBP4 production by BAT in conditions of thermogenic stress. In WAT, adrenergic induction of RBP4 gene expression and release might have a distinct physiological significance, as lipolysis in white fat is enhanced in response to starvation, a state of increased RBP4 levels in serum, and reduced insulin action..

Our present findings indicate that PPARγ activation induces RBP4 expression by acting directly on the RBP4 gene promoter. This is in agreement with reports on the action of the PPARγ-activating drugs pioglitazone and troglitazone in human adipocytes in culture (8, 21). The effects of treatment of patients with TZDs on RBP4 levels are controversial. Lowered serum RBP4 levels have been reported after treatment of diabetic (34,35) and HIV-1-infected (36) patients with pioglitazone or rosiglitazone. However, other studies did not record a significant reduction after pioglitazone treatment (37,38). In mice, chronic treatment with rosiglitazone lowers RBP4 serum levels, but only in insulin-resistant mice (3). In patients, a single recent report showed increased RBP4 levels in WAT from pioglitazone-treated patients (8). The apparently contradictory observations of a positive impact of TZDs on RBP4 gene expression in adipose tissues shown here, and the lowered RBP4 circulating levels in several reports of TZD-treated patients, may be due to the complex turnover processes determining RBP4 levels in serum, for which release from adipose tissues is just one more component. Indirect effects of TZDs on non-adipose sites of RBP4 release, or on degradation and renal clearance of RBP4, cannot be excluded and warrant further research. In any case, a direct anti-

diabetic action of TZDs via reduction of RBP4 release by adipose cells is unlikely in light of the present findings.

The present results identify a novel pathway of regulation of RBP4 gene expression by the recognition of PPARα, and the corresponding activating drugs, as a powerful activators of RBP4 gene expression and release by brown adipocytes, the type of adipose cell with preferential expression of PPARα. The fact that the RBP4 gene is part of the cluster of PPARα-regulated genes, usually encoding lipid catabolism proteins, may be related to the aforementioned need for dealing with the free retinol appearing in BAT as a consequence of a high lipolysis during thermogenic activation. On the other hand, the identification of the RBP4 gene as a target of PPARα in brown adipocytes should lead to exploration of whether this pathway is also active in liver, a main site of PPARα and RBP4 gene expression, and a major target of fibrates, PPARα-activating drugs of current use in some dyslipidemias. In a recent study, chronic treatment of obese mice with the PPARα agonist fenofibrate was reported to cause a reduction of RBP4 mRNA expression in WAT (39), although it is unclear whether this was a direct or indirect effect as a consequence of long-term treatment "in vivo". In that report, the action of fenofibrate causing a slight decline in RBP4 mRNA expression in 3T3-L1 adipocytes should be considered to the light of the present findings and other previous reports (40) indicating an extremely low basal expression of RBP4, close to detection limits, in 3T3-L1 adipocytes.

A close interplay between the cAMP and PPAR-dependent pathways of regulation of the RBP4 gene is shown in the present study, and most of the cAMP action on RBP4 gene transcription requires intact PPAR-dependent pathways. A role for PPARα in mediating noradrenergic, cAMP-dependent, regulation of gene transcription in BAT has been proposed (41), and it was attributed to the action of cAMP in promoting lipolysis and the intracellular generation of free fatty acid derivatives, natural activators of PPARs. However, modulation of PPARα activity via protein kinase-A dependent phosphorylation cannot be excluded (42). Nonetheless, our present data strongly support that it is PGC-1α induction by cAMP and the corresponding action of PGC-1α in co-activating PPARα and/or PPARγ what determines most of the cAMP-dependent RBP4 gene regulation.

In summary, we report that PPARγ and PPARα, as well as cAMP, control RBP4 gene expression in brown adipocytes. Induction of PGC-1α expression by cAMP and subsequent co-activation of PPARs play a pivotal role in mediating cAMP effects. Drugs activating PPARγ such as TZDs promote RBP4 gene up-regulation in adipocytes. Fibrates, which act on PPARα, also control RBP4 gene expression in brown adipocytes. Taking into account the recent recognition of active BAT in adult humans (12), further research will be required to establish the role of the induction of RBP4 gene expression and RBP4 protein release by BAT in the

context of the overall systemic responsiveness to these drugs when used in the treatment of insulin resistance and dylipidemias.

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FIGURE LEGENDS

Figure 1. RBP4 mRNA levels in brown and white adipose tissue. Effects of thermogenic stimulus and PPARα-dependent stimulation. A) RBP4 mRNA levels in BAT and WAT from Swiss mice maintained at thermoneutral temperature (28ºC) (TN) or exposed to 4ºC ambient temperature during 24h. B), RBP4 mRNA levels in BAT from lactating mice 6h after treatment with a single injection of Wy14,643 (50 μg/g body weight) or bezafibrate (100 μg/g body weight) (BZ). C) RBP4 mRNA levels in BAT from PPARα-null and wild-type mice exposed to 4ºC ambient temperature over 24h. Bars indicate the mean ± SEM of 6-7 mice from at least 3 different litters. Data are shown as fold-induction relative to control values for each panel. Significant differences between controls and cold-exposed (panels A and C) or with respect to drug-injected mice (panel B) are shown by * $p<0.05$, and the comparison between wild-type and PPARα-null mice by # $p<0.05$.

Figure 2. Effects of hormonal agents on RBP4 mRNA and RBP4 protein levels in brown adipocytes differentiated in culture. A) RBP4 mRNA levels in mouse brown adipocytes differentiated in primary culture and exposed to the indicated agents (see Materials and Methods). B) RBP4 protein in culture medium from brown adipocytes differentiated in culture. An example of immunoblot analysis of RBP4 (24 kDA) in cell culture medium is shown in the C) bottom panel. Cells were treated with 1 μM GW7647; 10 μM rosiglitazone (Rosi); 1 μM GW501516, 100 μM bezafibrate (BZ); 1 μM 9-cis retinoic acid; 10 μM AGN194202; 1 μM all-trans retinoic acid; 10 μM TTNPB; 0.5 μM norepinephrine (NE) or 1 mM dibutyryl-cAMP. Bars indicate the mean ± SEM of 4-6 independent cell cultures for each condition. Data are shown as fold-induction relative to control (untreated cells) values for each panel. Significant differences in comparison to controls are shown by * $p<0.05$.

Figure 3. Responsiveness of RBP4 gene expression to hormonal agents in brown adipocytes lacking PPARα . RBP4 mRNA levels in mouse brown adipocytes obtained from wild-type and PPARα-null mice and exposed to the indicated agents (see Fig2A). Bars indicate the mean ± SEM of 4-6 independent cell cultures for each condition. Data are shown as fold-induction relative to values in non-treated cells from wild-type mice. Significant differences between each treatment condition and non-treated cells for each genetic background

are indicated by * $p<0.05$, and those between wild-type and PPARα-null cells for each treatment condition by # $p<0.05$.

Figure 4. Effects of PPARα and PPARγ antagonists on RBP4 mRNA expression in response to norepinephrine in brown adipocytes. RBP4 mRNA levels in mouse brown adipocytes differentiated in primary culture and exposed to norepinephrine (NE) in the presence or absence of the PPARα antagonist GW6471 (10 μM) and/or the PPARγ-antagonist GW9662 (30 μM). Bars indicate the mean ± SEM of 4-6 independent cell cultures for each condition. Data are shown as fold-induction relative to control (untreated cells) values for each panel. Significant differences in comparison to controls are shown by * $p<0.05$ and those for the effects of NE in the presence of antagonist relative to its absence by # $p<0.05$.

Figure 5. Effects of PPARα and PPARγ activation, and of cAMP on RBP4 promoter A)**,** HIB-1B cells were transfected with the -1192-RBP4-Luc plasmid, the deleted plasmid construct -535-RBP4-Luc or the –1192 hRBP4-Mut-Luc mutated version (see C), and, when indicated, were co-transfected with the plasmid expression vectors for PPARα or PPARγ, and exposed to the PPARα activator GW7647, the PPARγ activator rosiglitazone, and 1 mM dibutyryl-cAMP (cAMP). Bars indicate the mean ± SEM of normalized luciferase activity (see Methods section). Significant differences ($p<0.05$) due to PPAR agonists or cAMP relative to controls for each construct and co-transfection setting are shown by *, those due to PPARα or PPARγ for each condition are shown by #, and those between wild-type and mutated forms of the promoter constructs at the same condition are shown by Δ. B), Chromatin immunoprecipitation of PPARα and PPARγ binding to RBP4 gene promoter. HIB-1B cells were transfected with the -1192-RBP4-Luc and the PPARα and PPARγ expression vectors, and treated with 1μM GW7647 and 10 μM rosiglitazone, respectively. PPARα or PPARγ antibodies or an unrelated IgG were used to immunoprecipitate the protein-DNA complexes. Bars are means ± SEM of relative enrichment of the PCR amplification signal due the PPAR antibodies respect to IgG (see Materials and Methods). Significant differences respect to IgG are shown by * $p<0.05$. C) Sequence of the PPAR responsive element in the RBP4 promoter; site-directed mutations to generate 1192-RBP4-Mut-Luc are shown in lower case, D) Chromatin immunoprecipitation of PPARα and PPARγ binding to the wild-type and mutant forms of the RBP4 gene promoter. HIB-1B cells were transfected and treated as in B), but including either the -1192-RBP4-Luc or -1192-RBP4-Mut-Luc plasmids. Bars are means ± SEM of relative enrichment of the PCR

amplification signal respect to input. Significant differences between the wild-type and the mutated promoter constructs are shown by * $p<0.05$.

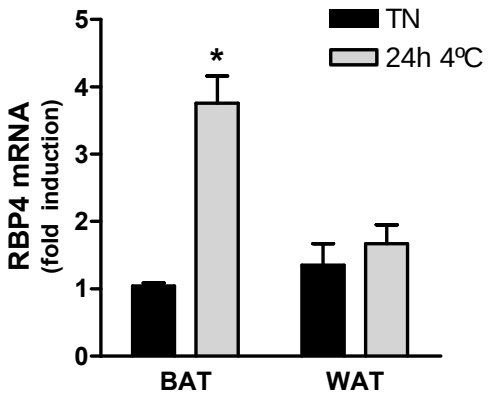
Figure 6. Effects of hormonal agents on RBP4 mRNA and protein levels in murine white adipocytes differentiated in primary culture and in differentiated human SGBS white adipocytes. A) RBP4 mRNA levels in mouse white adipocytes differentiated in primary culture and exposed to the indicated agents (see Materials and Methods). B) RBP4 protein in culture medium from white adipocytes differentiated in culture, C) RBP4 mRNA levels in human SGBS white adipocytes differentiated in culture and exposed to the indicated agents and concentrations (see Legend to Fig 2). D) RBP4 mRNA levels in human SGBS white adipocytes differentiated in culture and transduced with adenoviral vectors driving PPARα or green fluorescent protein (GFP) as a control, and treated or not with the PPARα activator GW7647 (1μM).. Bars indicate the mean ± SEM of 4-6 independent cell cultures for each condition. Data are shown as fold-induction relative to control (untreated cells) values for each panel. Significant differences in comparison to controls are shown by * $p<0.05$.

Figure 7. Regulation of PGC-1α gene expression and effects of PGC-1α on RBP4 gene expression. A) Effects of cycloheximide (5 μg/ml) treatment on the changes in RBP4 mRNA elicited by norepinephrine (0.5 μM). Data are expressed as fold induction relative to 0 hours, B) Time-course of changes in PGC-1α mRNA and RBP4 mRNA expression in brown adipocytes differentiated in primary culture after norepinephrine treatment (0.5 μM). Data are expressed as fold-induction relative to 0 hours. For A) and B), points are means from 2-3 independent experiments with triplicate plates, in which the variation within the experimental groups was less than 15%. C) Adipocytes derived from mouse embryonic fibroblasts from PGC-1α-null and wild-type littermates were exposed to 1 mM dibutyryl-cAMP for 3, 6 and 24 h and induction of RBP4 mRNA expression was expressed as means ± SEM. Statistical differences between PGC-1α-null and wild-type adipocytes are shown as * $p<0.05$; D) HIB-1B cells were transfected with the -1192-RBP4-Luc or –1192-RBP4-Mut-Luc plasmids and, when indicated, co-transfected with the plasmid expression vectors for PPARγ or PGC-1α, and exposed to the PPARγ activator rosiglitazone. Bars indicate the mean ± SEM of normalized luciferase activity. Significant differences ($p<0.05$) in comparison to controls are shown by *, those due to both PPARγ and PGC-1α are shown by #, and those between wild-type and mutated form of the promoter at the same condition are sown by Δ. E) RBP4 mRNA levels in SGBS human white

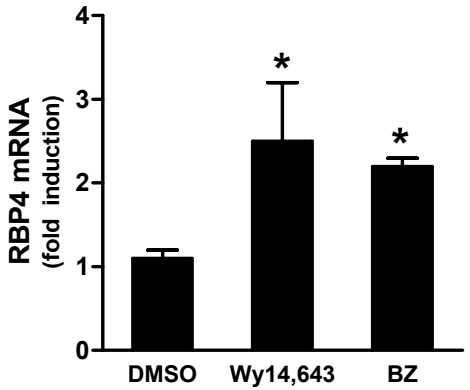
adipocytes differentiated in culture transduced with adenoviral vectors driving PGC-1α, or green fluorescent protein (GFP) as a control. Bars indicate the mean ± SEM of RBP4 mRNA levels for 4-5 independent cell cultures for each condition. Data are expressed as fold-induction relative to non-treated, non-adenoviral-transduced cells

Fig 1

A)


TN
24h 4ºC
RBP4 mRNA
(fold induction)
0
1
2
3
4
5
*
BAT
WAT

B)


RBP4 mRNA
(fold induction)
0
1
2
3
4
*
*
DMSO
Wy14,643
BZ

C)

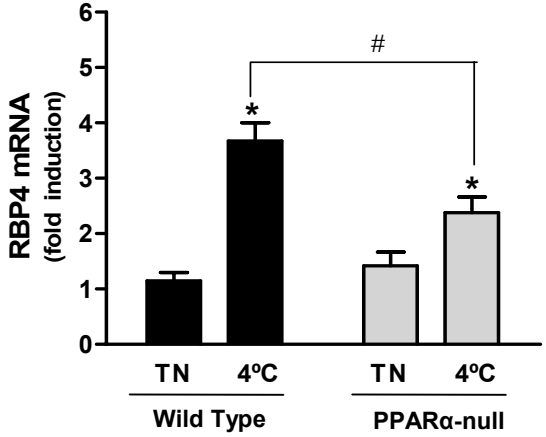

RBP4 mRNA
(fold induction)
0
1
2
3
4
5
6
#
*
*
TN
4ºC
TN
4ºC
Wild Type
PPARα-null

Fig 2

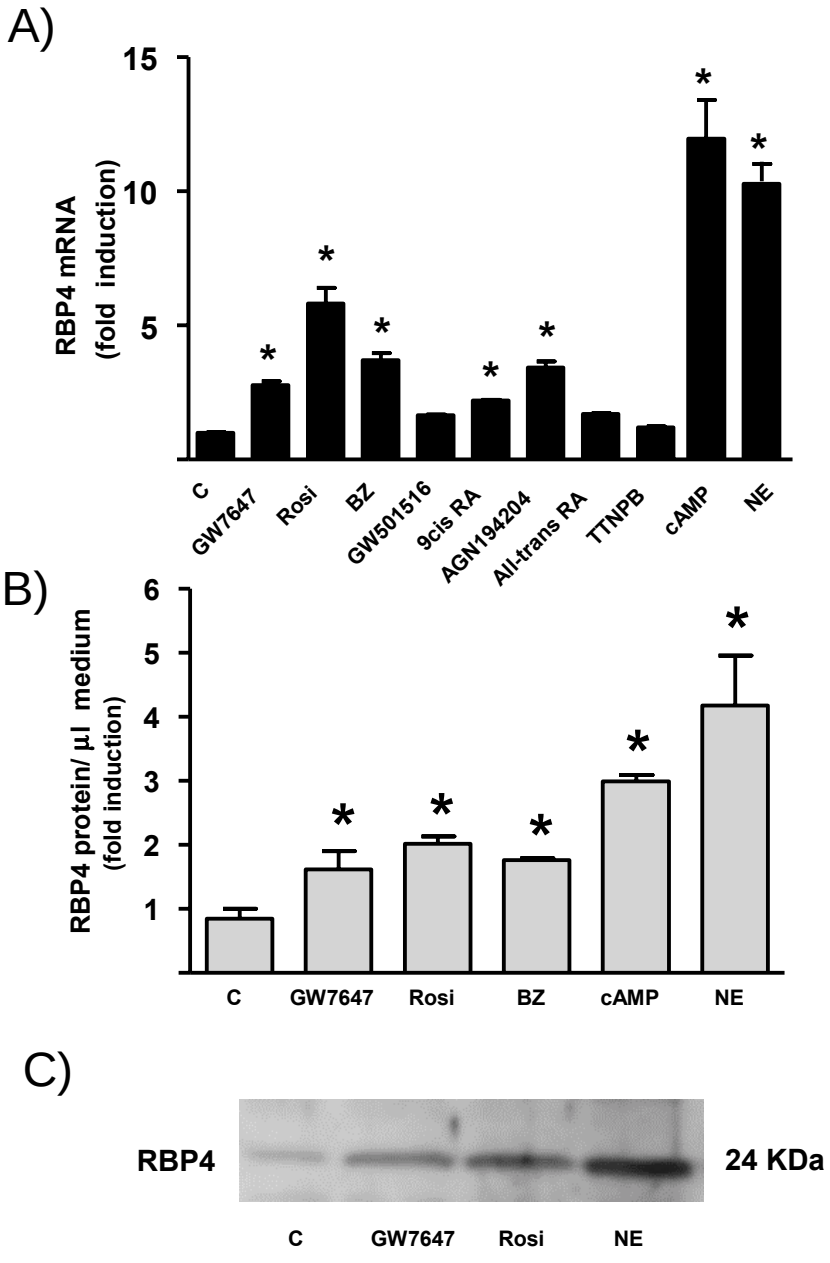

A)
RBP4 mRNA (fold induction)
15
10
5
C
GW7647
Rosi
BZ
GW501516
9cis RA
AGN194204
All-trans RA
TTNPB
cAMP
NE
B)
RBP4 protein/ µl medium (fold induction)
6
5
4
3
2
1
C
GW7647
Rosi
BZ
cAMP
NE
C)
RBP4
24 KDa
C
GW7647
Rosi
NE

Fig 3.

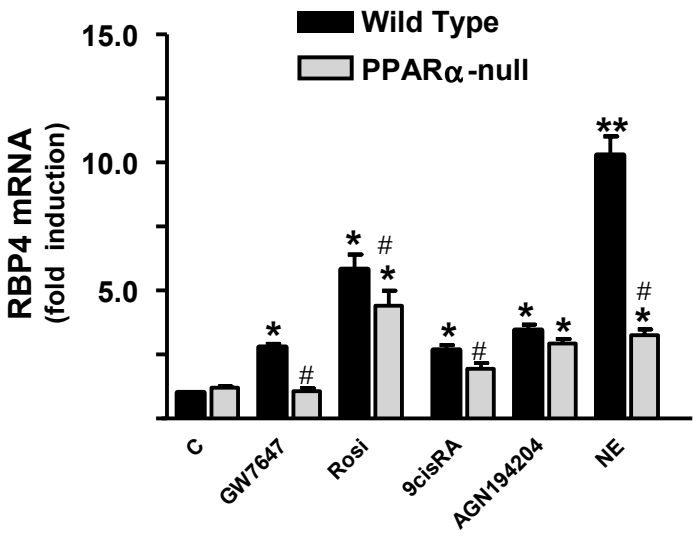

Wild Type
PPARα-null
RBP4 mRNA
(fold induction)
15.0
10.0
5.0
C
GW7647
Rosi
9cisRA
AGN194204
NE

Fig 4.

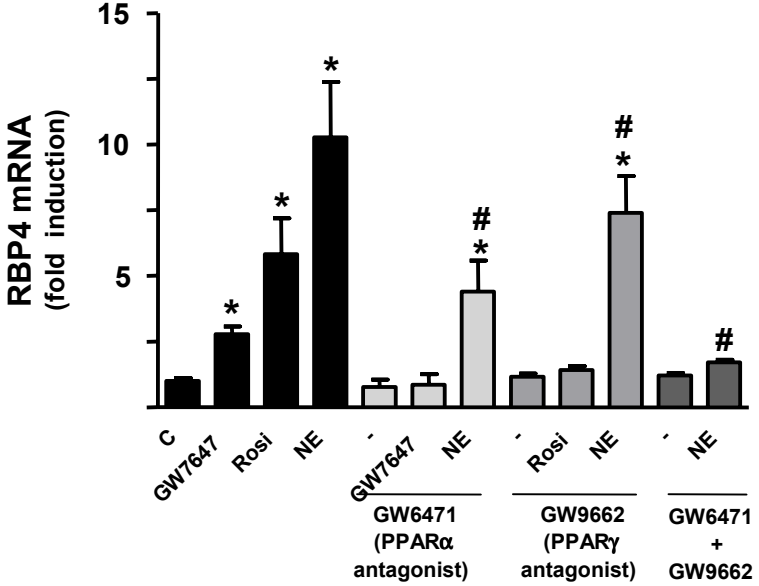

RBP4 mRNA
(fold induction)
15
10
5
C
GW7647
Rosi
NE
-
GW7647
NE
-
Rosi
NE
-
NE
GW6471
(PPARα
antagonist)
GW9662
(PPARγ
antagonist)
GW6471
+
GW9662

Fig 5.

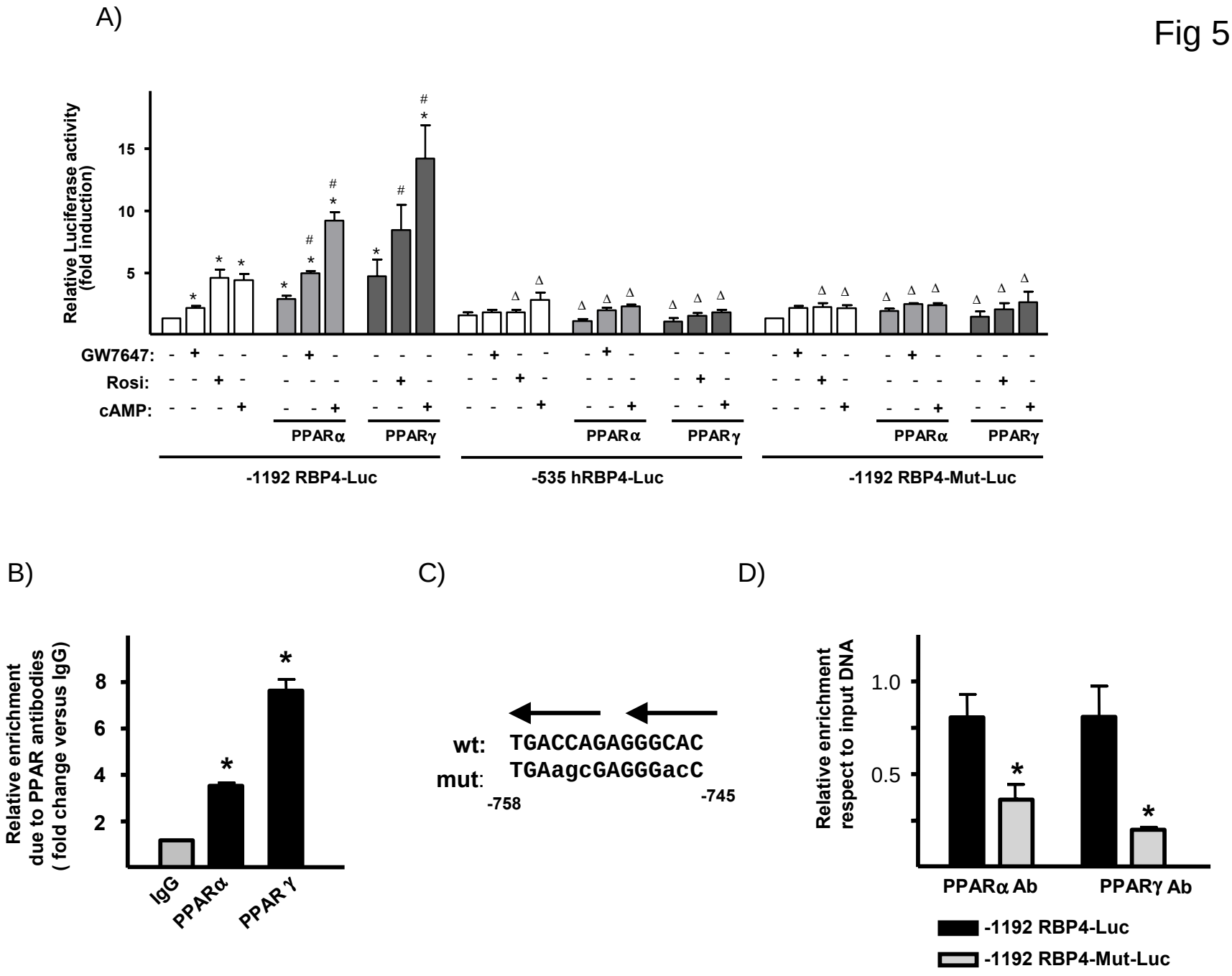

A)
Relative Luciferase activity (fold induction)
15
10
5
GW7647:
Rosi:
cAMP:
PPARα
PPARγ
-1192 RBP4-Luc
-535 hRBP4-Luc
-1192 RBP4-Mut-Luc
B)
Relative enrichment due to PPAR antibodies (fold change versus IgG)
8
6
4
2
IgG
PPARα
PPARγ
C)
wt: TGACCAGAGGGCAC
mut: TGAagcGAGGGacC
-758
-745
D)
Relative enrichment respect to input DNA
1.0
0.5
PPARα Ab
PPARγ Ab
-1192 RBP4-Luc
-1192 RBP4-Mut-Luc

Fig 6.

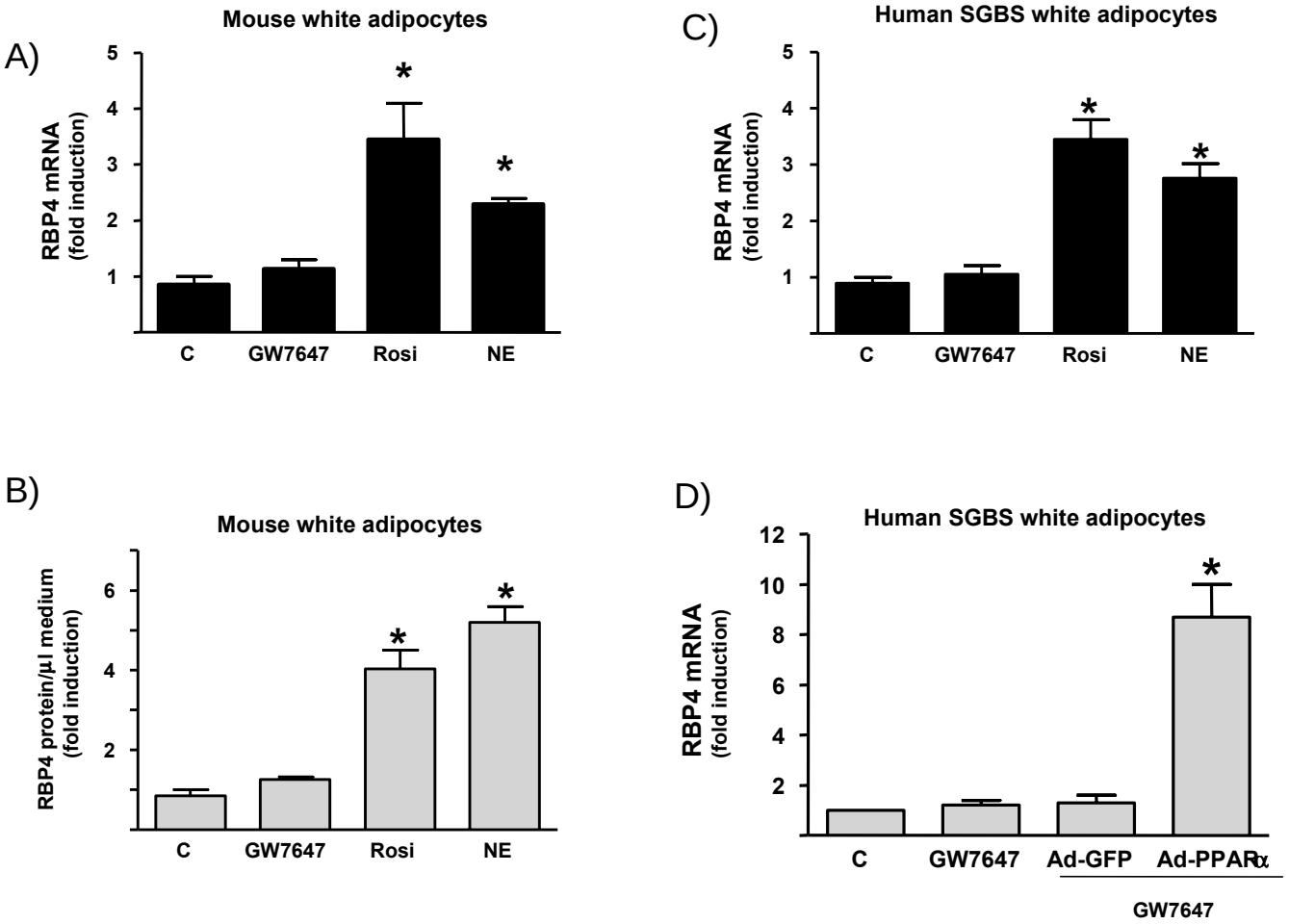
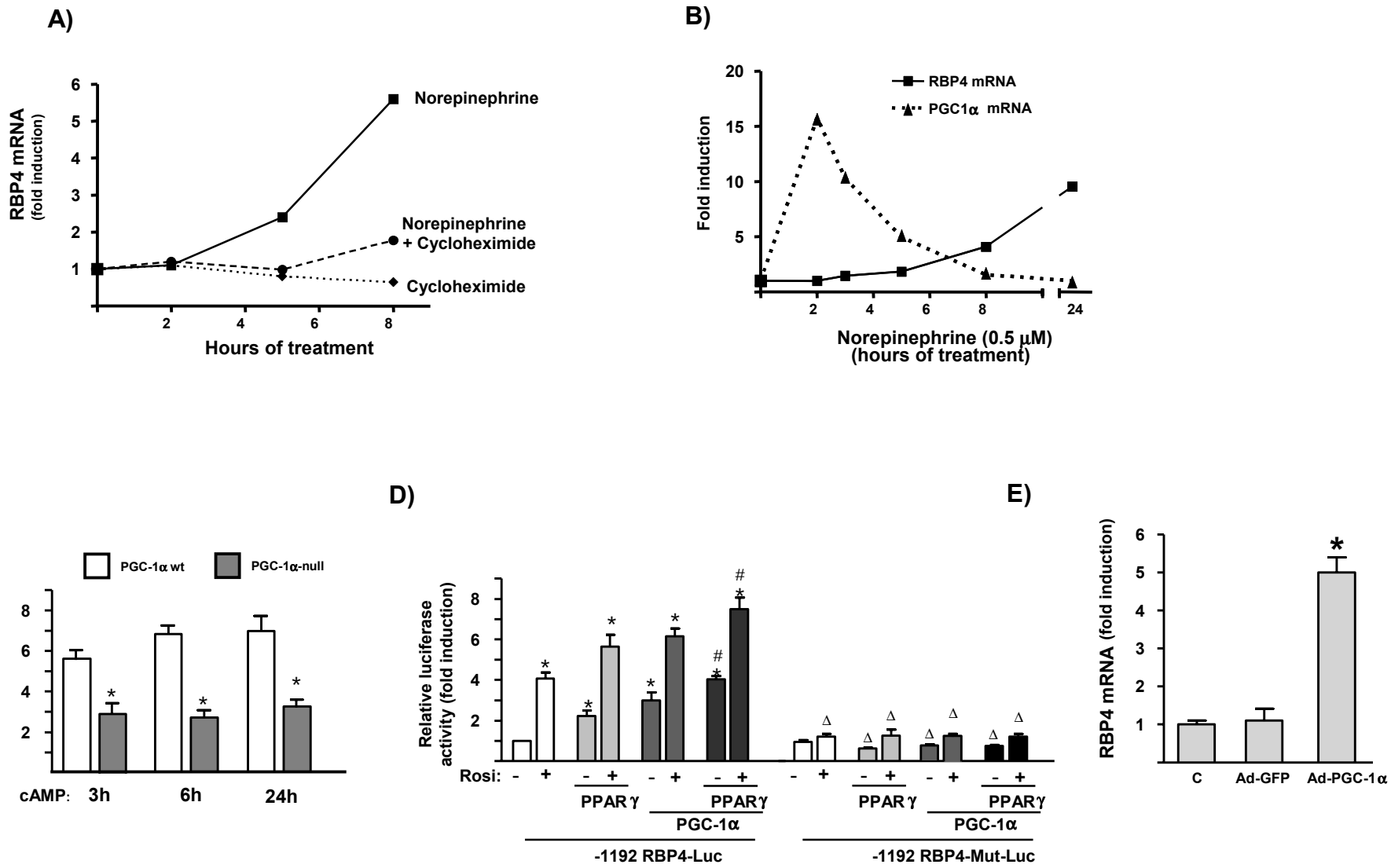

A)
Mouse white adipocytes
RBP4 mRNA (fold induction)
5
4
3
2
1
*
*
C
GW7647
Rosi
NE
C)
Human SGBS white adipocytes
RBP4 mRNA (fold induction)
5
4
3
2
1
*
*
C
GW7647
Rosi
NE
B)
Mouse white adipocytes
RBP4 protein/µl medium (fold induction)
6
4
2
*
*
C
GW7647
Rosi
NE
D)
Human SGBS white adipocytes
RBP4 mRNA (fold induction)
12
10
8
6
4
2
*
C
GW7647
Ad-GFP
Ad-PPARα
GW7647

Fig 7.


A)
RBP4 mRNA (fold induction)
Norepinephrine
Norepinephrine + Cycloheximide
Cycloheximide
Hours of treatment
B)
Fold induction
RBP4 mRNA
PGC1α mRNA
Norepinephrine (0.5 μM) (hours of treatment)
C)
PGC-1α wt
PGC-1α-null
RBP4 mRNA (fold induction)
cAMP: 3h 6h 24h
D)
Relative luciferase activity (fold induction)
Rosi:
PPARγ
PGC-1α
-1192 RBP4-Luc
-1192 RBP4-Mut-Luc
E)
RBP4 mRNA (fold induction)
C
Ad-GFP
Ad-PGC-1α