Noradrenaline represses PPAR (peroxisome-proliferator-activated receptor) γ 2 gene expression in brown adipocytes: intracellular signalling and effects on PPAR γ 2 and PPAR γ 1 protein levels

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PPAR (peroxisome-proliferator-activated receptor) γ is expressed in brown and white adipose tissues and is involved in the control of differentiation and proliferation. Noradrenaline stimulates brown pre-adipocyte proliferation and brown adipocyte differentiation. The aim of the present study was thus to investigate the influence of noradrenaline on PPAR γ gene expression in brown adipocytes. In primary cultures of brown adipocytes, PPAR γ 2 mRNA levels were 20-fold higher than PPAR γ 1 mRNA levels. PPAR γ expression occurred during both the proliferation and the differentiation phases, with the highest mRNA levels being found at the time of transition between the phases. PPAR γ 2 mRNA levels were down-regulated by noradrenaline treatment (EC₅₀, 0.1 μ M) in both proliferative and differentiating cells, with a lagtime of 1 h and lasting up to 4 h, after which expression gradually recovered. The down-regulation was β -adrenoceptor-induced and intracellularly mediated via cAMP and protein kinase A; the signalling pathway did not involve phosphoinositide 3-kinase, Src, p38 mitogen-activated protein kinase or extracellular-signal-regulated kinases 1 and 2. Treatment of the cells with the protein synthesis inhibi-

tor cycloheximide not only abolished the noradrenaline-induced down-regulation of PPAR γ 2 mRNA, but also in itself induced PPAR γ 2 hyperexpression. The down-regulation was probably the result of suppression of transcription. The down-regulation of PPAR γ 2 mRNA resulted in similar down-regulation of PPAR γ 2 and phosphoPPAR γ 2 protein levels. Remarkably, the level of PPAR γ 1 protein was similar to that of PPAR γ 2 (despite almost no PPAR γ 1 mRNA), and the down-regulation by noradrenaline demonstrated similar kinetics to that of PPAR γ 2; thus PPAR γ 1 was apparently translated from the PPAR γ 2 template. It is suggested that β -adrenergic stimulation via cAMP and protein kinase A represses PPAR γ gene expression, leading to reduction of PPAR γ 2 mRNA levels, which is then reflected in down-regulated levels of PPAR γ 2, phosphoPPAR γ 2 and PPAR γ 1.

Key words: brown adipocyte, cAMP, cycloheximide, noradrenaline, peroxisome-proliferator-activated receptor γ 2 (PPAR γ 2), protein kinase A.

INTRODUCTION

The PPAR (peroxisome-proliferator-activated receptor) γ is a ligand-dependent transcription factor that is predominantly expressed in adipose tissue, both white and brown [1,2]. In mouse, there are two PPAR γ isoforms, γ 1 and γ 2, which are derived from the same gene, but are transcribed from different promoters [2a]. The expression of the PPAR γ 2 isoform is practically restricted to adipose tissue [2]. PPAR γ plays an important promoting role in the differentiation of adipocytes [3]. PPAR γ heterodimerizes with the 9-*cis*-retinoic acid receptor, RXR α , and the heterodimer binds to the PPRE (PPAR-response element) [4]. PPRES have been characterized in the promoter/enhancer regions of several genes expressed in adipocytes, such as lipoprotein lipase, aP2 (i.e. fatty acid binding protein) and phosphoenolpyruvate carboxykinase (for review see [5]). Also the gene for the brown-adipose-tissue-specific UCP1 (uncoupling protein 1) contains a PPRE site [6,7].

Due to the importance of PPAR γ for cell differentiation [8], significant interest has developed for the use of PPAR γ activation as an anticancer treatment in different tissues [9]. Activation of PPAR γ in cancer cells is expected to promote differentiation and thus to decrease proliferation. Conversely, during physiologically

induced cell proliferation, the opposite effect may be necessary, i.e. to ensure a low activity of PPAR γ in order to allow for proliferation instead of differentiation. One such physiologically induced state of cell proliferation occurs during the recruitment process in brown adipose tissue [10]. In brown adipose tissue, noradrenaline promotes both cell proliferation and cell differentiation. From studies of cells in culture, it has been concluded that a switch in the responsiveness to noradrenaline occurs: in brown pre-adipocytes, noradrenaline stimulates proliferation, but in brown adipocytes, noradrenaline advances the differentiation process, both processes being mediated by cAMP [10]. It could thus be proposed that this switch in the ability of noradrenaline to promote these different processes may be associated with a switch in the ability of noradrenaline to influence PPAR γ gene expression, from repressing PPAR γ gene expression in brown pre-adipocytes to promoting PPAR γ gene expression in mature brown adipocytes.

To investigate whether and how noradrenaline influences the expression of the transcription factor PPAR γ in brown adipocytes, primary cultures of brown adipocytes were exposed to noradrenaline, the signalling process involved in the control of PPAR γ expression was analysed, and the relationship between PPAR γ mRNA and protein amounts was examined.

Abbreviations used: CREB, cAMP-response-element-binding protein; DMEM, Dulbecco's modified Eagle's medium; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PPAR, peroxisome-proliferator-activated receptor; PPRE, PPAR-response element; TFIIIB, transcription factor IIB; UCP1, uncoupling protein 1.

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EXPERIMENTAL

Animals, cell isolation and cell culture

Male NMRI mice, purchased from local suppliers (B&K or Eklunds, Stockholm, Sweden), were kept at room temperature (approx. 22 °C) for at least 24 h after arrival. At the age of 4 weeks, the mice were killed by CO₂, and the brown adipose tissue was isolated from the interscapular, cervical and axillary depots, principally as described in [11]. The pooled tissue pieces were minced in DMEM (Dulbecco's modified Eagle's medium) and transferred to a digestion solution with 0.2 % (w/v) collagenase (type II; Sigma) in a buffer consisting of 0.1 M Hepes (pH 7.4), 123 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 4.5 mM glucose and 1.5 % (w/v) BSA. The digestion was performed for 30 min at 37 °C with continuous vortex-mixing. The suspension was filtered through a 250 µm pore-size nylon filter (Sintab, Oxie, Sweden) into sterile 10 ml tubes. The filtered suspension was kept on ice for 30 min to let the mature adipocytes float up. The top layer of the suspension was removed, and the rest of the suspension was filtered through a 25 µm pore-size nylon filter (Sintab) and re-centrifuged at 700 *g* for 10 min, to pellet the precursor cells. The pellet was resuspended in 5 ml of DMEM and centrifuged at 700 *g* for 10 min. The pellet was then suspended in culture medium (0.5 ml/animal). The cells were cultured in six 10-cm²-well plates (Corning); 1.8 ml of culture medium was added to each well before 0.2 ml (cells corresponding to 0.4 animals) of cell suspension was added. The culture medium was DMEM with 10 % (v/v) newborn calf serum (Flow Laboratories, McLean, VA, U.S.A.), 4 nM insulin, 25 µg/ml sodium ascorbate, 10 mM Hepes, 4 mM glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin. The cells were grown at 37 °C in an atmosphere of 8 % CO₂ in air with 80 % humidity. The medium was changed on day 1 and then every second day. The medium was pre-warmed to 37 °C before changing. The medium was not changed on the same day as the cells were harvested. The experiments were performed on different days of culture, as indicated in each individual experiment.

Analysis of mRNA levels

After the experiments, the medium was discarded and the cells were harvested from each well with 1 ml of Ultraspec (Biotecx Laboratories, Houston, TX, U.S.A.) as described in the manufacturer's protocol. The RNA obtained was examined by PCR or Northern blotting.

For quantitative real-time PCR, the first-strand cDNA was first synthesized as described previously [12]. RNA expression was then quantified by real-time quantitative PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Each PCR reaction contained, in a final volume of 25 µl, 1.5 µl of first-strand cDNA, 12.5 µl of 2× SYBR Green PCR Master Mix (Applied Biosystems) and 7.5 pmol of each primer [for PPARγ1, forward, 5'-GGA CTG TGT GAC AGA CAA GAT TTG A-3' and reverse, 5'-CTG AAT ATC AGT GGT TCA CCG C-3' (GenBank® accession number U01841); for PPARγ2, forward, 5'-CTC TGT TTT ATG CTG TTA TGG GTG A-3' and reverse, 5'-GGT CAA CAG GAG AAT CTC CCA G-3' (GenBank® accession number U09138); for TFIIIB (transcription factor IIB), forward, 5'-GTT CTG CTC CAA CTT TTG CCT-3' and reverse, 5'-TGT GTA GCT GCC ATC TGC ACT T-3' (GenBank® accession number NM_145546); primers from DNA Technology A/S, Aarhus, Denmark]. All reactions were performed using the following cycling conditions: 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. PCR was carried out in 96-well plates and in triplicate.

The relative amounts of all mRNAs were calculated using the comparative CT method.

For Northern blotting, total RNA was separated on an agarose gel (1.25 %) containing 20 mM Mops (pH 7.0) and 6.7 % formaldehyde. Ethidium bromide (1 mg/ml) was added to the gel to examine the distribution of RNA in UV light. The gel was run for 2–3 h at 100 V. The RNA was transferred on to a Hybond-N membrane (Amersham Biosciences) by capillary blotting overnight. The membrane was prehybridized at 42 °C for at least 1 h in 10 ml of hybridization solution containing 5× SSC (pH 7.0) (1× SSC is 0.15 M NaCl/0.015 M sodium citrate), 5× Denhardt's (1× Denhardt's is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.02 % BSA), 0.5 % (w/v) SDS, 50 mM sodium phosphate (pH 6.5), 50 % formamide and 100 µg/ml degraded herring sperm DNA. Hybridization of the membranes was performed overnight at 42 °C in a fresh hybridization solution with the addition of denatured probe, [³²P]dCTP-labelled cDNA, corresponding to mouse PPARγ mRNA, labelled by random priming (Amersham Biosciences). The PPARγ cDNA contained 454 bp corresponding to positions 127–581 in the published PPARγ2 sequence with GenBank® accession number U09138 (a gift from Dr Bruce Spiegelman, Dana Faber Cancer Research Institute, Boston, MA, U.S.A.). The identity of the probe was confirmed by sequencing. The probe matched the mouse mRNA regions that correspond to exon 1 and exon 2, which are exons common for PPARγ1 and γ2. The probe was thus not specific for PPARγ2, but based on the results in Figure 1(C), the outcome was referred to as PPARγ2. After hybridization, the solution was removed and the membranes were washed twice for 30 min at room temperature in a solution of 2× SSC and 0.2 % (w/v) SDS, followed by washing twice for 60 min at 45 °C in 0.1× SSC and 0.2 % (w/v) SDS. The membranes were sealed in a plastic envelope and then exposed to a PhosphorImager screen and scanned in a MolecularDynamic's PhosphorImager 425 S. Specific mRNA signals were analysed with the ImageQuant software. For analysis, the curve-fitting option of the KaleidaGraph 3.0 application was used as indicated in the legends to the Figures. The uncertainties given are those of the KaleidaGraph estimates.

Analysis of PPAR protein levels

Brown adipocytes were treated with 1 µM noradrenaline for the specified times. After the treatment, the cell cultures were washed twice in PBS and then harvested in a lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2 % (w/v) SDS, 10 % (v/v) glycerol and a protease inhibitor cocktail (Complete Mini; Roche). After sonication, the concentration of the soluble proteins was determined (using the method of Lowry) in the sample, and 1/10 vol of lysis buffer supplemented with 0.5 M dithiothreitol and 1 % (w/v) Bromophenol Blue was added to each sample. Proteins (30 µg) were separated in 12 % polyacrylamide gels containing SDS, and transferred on to a PVDF membrane (Amersham Biosciences) in 48 mM Tris/HCl, 39 mM glycine, 0.037 % (w/v) SDS and 15 % (v/v) methanol using a semi-dry electrophoretic transfer cell (Bio-Rad). Following transfer, the membrane was stained with Ponceau S for examination of equal loading of proteins. After washing, the membrane was blocked in 5 % (w/v) dried milk overnight at 4 °C. The membrane was then incubated in 1:500 diluted primary antibody. The antibody against PPARγ (sc-7273) was obtained from Santa Cruz Laboratories, while the antibody against PPARα (MA1-822) was from Affinity BioReagents. The immunoblots were visualized with horseradish-peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and ECL® (enhanced chemiluminescence; Amersham Biosciences) in

Figure 1 PPAR γ 2 mRNA levels in brown adipocytes during differentiation

Primary cultures of brown adipocytes were grown in culture for the indicated number of days. Where indicated, 0.1 μ M noradrenaline (NA) had been added 3 h before harvest. Total RNA was isolated and analysed as described in the Experimental section. **(A)** Northern blot. Total RNA (5 μ g) was used for each lane and the blot was hybridized with the PPAR γ probe described in the Experimental section. **(B)** PPAR γ mRNA levels during differentiation. Experiments were performed as illustrated in **(A)**. PPAR γ values ($\circ$) with thick lines are from cultures not treated with noradrenaline and are means $\pm$ S.E.M. for four independent experiments, each performed in duplicate. The value at day 5 was in each experiment set to 100 %, and the PPAR γ mRNA levels on the other days were expressed relative to this value in each individual experiment. Values with broken lines are from an additional experiment in which PPAR γ mRNA levels ($\circ$) and noradrenaline-induced UCP1 mRNA levels ($\blacksquare$) were determined in parallel (means from duplicate samples, normalized to 18 S RNA); these data were also adjusted to 100 % for the day of highest mRNA level. **(C)** PPAR γ 1 and PPAR γ 2 mRNA levels in brown adipocytes. Cultures were grown as in **(A)** and then treated with 1 μ M noradrenaline or not for 2 h. RNA was extracted and PPAR γ 1, PPAR γ 2 and TFIIIB mRNA levels were determined by real-time PCR as described in the Experimental section. The PPAR γ 1 and PPAR γ 2 mRNA levels were normalized to the TFIIIB mRNA levels in each sample. The mean total amount of PPAR γ mRNA in untreated cells was set to 100 %. Results are means $\pm$ S.E.M. of triplicate samples; * indicates that the effect of noradrenaline on PPAR γ 2 was significant ($P \leq 0.05$), whereas there was no significant effect on PPAR γ 1 levels. **(D)** Relative effect of noradrenaline on the PPAR γ 2 mRNA level during cell differentiation. Values were calculated from the data in **(A)**, adjusting the non-treated PPAR γ 2 mRNA to 100 % for each day and indicating the relative effect of noradrenaline for each day.

a CCD (charge-coupled device) camera. The blots were quantified using the Image Gauge V3.45 program (Fuji Film).

Chemicals

Noradrenaline bitartrate (Arterenol), A23187, PMA, pertussis toxin, cycloheximide, actinomycin D, forskolin, isoprenaline, 8-bromo-cAMP, CGP-12177, cirazoline, CL-316243 and Ly-294002 were obtained from Sigma/RBI; H89, PP2, PD-98059 and SB-202190 from Calbiochem. PMA, Ly-294002, H89, PP2, PD-98059, SB-202190 and forskolin were dissolved in DMSO.

RESULTS

PPAR γ mRNA levels in spontaneously differentiating brown adipocytes

To examine whether noradrenaline differentially influences PPAR γ gene expression in brown pre-adipocytes compared with differentiated brown adipocytes, brown pre-adipocytes were grown and developed in culture. Under the conditions used, the fibroblast-like pre-adipocytes proliferate until they are confluent

at day 5–6 and then differentiate to mature brown adipocytes in a spontaneous, but highly temporally and qualitatively reproducible, way [10,11,13].

Significant levels of PPAR γ mRNA were observed already in the morphologically undifferentiated cells at day 3 (Figure 1A). In a compilation of a series of experiments (Figure 1B), it is seen that the level of PPAR γ mRNA increased somewhat during the cell culture, to reach a maximum on day 5 of culture. The expression of the established brown adipocyte differentiation marker, noradrenaline-induced UCP1 mRNA, is also displayed in Figure 1(B), together with the corresponding PPAR γ levels; clearly, the maximal PPAR γ expression coincides with the maturation phase of the cells. The presence of PPAR γ already in brown pre-adipocytes and the small increase slightly before the accelerated expression of differentiation markers is in accordance with earlier observations [13]. PPAR γ expression is thus maximal at the time of the switch from the brown pre-adipocyte to the mature brown adipocyte stage.

To examine which PPAR γ transcripts were expressed in these cells, RNA samples from the cell cultures with maximal PPAR γ expression level were analysed by quantitative real-time PCR (Figure 1C, grey bars). As seen, practically no PPAR γ 1 mRNA

was found in the cell cultures; $\geq 95\%$ of the PPAR γ transcript present was PPAR γ 2 mRNA. For comparison, we also examined the PPAR γ 1/PPAR γ 2 mRNA ratio in some mouse tissues. Similarly to the case in the brown adipocytes in culture, PPAR γ 2 was the dominant isoform both in mouse brown adipose tissue ($> 70\%$) and white adipose tissue ($> 75\%$), whereas in liver, the PPAR γ 1 isoform was dominant ($> 90\%$) (results not shown). Based on the results in Figure 1(C), we refer in the following to the detected species on Northern blots as PPAR γ 2.

Effect of noradrenaline on PPAR γ 2 mRNA levels

As shown in Figures 1(A) and (D), PPAR γ mRNA levels were decreased markedly upon noradrenaline treatment. Real-time PCR established that the reduction observed was due to a decrease in the levels of PPAR γ 2, and was not counteracted by an increase in PPAR γ 1 (Figure 1C). The noradrenaline-induced down-regulation of PPAR γ 2 mRNA levels was observed in proliferative as well as in differentiating cells (Figures 1A and 1D); there was thus no switch in the qualitative response between pre-adipocytes and mature adipocytes concerning adrenergic regulation of PPAR γ 2 mRNA levels. The absence of a switch is in contrast with the qualitative switch from noradrenaline promoting cell proliferation in brown pre-adipocytes to noradrenaline promoting cell differentiation in mature brown adipocytes, to the switch from β_1 -adrenoceptors being coupled to adenylate cyclase to β_3 -adrenoceptors being the coupled ones [14], and to the switch from noradrenaline decreasing C/EBP α (CCAAT/enhancer-binding protein α) gene expression to noradrenaline increasing it [15].

For further investigations of the effect of noradrenaline, cells at day 5 in culture were used. To examine the time course of the noradrenaline effect, the cell cultures were treated with noradrenaline, and the levels of PPAR γ 2 mRNA were determined after various lengths of time. The combined results from a series of such experiments are presented in Figure 2(A). There was a marked lagtime of nearly 1 h before a noradrenaline effect was observable, an indication that a complex mediatory system was involved. The lagtime was followed by a marked successive decrease in mRNA, lasting for about 1 h. The PPAR γ 2 mRNA levels then remained low for about 2 h, and then successively increased. The mRNA levels regained control values after about 24 h; with a higher dose of noradrenaline, a doubling of mRNA levels has been reported after 24 h of treatment [16].

To obtain a dose-response curve for the noradrenaline-induced decrease in PPAR γ 2 mRNA levels, cultures were treated for 3 h with different concentrations of noradrenaline (Figure 2B). The effect of noradrenaline was clearly dose-dependent, monophasic and saturable. The PPAR γ 2 mRNA levels were reduced maximally to approx. 50%, even with high concentrations of noradrenaline. The calculated EC_{50} value for noradrenaline was 80 ± 11 nM. This is similar to that of the noradrenaline-induced increase in the gene expression of VEGF (vascular endothelial growth factor) A [17] and ribonucleotide reductase subunit R2 [18], but higher than the EC_{50} (approx. 10 nM) for stimulation of gene expression of UCP1 [11] and the β_1 -adrenergic receptor [19], and much higher than the EC_{50} for the noradrenaline-induced decrease in β_3 -adrenoceptor mRNA (approx. 1 nM) [19,20].

The effects of noradrenaline-induced repression of PPAR γ 2 gene expression on PPAR γ protein levels

To study whether the norepinephrine-induced repression of PPAR γ 2 gene expression resulted in lower levels of the corresponding protein, we examined samples of norepinephrine-treated brown adipocytes by immunoblotting.

Figure 2 Effect of noradrenaline (NA) on PPAR γ 2 mRNA levels

(A) Time curve. Primary cultures of brown adipocytes on day 5 were treated with $0.1 \mu\text{M}$ noradrenaline for the indicated lengths of time, and analysed for PPAR γ 2 mRNA as in Figure 1(A). The curve is based on two to nine independent experiments (except at 21 h, where only one was performed) in duplicate wells. In each experiment, the PPAR γ 2 mRNA value from non-treated cells was set to 100% and the other values were expressed relative to this. Results are means $\pm$ S.E.M. The curve was drawn by the 'smooth' function of KaleidaGraph. (B) Dose-response curve. Brown adipocyte cultures (day 5) were treated with the indicated concentrations of noradrenaline for 3 h and analysed as in Figure 1(A). Results are means $\pm$ S.E.M. for five independent experiments. In each experiment, the control level was set to 100% and the other values were expressed relative to this. The results were analysed for adherence to Michaelis-Menten kinetics with the formula: $\text{mRNA}_{\text{NA}} = 100\% - \Delta\text{mRNA}_{\text{max}} \cdot [\text{noradrenaline}] / (EC_{50} + [\text{noradrenaline}])$ (excluding the $100 \mu\text{M}$ value). The EC_{50} value thus obtained was 80 ± 11 nM noradrenaline and the maximal decrease ($\Delta\text{mRNA}_{\text{max}}$) was $55 \pm 1\%$.

Using a commercial antibody against PPAR γ (sc-7273), which can recognize both PPAR γ 2 and PPAR γ 1, we observed that the brown adipocyte protein extracts displayed three bands (Figure 3A). The upper two bands were identified as the phosphorylated and non-phosphorylated forms of PPAR γ 2. This was based on the lower of these two having the expected molecular mass of PPAR γ 2 (56 kDa) [21], and in a parallel immunoblot, protein extracts from 3T3-L1 adipocytes and from cells over-expressing PPAR γ 2 protein displayed similar bands (results not shown). As a negative control (Figure 3A), we used a protein extract from mouse heart, as heart does not express PPAR γ 2 [22]. Treatment of brown adipocyte protein extracts with calf intestinal phosphatase before electrophoresis resulted in the disappearance of the upper of these bands (results not shown), confirming the phosphoprotein nature of this band. The intensities of both these bands were decreased markedly after noradrenaline stimulation (Figure 3A, and see below).

Figure 3 Effect of noradrenaline (NA) on PPAR γ and PPAR α protein levels

Primary cultures of brown adipocytes were treated essentially as indicated in Figure 2, and protein was extracted and analysed by immunoblotting as described in the Experimental section; each lane contained 30 μ g of protein from brown adipocytes (b.a.) non-treated (–) or treated for 4 h with 1 μ M noradrenaline, or from mouse heart homogenate. In (A), an anti-PPAR γ antibody was used; in (B), an anti-PPAR α antibody was used. The sizes (in kDa) indicated to the left are from protein size markers; the arrows to the right indicate the suggested nature of the bands (see text).

The anti-PPAR γ antibody also recognized a band of a molecular mass of 52 kDa. The intensity of this band was also decreased markedly after noradrenaline treatment (Figure 3A). The molecular mass of both PPAR γ 1 and PPAR α is approx. 52 kDa, and due to the similarity between PPAR γ and PPAR α , the possibility existed that the anti-PPAR γ antibody also recognized PPAR α . PPAR α is also expressed in brown adipocytes [13], but as demonstrated above (Figure 1C), PPAR γ 1 mRNA is nearly absent from the brown adipocytes examined in the present study, and PPAR γ 1 protein would therefore not *a priori* be expected. Attempts to separate PPAR α and PPAR γ 1 were made with larger gels and utilizing protein from cell lines retrovirally transduced with PPAR α and PPAR γ , but a separation was not achieved (results not shown).

Therefore, to examine the possibility that the 52 kDa band resulted from non-specific interaction of the anti-PPAR γ antibody with PPAR α , we analysed samples using a commercial antibody (MA1-822) against PPAR α . As seen (Figure 3B), this antibody recognized a band at $\approx$ 52 kDa in heart preparations, in accordance with the reported high expression of PPAR α in this tissue [22]. Similarly, this anti-PPAR α antibody recognized a protein in the brown adipocyte extracts, principally in agreement with brown adipocytes expressing the mRNA of PPAR α [13]. Notably, the band recognized by the anti-PPAR α antibody did not diminish in intensity as an effect of noradrenaline treatment (Figure 3B).

However, since the anti-PPAR γ antibody could not recognize any protein of 52 kDa in the heart preparation (Figure 3A) that clearly contained very significant amounts of PPAR α (Figure 3B), we conclude that the band recognized by the anti-PPAR γ antibody at 52 kDa cannot be PPAR α ; it is therefore probably PPAR γ 1. This conclusion is unexpected, considering the >20 -fold predominance of PPAR γ 2 mRNA levels over PPAR γ 1 mRNA levels in this system (Figure 1C). It is, of course, possible that the translation efficiency is 20-fold higher for PPAR γ 1 than for PPAR γ 2 mRNA. However, alternatively, since the mRNA

Figure 4 Time course of noradrenaline (NA) effect on PPAR γ 1 and PPAR γ 2 levels

Primary cultures of brown adipocytes were treated essentially as indicated in Figure 2, and protein was extracted and analysed for PPAR γ by immunoblotting as described in Figure 3. (A) Example of time curve. (B) Compilation of results from experiments as those illustrated in (A). For each time point, the level of PPAR γ 1 and PPAR γ 2 was set to 100% and the corresponding level in NA-treated cultures was calculated; values at each time point are from one culture or means $\pm$ S.E.M. from two cultures, each analysed singly or duplicate. For the PPAR γ 2 levels, the line connecting the data points has been drawn ignoring the 4 h data point.

template for PPAR γ 2 also contains the full template for PPAR γ 1, including an undisturbed translation-initiation sequence, we suggest that the PPAR γ 1 is formed from the PPAR γ 2 template. The levels of PPAR γ 1 and PPAR γ 2 are about the same, as judged from the intensities on the immunoblot (Figure 3A). This may be interpreted that the translation machinery, with equal probability, commences at the PPAR γ 2 and PPAR γ 1 initiation sites on the PPAR γ 2 mRNA; alternatively, each protein could be translated in parallel from both the PPAR γ 2 and the PPAR γ 1 initiation sites, as has been discussed for other proteins [23].

In Figure 4(A), an example of a time curve of the effect of noradrenaline on PPAR γ protein levels is shown, and in Figure 4(B), a compilation of results from several experiments with different, overlapping, time points is displayed. It is seen that both PPAR γ 2 and PPAR γ 1 protein amounts decrease, finally reaching a level of approx. 50% of the starting value; this level is not statistically significantly different for PPAR γ 2 and PPAR γ 1 (in the analysis, the phosphoPPAR γ 2 and non-phosphorylated PPAR γ 2 bands were combined). A 50% reduction is what would be expected from the 50% reduction in PPAR γ 2 mRNA observed after

noradrenaline treatment (Figures 1C, 2A and 2B). The curve shapes adhere to being delayed reflections of the kinetics of the changes in PPAR γ 2 mRNA levels (Figure 2A). Remarkably, the decrease in PPAR γ 1 protein level was somewhat more rapid than the decrease in PPAR γ 2 level (the half-lives of PPAR γ 2 protein and PPAR γ 1 protein do not have to be identical even if they are both translated from the same template). Taken together, these kinetic data support the suggestion that PPAR γ 2 and PPAR γ 1 are both translated from PPAR γ 2 mRNA.

We therefore conclude that the noradrenaline-induced repression of PPAR γ gene expression that leads to a reduction in PPAR γ 2 mRNA levels, and also results in a parallel, but delayed, decrease in the protein levels of PPAR γ 2 and PPAR γ 1. The mechanism behind the repression of PPAR γ gene expression was therefore studied further.

The half-life of PPAR γ 2 mRNA

The decrease in PPAR γ 2 mRNA levels induced by noradrenaline could be due either to an increase in the degradation rate of PPAR γ 2 mRNA or to an inhibition of the transcription of the PPAR γ 2 gene. To discriminate between these possibilities, the effects of noradrenaline were compared with those of the RNA synthesis inhibitor actinomycin D. The inhibitor was added to the cultures at a concentration that fully blocks noradrenaline-induced expression of the UCP1 gene [24]. The results of these experiments are shown in Supplementary Figure 1 (see <http://www.BiochemJ.org/bj/382/bj3820597add.htm>). The effect of noradrenaline addition in itself was in accordance with the time curve in Figure 2(A), with a lagtime of approx. 1 h and with a successive decrease thereafter, analysed here to correspond to a half-life of 42 min (range 38–47 min). The effect of actinomycin D was similar: the PPAR γ 2 mRNA levels decreased with a half-life of 35 min (range 27–50 min). When the cells were exposed to actinomycin D in combination with noradrenaline, the mRNA half-life was 43 min (range 39–46 min). As there was no significant difference between the half-lives under these three conditions, these experiments indicate that cessation of transcription is an adequate explanation for the decrease in PPAR γ 2 mRNA levels after noradrenaline stimulation.

Identification of the adrenergic receptors mediating the noradrenaline effect

To identify the adrenergic receptors involved in the mediation of the noradrenaline-induced down-regulation of PPAR γ 2 mRNA levels, cell cultures were treated with various adrenergic agents.

To examine if the α_1 -adrenergic receptor could be involved, the cultures were treated with the α_1 -adrenergic receptor agonist cirazoline. However, cirazoline was not able to mimic the noradrenaline-induced decrease in PPAR γ 2 mRNA levels (Figure 5A). Effects of the second messengers known to mediate the α_1 -adrenergic effects in these cells, i.e. calcium [25,26] and protein kinase C [27], were also investigated. The intracellular levels of calcium in the brown adipocytes were elevated by the calcium ionophore A23187 [28], but this did not lead to repression of PPAR γ 2 gene expression (Figure 5A). Similarly, protein kinase C was activated by the phorbol ester PMA [27], but this did not influence PPAR γ 2 gene expression (Figure 5A). Thus the noradrenaline effect on PPAR γ 2 mRNA levels is not mediated via the α_1 -adrenoceptor or α_1 -associated pathways.

To examine whether stimulation of the β -adrenergic receptors was involved, the effect of the general β -agonist isoprenaline was investigated (Figure 5B). Isoprenaline could mimic the effect of noradrenaline, indicating that this effect of noradrenaline was

Figure 5 Pharmacological characterization of adrenoceptors mediating the noradrenaline (NA) effect on PPAR γ 2 gene expression

Primary cultures of brown adipocytes were grown in culture. On day 5, the indicated agonists were added to the cell cultures 3 h before harvesting. Total RNA was isolated and the PPAR γ 2 mRNA levels were analysed as in Figure 1(A). **(A)** α_1 -Adrenergic pathways were analysed by treatment with noradrenaline (0.1 μ M; $n=7$), the α_1 -adrenergic receptor agonist cirazoline (cir; 1 μ M; $n=7$), the calcium ionophore A23187 (A23; 1 μ M; $n=2$), and the protein kinase C agonist PMA (50 nM; $n=2$). The mean PPAR γ 2 mRNA level in untreated cells (C) was set to 100 % in each series. **(B)** β -Adrenergic pathways were analysed by treatment with noradrenaline (1 μ M; $n=7$), with the β -adrenergic receptor agonist isoprenaline (iso; 1 μ M; $n=3$), the β_3 -adrenergic receptor agonists CGP-12177 (CGP; 10 μ M; $n=4$) and CL-316243 (CL; 1 μ M; $n=3$), with forskolin (forsk; 5 μ M; $n=4$) or 8-bromo-cAMP (8-Br; 0.1 μ M; $n=1$). In **(A)** and **(B)**, ***, ** and * indicate $P < 0.001$, $P < 0.01$ and $P < 0.05$ respectively for effect of treatment (Student's paired t test). Results are means $\pm$ S.E.M.

mediated via β -receptors. In these brown adipocytes on day 5, both β_1 - and β_3 -adrenergic receptors are present, but no β_2 -adrenergic receptors [14,19]. To test whether the β_3 -adrenergic receptors could mediate the signal, the β_3 -adrenoceptor agonists CGP-12177 and CL-316243 were used. These β_3 -adrenergic receptor agonists gave similar results to those of isoprenaline or noradrenaline (Figure 5B). Thus the β_3 -adrenoceptors were competent in mediating the adrenergic signal. However, the β_3 -adrenergic receptors as such (as compared with the β_1 -adrenergic receptors) were not essential for the adrenergic effect, because noradrenaline was able to decrease PPAR γ 2 mRNA levels already in cells that had only been 3 days in culture (Figure 1), i.e. before the β_3 -adrenoceptors are expressed and when only β_1 -adrenoceptors are found [14].

Classically, β -adrenergic receptors are considered to be coupled to G $_s$ -proteins that stimulate adenylate cyclase, leading to an increase in the level of the second messenger cAMP. To investigate whether stimulation of this pathway could mimic the effects of noradrenaline stimulation, the brown adipocytes were exposed to the adenylate cyclase activator forskolin or to the cAMP analogue 8-bromo-cAMP. Both agents induced a down-regulation of the PPAR γ 2 mRNA levels to about the same degree as did noradrenaline treatment (Figure 5B). Thus the β -adrenergic effect is fully mimicked by cAMP and does not require receptor activation. As the cAMP level obtained after forskolin is markedly higher than that after noradrenaline [26], the extent of repression of PPAR γ 2 gene expression (to approx. 50 % of control) was apparently not limited by the amount of cAMP, but by a process downstream from cAMP.

Investigation of possible β -adrenergic-receptor-associated signalling pathways

The intracellular pathways involved in the noradrenaline-induced down-regulation in PPAR γ 2 mRNA levels were investigated further by studying the effects of pathway-specific intracellular inhibitors. For this, the cells were pre-treated with these inhibitors 1 h before and during a 3 h exposure to noradrenaline (see Supplementary Figure 2 at <http://www.BiochemJ.org/bj/382/bj3820597add.htm>). Again, exposure to noradrenaline in otherwise untreated cultures led to a reduction in PPAR γ 2 mRNA levels to about 50% (Supplementary Figure 2A). In Supplementary Figure 2(B), the effect of noradrenaline, i.e. the Δ PPAR γ 2 mRNA value, is depicted, for easier comparison of this parameter between the different inhibitors.

In 3T3-F442A adipocytes, β_3 -adrenoceptors have been suggested to be able to directly stimulate G_i -proteins [29,30]. If a β_3 -adrenergic-receptor-coupled G_i -protein pathway was involved in the mediation of the noradrenaline-induced repression of PPAR γ 2 gene expression in the brown adipocytes studied here, inhibition of G_i -proteins by pertussis toxin should inhibit the noradrenaline effect; this was clearly not the case (Supplementary Figure 2). This postulated β_3 -adrenoceptor-coupled G_i -protein pathway has also been proposed to directly stimulate PI3K (phosphoinositide 3-kinase) in adipocytes [29], and this kinase, stimulated either in this way or through the classical β -adrenergic receptor/ G_s /cAMP pathway, could then mediate the repression. However, when the cultured brown adipocytes were treated with the PI3K inhibitor Ly-294002 at a concentration that inhibits insulin-induced glucose uptake in cultured brown adipocytes [31], the noradrenaline effect on PPAR γ 2 mRNA levels was unchanged (Supplementary Figure 2).

In cultured brown adipocytes, PKA (protein kinase A) further mediates the cAMP signal towards UCP1 gene expression [32]. To investigate whether PKA also mediates the noradrenaline effect on PPAR γ 2 gene expression, the PKA inhibitor H89 was used at a concentration that abolishes noradrenaline-induced UCP1 gene expression [32]. H89 abolished the noradrenaline-induced decrease in PPAR γ 2 mRNA levels (Supplementary Figure 2B). This outcome is thus compatible with the noradrenaline-induced decrease in PPAR γ 2 mRNA levels being PKA-mediated, but non-specific effects of inhibitors can never be totally excluded.

β_3 -adrenoceptor pathways (as well as α_1 -adrenoceptor pathways) activate the cytosolic tyrosine kinase Src in brown adipocytes [33]. Therefore the Src inhibitor PP2 was used at a concentration that fully abolishes noradrenaline-induced and Src-mediated phosphorylation of ERK1/2 (extracellular-signal-regulated kinase 1 and 2) in cultured brown adipocytes [17]. However, PP2 had only a marginal effect on the noradrenaline-induced decrease in PPAR γ 2 mRNA levels.

In brown adipocytes, noradrenaline activates the MAPK (mitogen-associated protein kinase) ERK1/2 through MEK (MAPK/ERK kinase) activation [34]. That activation of ERK1/2 may be mediatory for the effect of noradrenaline on PPAR γ 2 levels may be suggested, since PPAR γ 2 is phosphorylated by MAPK in adipocyte cell lines, and the phosphorylated PPAR γ 2 suppresses the transcriptional activity of PPAR γ 2 and thus adipogenesis [35]. However, the MEK inhibitor PD-98059, at a concentration that inhibits the noradrenaline effect on ERK1/2 phosphorylation [34], had no effect on the noradrenaline-induced decrease on PPAR γ 2 mRNA levels (Supplementary Figure 2).

In brown adipocytes, noradrenaline also activates p38 MAPK [36]. Therefore the p38 inhibitor SB-202190 was added to the cells (Supplementary Figure 2B), but this did not have a significant effect on the noradrenaline-induced repression of PPAR γ 2 gene expression (Supplementary Figure 2).

Thus the data observed were compatible with a β -adrenergic pathway, including cAMP and PKA activation, but not involving G_i , PI3K, Src, ERK1/2 or p38 MAPK.

Further mediation of the adrenergic signal requires protein synthesis

As a lagtime was observed for the noradrenaline-induced repression of PPAR γ 2 gene expression (Figure 2A), it is feasible that the effect requires the synthesis of a new protein, rather than being due to a direct effect on existing proteins. The cultures were therefore treated with the protein synthesis inhibitor cycloheximide at a concentration which blocks translation in differentiated brown adipocytes in culture [37]. Cycloheximide fully blocked the ability of noradrenaline to decrease PPAR γ 2 mRNA levels (Supplementary Figure 3 at <http://www.BiochemJ.org/bj/382/bj3820597add.htm>). Additionally, after a lagtime of about 1 h, it evoked in itself (or equally in the presence of noradrenaline) an increase in PPAR γ 2 mRNA levels. The levels were doubled after a further 1 h and may apparently increase further during the next 6–12 h [13]. These results indicate that protein synthesis is required for mediation of the adrenergic signal and could also imply that a continually synthesized protein with a rapid turnover leads to tonic repression of PPAR γ 2 mRNA synthesis.

DISCUSSION

In the present study, we found that the PPAR γ mRNA isoform expressed in brown adipocytes was predominantly PPAR γ 2. In primary cultures of brown adipocytes, PPAR γ 2 mRNA was found already in the undifferentiated brown pre-adipocytes. The expression peaked at day 5, just as the cells reached confluence and entered differentiation. Noradrenaline induced a transient decrease in PPAR γ 2 mRNA levels in both brown pre-adipocytes and in brown adipocytes, and this resulted in parallel decreases in the protein levels of phosphoPPAR γ 2, PPAR γ 2 and PPAR γ 1. A tentative pathway for the intracellular mediation of the noradrenaline-induced repression of gene expression may be formulated (see Supplementary Figure 4 at <http://www.BiochemJ.org/bj/382/bj3820597add.htm>).

PPAR γ 2 mRNA dominance in brown adipocytes

We found that the PPAR γ 2 mRNA levels exceeded the PPAR γ 1 mRNA levels approx. 20-fold in the cultured brown adipocytes. The relative levels of PPAR γ 1 mRNA compared with those of PPAR γ 2 mRNA have not previously been determined in brown adipocytes, but a predominant expression of PPAR γ 2 (compared with that of PPAR γ 1) in mouse adipocyte cell lines has been mentioned previously [2]. In mouse brown and white adipose tissue, we also observed a clear predominance of PPAR γ 2 mRNA (as mentioned in the Results section), and, similarly, in human white adipose tissue, a 20-fold excess of PPAR γ 2 mRNA has been found [38]. However, there are also reports that roughly equal levels of PPAR γ 1 and PPAR γ 2 mRNA, or a predominance of PPAR γ 1, are found in mouse [39] and human [40] white adipose tissue. These qualitative differences in the PPAR γ 1/PPAR γ 2 mRNA ratios reported (if not methodological) may be related to the prevailing hormonal status.

The noradrenaline effect

When brown pre-adipocytes or adipocytes were treated with noradrenaline, down-regulation of the PPAR γ 2 mRNA levels occurred within the first 2–4 h after treatment (Figures 1 and 2).

The down-regulation was transient. In the brown adipocyte-like cell line HIB-1B, both a noradrenaline-induced decrease [6] and a noradrenaline-induced increase [13] in PPAR γ 2 mRNA levels have been noted (4–5 h after stimulation), perhaps reflecting variability in the characteristics of the cell line and phenotypic drift in the cell line. The noradrenaline-induced repression observed here in brown adipocyte primary cultures may be related to reported effects of exposure to cold on PPAR γ 2 gene expression in brown adipose tissue. Cold exposure leads to increased release of noradrenaline *in situ* in brown adipose tissue, and cold exposure has been reported to induce a decrease in PPAR γ 2 mRNA levels [41,42].

Noradrenaline stimulates proliferation in brown pre-adipocytes and differentiation in mature brown adipocytes. Therefore, in accordance with present formulations concerning a positive role of PPAR γ 2 for cell differentiation and a concurrent inhibitory role in cell proliferation, a shift from a noradrenaline-induced repression of transcription in brown pre-adipocytes to a noradrenaline-induced augmentation of transcription in differentiated brown adipocytes would have been expected. A notable observation here was therefore the lack of a switch in the qualitative response to noradrenaline between brown pre-adipocytes and brown adipocytes (Figure 1).

Cellular mediation of the noradrenaline effect

Based on the observations reported here, we suggest a pathway for the intracellular signalling from adrenergic stimulation to repression of PPAR γ 2 gene expression and decreased levels of PPAR γ 2 and PPAR γ 1, as depicted in Supplementary Figure 4. The basis for this pathway is as follows.

Although α_1 -, α_2 -, β_1 - and β_3 -adrenoceptors are present in brown adipocytes, the experiments with adrenergic agonists and inhibitors or activators of the corresponding signalling processes (Figure 5 and Supplementary Figure 2) demonstrated that only β -adrenoceptors (both β_1 - and β_3 -adrenoceptors) could mediate the repression. cAMP levels are increased after noradrenaline stimulation in these cell cultures [26,43]. The repression of PPAR γ 2 mRNA levels is probably mediated via this cAMP, as a repression could also be induced by forskolin or 8-bromo-cAMP (Figure 5B). In contrast, in HIB-1B pre-adipocytes [6] and in rabbit type II pneumonocytes [44], dibutyryl-cAMP increased PPAR γ 2/ γ 1 mRNA levels. Thus no general response of the PPAR γ 2 gene to cAMP increases can be formulated.

PKA is activated by noradrenaline/cAMP in brown adipocytes [32]. Based on investigation of the effects of different kinase inhibitors (Supplementary Figure 2), it seems likely that the noradrenaline-induced decrease in PPAR γ 2 mRNA levels is mediated by PKA, since the effect was totally abolished by the inhibitor H89, but not by any other inhibitor tested.

The response could proceed further via CREB (cAMP-response-element-binding protein) phosphorylation. Noradrenaline, through PKA activation, induces phosphorylation of the transcription factor CREB also in primary cultures of brown adipocytes [45,46]. However, in other cell types (3T3-L1 cells), it has been observed that a constitutively active CREB construct induces (not represses) the expression of PPAR γ 2 [47], and dominant-negative CREB constructs inhibit PPAR γ 2 gene expression [47]. As noradrenaline inhibits PPAR γ 2 transcription in the brown adipocytes used in the present study, it is unlikely that CREB is a direct activator of PPAR γ 2 gene expression.

In this context, it may be noted that the noradrenaline effect has a lagtime (Figure 2A), suggesting that an activation/inactivation of an existing transcription factor cannot easily explain the mediation; instead there could be a requirement for protein

synthesis in the mediation process. Inhibition of protein synthesis indeed abolished the effect of noradrenaline (Supplementary Figure 3). As inhibition of protein synthesis not only abolished the noradrenaline effect, but also led to superinduction of PPAR γ 2 (Supplementary Figure 3) [13], the results can be interpreted as indicating the existence of a mediatory protein. Although there could be other effects of protein synthesis inhibition [48], the data would be compatible with a mediatory protein being expressed at a basal level, having a relatively short half-life (< 1 h) and acting as a transcriptional repressor on the PPAR γ 2 gene. When protein synthesis is inhibited, the regulatory protein would successively disappear, and so thus would repression, leading to increased levels of PPAR γ 2 mRNA. On the other hand, noradrenaline stimulation (e.g. through CREB phosphorylation) could enhance the rate of synthesis of the mediatory repressor protein, leading to an increased repression of PPAR γ 2 transcription.

Both PPAR γ 2 and PPAR γ 1 protein levels are repressed by noradrenaline

Although noradrenaline stimulation led to a marked reduction in PPAR γ 2 mRNA levels, this reduction could be without significant effects on cellular functions, if it were not reflected in an altered level of PPAR γ 2 protein. As the repression is transient (Figure 2A), the protein levels could remain nearly unchanged if the half-life of PPAR γ 2 was long. However, direct investigation of PPAR γ 2 protein levels clearly demonstrated that the decreased mRNA level was fully reflected in a decreased PPAR γ 2 level (Figures 3 and 4). Thus regulatory effects of the altered expression may be envisaged.

Although only PPAR γ 2 mRNA is found in significant amounts in the brown adipocytes used in the present study, the immunoblots revealed that PPAR γ 1 protein was present in equal amounts to PPAR γ 2 protein (Figures 3 and 4). The most likely explanation for this phenomenon is that the PPAR γ 2 mRNA is translated with equal probability from the PPAR γ 2 initiation site and from the PPAR γ 1 initiation site (that is found unperturbed in the PPAR γ 2 mRNA). Whether this is a specific phenomenon for the brown adipocytes used in the present study is unknown, but the potential for the dual outcome of translation of the PPAR γ 2 mRNA has been evident since the first characterization of the PPAR γ 2 splice variant [2].

The physiological function of noradrenaline-induced repression of PPAR γ 2 gene expression

The physiological role of the noradrenaline-induced repression of PPAR γ 2 gene expression reported in the present paper, and the ensuing decreases in PPAR γ 2 and PPAR γ 1 protein, is only partially understandable. A repression of PPAR γ 2 gene expression in brown pre-adipocytes is functionally explainable based on the proliferative effect of noradrenaline [43] and the anti-proliferative role ascribed to PPAR γ . However, the persistence of the effect in mature brown adipocytes, in which cell proliferation is not induced by noradrenaline [43], makes this explanation for the noradrenaline-induced repression less probable.

If the differentiation-promoting effect of PPAR γ is accepted, the suppressed expression of PPAR γ 2 by noradrenaline should have a de-differentiating effect on brown adipocyte characteristics. However, a general differentiation-promoting effect of noradrenaline is seen in the brown adipocytes. For instance, even though there is a PPRE site, e.g. in the UCP1 promoter [6,7], UCP1 gene expression is markedly induced, and not even transiently repressed during noradrenaline stimulation [11]; the explanation concerning UCP1 may be that this PPRE site could

be stimulated primarily by PPAR α [7]; PPAR α is not decreased by noradrenaline treatment (Figure 3A). Although brown adipocytes share traits with white adipocytes and adipocyte-like cell lines, the final differentiation process of brown adipocytes must necessarily be distinct from that of white adipocytes. Thus, whereas PPAR γ is necessary for adipose conversion, the possibility exists that temporal repression of PPAR γ 2 expression is a part of the events that promote acquisition of brown adipose traits (as compared with general adipose traits) during chronic noradrenaline stimulation of maturing brown adipocytes.

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