

Thiazolidinediones Inhibit the Expression of β_3 -Adrenergic Receptors at a Transcriptional Level

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The effect of the thiazolidinediones (TZDs) darglitazone and troglitazone on β_3 -adrenergic receptor (AR) expression was studied in cultured cell lines representing several tissues. After 24 h of exposing HIB-1B brown adipocytes to 30 $\mu\text{mol/l}$ darglitazone or 20 $\mu\text{mol/l}$ troglitazone, β_3 -AR mRNA levels were reduced by 75%. This effect was significant within 1 h of exposure to a maximal dose of these drugs, with the full effect obtained within 10 h. The darglitazone ID_{50} was ~ 10 nmol/l , similar to the K_d of TZDs binding to peroxisome proliferator-activated receptor- γ (PPAR- γ). These drugs also decreased β_3 -AR mRNA in 3T3-F442A white adipocytes, but not in SK-N-MC cells, which lack PPAR- γ 2. A luciferase reporter gene containing 1.4 kb of 5' flanking sequence of the mouse β_3 -AR was transiently transfected, with or without PPAR- γ 2, in SK-N-MC cells. The vigorous expression of luciferase driven by the β_3 -AR gene sequence was inhibited by TZDs in a PPAR- γ 2-dependent manner. The half-lives of β_3 -AR precursor RNA and mRNA were short, ~ 40 and ~ 100 min, respectively, and remained unaffected by TZD treatment. Exposure of HIB-1B cells to 30 $\mu\text{mol/l}$ darglitazone was associated with reduced β_3 -AR mRNA levels, as well as decreased response of uncoupling protein 1 to norepinephrine + propranolol (a β_1 , β_2 -AR antagonist) or the specific β_3 -AR agonist CL 316, 243. Both the β_3 -AR mRNA level and response to these stimuli fully recovered by 24 h of removing the drug, indicating that the β_3 -AR protein and its coupling to adenylyl cyclase rapidly followed the changes in mRNA. Thus, TZDs can rapidly reduce β_3 -AR expression at the transcriptional level, acting through PPAR- γ 2. The rapid turnover and responses of β_3 -AR to perturbations, along with numerous other factors reported to regulate its expression, suggest a tight control of β_3 -AR and function. Lastly, leptin being the only other known gene suppressed by TZDs, the present studies support a concerted lipogenic effect of these drugs. *Diabetes* 49:2108–2115, 2000

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AR, adrenergic receptor; BAT, brown adipose tissue; cds, charcoal dextran-stripped; CL, CL 316, 243; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MAP, mitogen-activated protein; MMLV, Moloney murine leukemia virus; NE, norepinephrine; PCR, polymerase chain reaction; PPAR, peroxisome proliferator-activated receptor; RT, reverse transcription; TZD, thiazolidinedione; UCP, uncoupling protein; WAT, white adipose tissue.

In mammals, there are two distinct types of adipose tissues: white adipose tissue (WAT) and brown adipose tissue (BAT). Both play important, but opposite, roles in energy balance. Whereas WAT stores energy in the form of triglycerides, BAT dissipates energy as heat in response to cold or excessive caloric intake. Their function is coordinated by the sympathetic nervous system and modulated by several hormones and metabolic signals. Norepinephrine (NE), released by sympathetic nerve terminals, stimulates lipolysis in BAT and WAT, as well as thermogenesis in BAT. These actions of NE are mediated by β -adrenergic receptors (ARs), which belong to the family of G protein-coupled transmembrane receptors that use cAMP as main second messenger.

The β_3 -AR is predominantly expressed in WAT and BAT (1–4), in contrast to the other β -AR (β_1 - and β_2 -AR), which are present in several other tissues. The restricted presence of β_3 -AR in adipose tissues, its ability to mobilize free fatty acids, and its capacity to stimulate BAT thermogenesis have together made it a very attractive target for the development of antiobesity drugs (5). Such β_3 -AR agonists have been developed based on the rodent β_3 -AR, and indeed when given chronically to these animals, they enhance lipolysis and thermogenesis, reduce fat accumulation in response to overfeeding (6), and improve insulin sensitivity (7). In humans, however, these particular compounds appear to be less effective and not as specific for the β_3 -AR as they are in rodents (8). An additional problem is the low level of expression of these receptors in human adipose tissues (9–11), which may ultimately limit the clinical use of stronger and more specific agonists. It is, therefore, important to identify and characterize the factors that modulate the expression of these receptors.

Thiazolidinediones (TZDs) are novel antidiabetic drugs that increase the responsiveness to insulin in both human and animal models of type 2 diabetes (12). They exert most, if not all, of their metabolic effects through the specific binding and activation of peroxisome proliferator-activated receptor- γ (PPAR- γ) (13), a member of the nuclear receptor superfamily of ligand-activated transcription factors. Of the two alternate splicing forms of the receptor, PPAR- γ 2 is specifically expressed at high levels in adipose tissues, where it controls the expression of many adipocyte-specific genes (14–16). Given the involvement of PPAR- γ in adipocyte differentiation and function, it is not surprising that TZDs promote adipocyte differentiation or adipogenesis (17). But they also increase BAT differentiation (18–20) and stimulate uncoupling protein (UCP) 2 and 3 expression (21,22), suggesting that the lipogenic effects may be coupled to the activation of mechanisms involved in energy dissipation.

Because β_3 -ARs may become a limiting factor for lipolysis and thermogenesis, and because they may also mediate an improvement in insulin sensitivity (7), it was of interest to investigate the effect of TZDs on β_3 -AR expression. We report here that TZDs rapidly and reversibly inhibit the expression of β_3 -AR mRNA acting at the transcriptional level.

RESEARCH DESIGN AND METHODS

Cell culture. The mouse brown adipose HIB-1B cells were a gift from Dr. B. Spiegelman; the mouse white adipose 3T3-F442A cells were provided by Dr. M. Lavau (INSERM, Paris); and human neuroblastoma SK-N-MC cells were purchased from American Tissue Culture Collection. Both adipose cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% CO₂, and SK-N-MC were cultured in minimal essential medium in 5% CO₂. All cells were plated at high density in medium containing 10% fetal bovine serum (FBS) (Wysent, St-Bruno, Quebec) to get confluence rapidly. After reaching confluence, HIB-1B cells were placed in medium supplemented with 10% charcoal dextran-stripped (cde) FBS for 3 days, which sufficed to obtain a good and stable level of expression of β_3 -AR. The 3T3-F442A cells were kept in DMEM 10% normal FBS supplemented with 100 nmol/l insulin for 7 days, after which >90% had acquired a mature adipocyte phenotype. At this time, they were switched to medium containing 10% cde-FBS. Unless otherwise indicated, cells were cultured in 6-well plates (9.6 cm² wells). Once cells reached the desired level of differentiation, they were exposed to TZD or its solvent, DMSO (final \leq 0.1%), in fresh medium containing 10% cde-FBS. In some experiments, HIB-1B cells were plated and grown to confluence, as mentioned above, and then incubated for 3 days in 30 μ mol/l darglitazone, which is associated with differentiation and expression of UCP-1 responsive to NE (20).

RNA analysis. Total RNA was isolated by two cycles of extraction with acid guanidinium-phenol-chloroform (23). After the first extraction, samples were treated with 0.2 U of RNase-free DNase I (RQ1 DNase; Promega) per microgram of nucleic acid in 40 mmol/l Tris-HCl, 10 mmol/l NaCl, 6 mmol/l MgCl₂, 10 mmol/l CaCl₂, pH 7.9 in the presence of ribonuclease inhibitor (Rnasin; Promega) for 1 h. This mixture was then re-extracted, and RNA was recovered by isopropanol precipitation. Total RNA concentration was quantified spectrophotometrically, and quantity and quality were verified by videodensitometry (Scion Image) after electrophoresis in 1% agarose stained with ethidium bromide. β_3 -AR and UCP-1 mRNAs were quantified by reverse transcription (RT) followed by competitive polymerase chain reaction (PCR) using the primers and competitors described below.

The DNA competitor used to quantify mouse β_3 -AR mRNA was constructed using the PCR MIMIC kit as recommended by the manufacturer (Clontech, Palo Alto, CA). Briefly, the β_3 -AR PCR competitor was generated from a provided template (v-erb B gene), which was amplified with composite primers containing a 5' 18-mer segment corresponding to the chosen segment of the β_3 -AR cDNA (see below) fused to a 20-mer sequence to span the desired size of the v-erb B gene. A 336-bp PCR product containing the β_3 -AR specific primer sequences resulted. Appropriate quantities of this competitor were generated and purified as recommended by the manufacturer. Primers were as follows: mouse β_3 -AR sense: ATGGCTCCGTGGCCTCAC; mouse β_3 -AR antisense: CTGGCTCATGATGGCGCG; sense composite primer: ATGGCTCCGTGGCCTCACCGCAAGTGAAATCTCCTCCG; and antisense composite primer: CTGGCTCATGATGGCGCGGACAAGATACTCATCTGC. The 454-bp UCP-1 competitor was also generated following the same protocol, as well as the following primers: UCP-1 sense: AAGCCAGGCTTCCAGTACTATTAGGT; UCP-1 antisense: GGTTTGATCCC ATGCAGATGGCTCTC; sense composite primer: AAGCCAGGCTTCCAG TACTATTAGGTCGCAAGTGAAATCTCCTCCG; and antisense composite primer: GGTTTGATCCCATGCAGATGGCTCTGTCTGTCAATGCAGTTTGTAG. The mouse β_3 -AR primers were derived from the corresponding gene sequence (24) located in the translated area of the first exon. The UCP-1 primers were derived from mouse UCP-1 gene sequences located in exons 2 and 3. Common PPAR- γ and PPAR- γ 2 mRNAs were also detected by semiquantitative RT-PCR using the following primers: common PPAR- γ mRNA sense: GTTCATGCTTGTGAAGGATGC, and antisense: ACTCTGGATTCAGCTGGTCCG; and PPAR- γ 2 sense: GCGATTCCTTCACTGATAC, and antisense: GCATTATGAGACATCCCCAC, as described elsewhere (25,26).

RT was carried out on 1 μ g of total RNA with 200 U of Moloney murine leukemia virus (MMLV) (Promega), as recommended by the manufacturer, in the presence of 0.6 μ g random hexanucleotides (Promega). A sham reaction without MMLV RT was routinely performed to ensure that PCR amplification did not arise from contaminating genomic DNA. Some 3 μ l of the RT reaction was denatured and amplified by PCR over 30 cycles of amplification (60°C, 1 min; 72°C, 1 min), ending with a final extension of 10 min at 72°C. The reaction mix-

ture contained 2 U of Taq DNA polymerase (Pharmacia) in 47 μ l of 50 mmol/l KCl, 1.5 mmol/l MgCl₂, 10 mmol/l Tris-HCl (pH 9), 200 μ mol/l of each deoxynucleotide triphosphate, 15 pmol of primers (β_3 -AR or UCP-1), and 0.1 μ mol of the corresponding competitor DNA template. PCR products were electrophoretically separated in a 1% agarose gel, visualized with ethidium bromide, and quantified by videodensitometry (Scion Image) scanning. Densitometric areas were corrected by the size difference and the ratio of target mRNA to competitor used to calculate the moles of target mRNA per microgram of total RNA. In pilot experiments introducing known amounts of chloramphenicol acetyl transferase RNA in the RT reaction, we established that this latter effect was highly reproducible, with minimal intra- and interexperiment variation.

β_3 -AR mature and precursor mRNA half-lives. The effect of TZDs on mature and precursor β_3 -AR mRNA stability was tested in cells treated with, respectively, 5 μ g/ml actinomycin D or 2 μ g/ml α -amanitin and harvested for RNA analysis at several times thereafter (27,28). Precursor β_3 -AR mRNA (hnRNA) was assayed as reported elsewhere (29), in 1 μ g of total RNA. RT was carried out as described earlier but using 10 pmol of an antisense primer, AGGGCCCGTTTGTAGTTCCCTA, encoding a region located in the first intron of the mouse β_3 -AR gene instead of random primers. PCR was performed as described above.

Transient transfection experiments. The 1.4 kb of 5' flanking region (-1,400 to -131) contained a murine β_3 -AR genomic clone (A.D. Strosberg and [4]) was PCR-amplified and inserted into the Hind III site of the pGL3-BASIC (enhancerless/promoterless) luciferase reporter vector (Promega), which we called 1.4 β_3 -AR-Luc. Murine PPAR- γ 2 cDNA cloned in the expression vector pSV-Sport 1 was a gift from Dr. B. Spiegelman (30). Experiments were performed in SK-N-MC cells, grown in 12-well plates (3.8 cm² wells) and transfected using the calcium phosphate precipitation method when they were 60–80% confluent. Calcium phosphate precipitate in fresh medium contained 3 μ g of 1.4 β_3 -AR-Luc or its empty vector, 0.5 μ g of pCMV β -galactosidase (Clontech) as an internal control, and either 0.5 μ g of mPPAR- γ 2 pSV-Sport or the corresponding empty vector. After 16 h the medium was removed, cells were washed with phosphate-buffered saline, and fresh medium containing TZDs or DMSO was added. Luciferase activity was measured 24 h later, as recommended by the manufacturer of the assay kit (Luciferase Assay System; Promega).

Chemicals. Darglitazone and troglitazone were provided by Pfizer (Dr. E. Pagani) and Parke-Davis (B. Jared), respectively. NE, propranolol, actinomycin D, and α -amanitin were purchased from Sigma (St. Louis, MO). The β_3 -AR receptor agonist CL 316, 243 (CL) was kindly donated by American Cyanamid (Dr. T.H. Claus).

Statistical analysis. Results are expressed as means \pm SE. Statistical significance was obtained by Student's *t* test or ANOVA followed by Neuman-Keuls or Dunnett's test for multiple comparisons. Curve fitting and correlation analysis were performed using GraphPad 3.0 software (San Diego, CA).

RESULTS

Effect of TZDs on β_3 -AR expression in HIB-1B cells. The addition of 30 μ mol/l darglitazone or 20 μ mol/l troglitazone to these cells caused a 70–80% decrease in β_3 -AR mRNA levels in 24 h (Fig. 1). In a similar experiment in 3T3-F442A cells, darglitazone induced a 58 \pm 5% reduction in β_3 -AR mRNA levels (data not shown). The time course of the inhibition was examined in HIB-1B cells using a maximal concentration of 100 μ mol/l of darglitazone. As shown in Fig. 2, the decrease in β_3 -AR mRNA was significant within 1 h and maximal 10–15 h after the addition of the TZD. Exposure of cells to graded doses of darglitazone for 24 h revealed a concentration-dependent inhibition, with the minimal concentration having a significant effect at 3 nmol/l, a maximal effect at 300 nmol/l, and an half-maximal inhibitory dose (ID₅₀) of \sim 10 nmol/l (Fig. 3). Notably, this latter value is similar to K_d for the most potent TZDs binding to PPAR- γ , which is \sim 40 nmol/l (13), and to the affinity of darglitazone for PPAR- γ (A. Swick, personal communication).

Molecular mechanism of TZD action on β_3 -AR mRNA expression. For further evidence that β_3 -AR mRNA suppression is a PPAR- γ -mediated effect, we also examined the effect of TZDs on other cell lines. Adipose cell lines, HIB-1B, 3T3-L1, and 3T3-F442A are known to contain both PPAR- γ 1

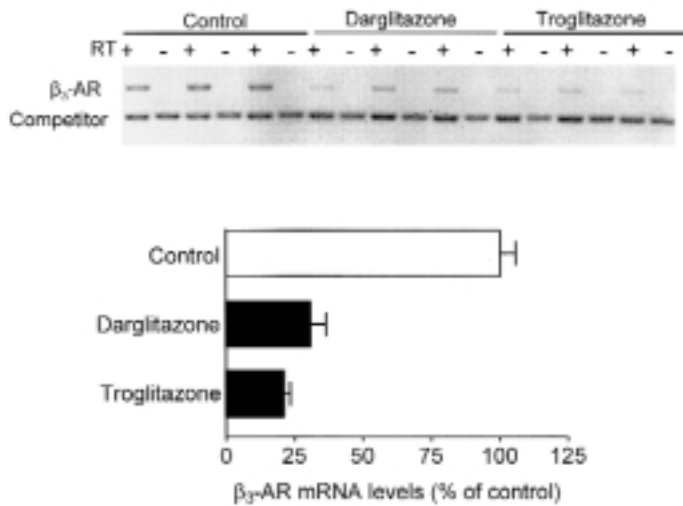


FIG. 1. Effect of TZDs on β_3 -AR mRNA levels in HIB-1B cells. HIB-1B cells were treated for 24 h with 30 $\mu\text{mol/l}$ darglitazone, 20 $\mu\text{mol/l}$ troglitazone, or vehicle (DMSO, control). Total RNA prepared for each sample was treated (+) or not (-) with MMLV RT to verify the absence of genomic DNA contributing to the PCR products. These were resolved and analyzed as described in RESEARCH DESIGN AND METHODS. The upper panel represents the negative image of the gel in which PCR products were resolved. The lower panel represents the mean \pm SE of the videodensitometric and quantitative analysis of the bands, expressed as a percent of the mean value obtained in control cells.

and PPAR- γ 2 (19,31). It was, therefore, of interest to examine the effects of these drugs on β_3 -AR mRNA in nonadipose cells. One such cell line is the human neuroblastoma SK-N-MC line. As previously reported (32), these cells clearly express β_3 -AR mRNA. This level of expression, \sim 17 times higher than that in HIB-1B cells, was not reduced by either darglitazone or troglitazone (Fig. 4A). Interestingly, these cells express only PPAR- γ 1, and not PPAR- γ 2, mRNA (Fig. 4B). To investigate whether the negative effect of TZDs on β_3 -AR expression could be mediated by PPAR- γ 2 receptors, we performed transient transfection studies in SK-N-MC cells. In preliminary experiments with the mouse β_3 -AR gene (E.B., J.E.S., unpublished observations), we found that a luciferase construct containing -1,400 to -131 (relative to the translation start site) of 5' flanking sequence exhibited vigorous promoter activity

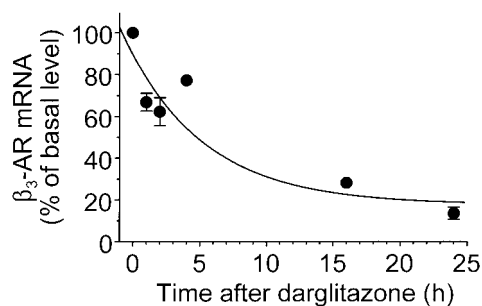


FIG. 2. Time-course of the reduction in β_3 -AR mRNA induced by darglitazone in HIB-1B cells. HIB-1B cells were exposed to 100 $\mu\text{mol/l}$ darglitazone and RNA was extracted from triplicate wells at the indicated times. Results are expressed as the mean percent \pm SE of the β_3 -AR mRNA level detected at time zero. Missing error bars are smaller than the dots indicating data points. Control cells exposed for the same time to DMSO showed no change in mRNA (not shown).

when transfected into SK-N-MC cells. As shown in Fig. 5A, troglitazone did not reduce the expression of 1.4 β_3 -AR-Luc unless this was cotransfected with 0.5 μg of PPAR- γ 2-containing vector. Similar results (not shown) were obtained with darglitazone. The relatively modest inhibition proved to be due to limiting amounts of PPAR- γ 2 expressed, since in a subsequent experiment with increasing transfected doses of PPAR- γ 2 cDNA, the inhibition was clearly dose-dependent ($r = -0.91$, $P < 0.001$; Fig. 5B).

To further document the transcriptional inhibition by TZDs and estimate the extent of possible actions on the stability of the β_3 -AR mRNA, we examined the effect of these drugs on both mature and precursor β_3 -AR mRNA. In HIB-1B cells exposed to actinomycin D in the absence or presence of darglitazone, β_3 -AR mRNA decreased rapidly, following first-order kinetics, with a half-life of 98 ± 21 min in control cells and 105 ± 35 min in darglitazone treated cells (NS; Fig. 6A). This comparatively short half-life in HIB-1B cells is not different from that previously reported in 3T3-F442A cells by others (33,34).

Examined under the appropriate conditions, changes in specific precursor mRNA (hnRNA) may reveal changes in the transcription of the corresponding gene or in precursor processing (29). Under our study conditions in HIB-1B cells (Fig. 6B), β_3 -AR hnRNA levels remain constant, indicating a steady state, whereas the addition of darglitazone caused a decline in β_3 -AR hnRNA with a half-life of 36 ± 11 min. To exclude an effect of darglitazone on the processing or degradation of precursor β_3 -AR hnRNA, cells were then examined under the presence of α -amanitin (27,28). As shown in Fig. 6C, β_3 -AR hnRNA disappeared with a similar half-life in control and darglitazone-treated cells (42 ± 15 min and 46 ± 13 min, respectively), indicating no effect of the drug in the stability of the β_3 -AR precursors. Moreover, since these values are not significantly different from those obtained with darglitazone alone (Fig. 6B), these results indicate that the inhibition of transcription by the TZD was complete.

Functional consequences of TZD-induced decrease in β_3 -AR mRNA. TZDs have been reported to induce or accelerate the acquisition of BAT phenotype by HIB-1B cells and isolated brown preadipocytes from rodents (18,19). We investigated whether the TZD-induced differentiation was associated with reduced expression of the β_3 -AR, and if so, whether this had functional consequences. As we previously

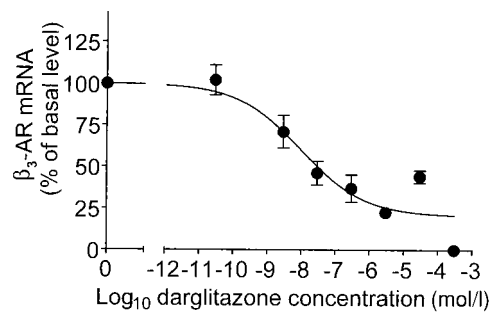


FIG. 3. Dose-dependent effect of darglitazone on β_3 -AR mRNA levels in HIB-1B cells. HIB-1B cells were treated in triplicates for 24 h with the indicated concentrations of darglitazone. Results are expressed as the mean percent \pm SE of the β_3 -AR mRNA level of untreated cells. Missing error bars are smaller than the dots indicating data points. Control cells exposed for 24 h to DMSO showed no change in mRNA (not shown).

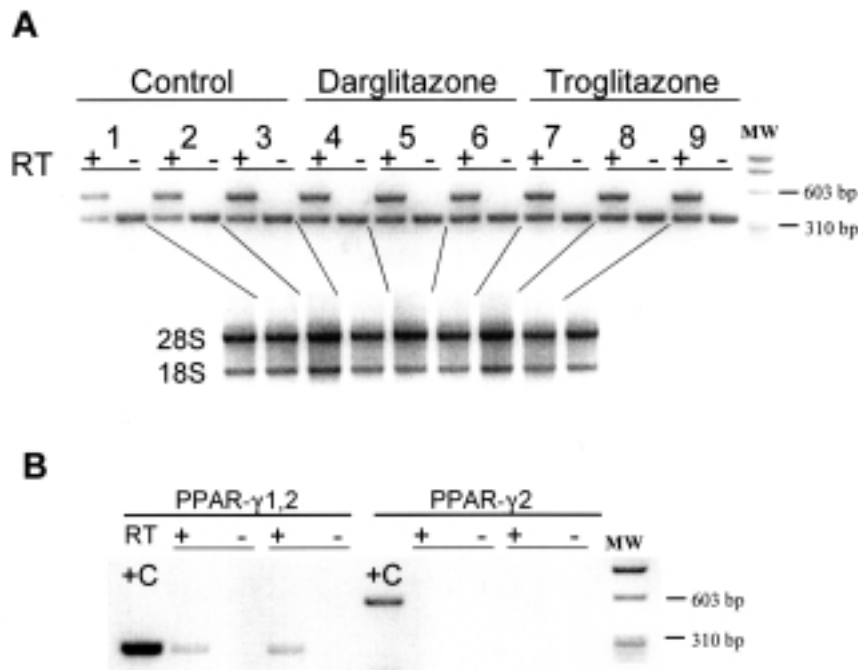


FIG. 4. Lack of effect of TZDs on β_3 -AR mRNA in human neuroblastoma SK-N-MC cells. SK-N-MC cells were treated for 24 h with 30 $\mu\text{mol/l}$ darglitazone or 20 $\mu\text{mol/l}$ troglitazone. **A:** β_3 -AR mRNA was analyzed and presented as in Fig. 1. Ribosomal RNA is shown in the lower portion of **A** to document the integrity and uniformity of the RNA. **B:** SK-N-MC cell RNA from duplicate wells was analyzed for PPAR- γ 1, - γ 2, and PPAR- γ 2 mRNAs using primers as described in RESEARCH DESIGN AND METHODS. +C, positive controls: cloned PPAR- γ 2 cDNA in the case of PPAR- γ 1, - γ 2, and reverse-transcribed mRNA from human WAT in the case of PPAR- γ 2. MW, molecular weight.

reported (20), exposure of HIB-1B cells to 30 $\mu\text{mol/l}$ darglitazone causes the UCP-1 gene to become responsive to adrenergic stimulation within 24 h, whereas within 3 days the cells become round and acquire refracting droplets by phase contrast, which are morphological indicators of differentiation (data not shown). When exposed for 3 days to darglitazone in such an experiment, cells displayed an ~70% lower β_3 -AR mRNA expression compared with control cells treated identically but not exposed to darglitazone ($P < 0.001$; Fig. 7). This effect was rapidly reversed, however, since β_3 -AR mRNA was restored to the levels of the control cells within 24 h of removal of darglitazone. Note how stable the β_3 -AR level of expression is in control cells.

As mentioned, TZDs induce the differentiation of HIB-1B cells but do not directly stimulate UCP-1 expression. Typically, cells exposed to these drugs will have little or no basal expression of UCP-1, but this gene becomes responsive to adrenergic and cAMP stimulation (20). Interestingly, in these studies, we observed that HIB-1B cells differentiated with TZDs responded better to adrenergic stimulation, not immediately, but 24 h after ending differentiation with darglitazone. This prompted us to investigate the possibility that this observation reflected the repression of β_3 -AR by darglitazone and its subsequent recovery upon removing the drug. Accordingly, we examined the responses of UCP-1 mRNA levels to adrenergic stimulation immediately and 24 h after exposing HIB-1B cells to darglitazone. Adrenergic stimulation consisted of a 4-h exposure to 10 $\mu\text{mol/l}$ NE \pm 0.5 $\mu\text{mol/l}$ propranolol, or 1 $\mu\text{mol/l}$ of the β_3 -AR specific agonist CL. That concentration of propranolol is sufficient to block β_1 -AR and β_2 -AR, but not β_3 -AR, at 10 $\mu\text{mol/l}$ NE (35), and it was included to estimate the contribution of non- β_3 -AR pathways and post-receptor mechanisms on the effect of NE. As shown in Fig. 8, the UCP-1 mRNA expression in response to NE increased by a factor of 4, whereas that by CL was 12 times greater 24 h after the removal of darglitazone. When propranolol was added, the absolute reduction in UCP-1 stimulation

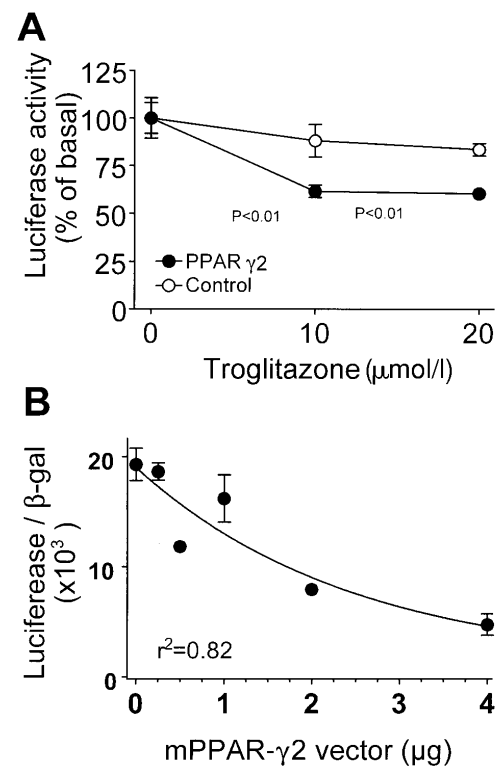


FIG. 5. Expression of β_3 -AR mRNA promoter activity and inhibition by TZDs in SK-N-MC cells. SK-N-MC cells were transiently transfected with a luciferase reporter vector containing 1.4 kb of 5' flanking sequence of the mouse β_3 -AR gene, as described in RESEARCH DESIGN AND METHODS, with or without cotransfection of mPPAR- γ 2 or its empty expression vector, pSV-Sport 1. Luciferase activity was corrected for β -galactosidase to account for variations in transfection efficiency. Each point represents the mean \pm SE of triplicate wells. Missing error bars are smaller than the dots indicating data points. **A:** Effect of troglitazone on the luciferase activity expressed as percent of basal luciferase levels. Cells were cotransfected with 0.5 μg pSV-Sport 1-mPPAR- γ 2 or the empty vector. **B:** Cells cotransfected with the indicated amounts of pSV-Sport 1-mPPAR- γ 2 and treated with 30 $\mu\text{mol/l}$ darglitazone.

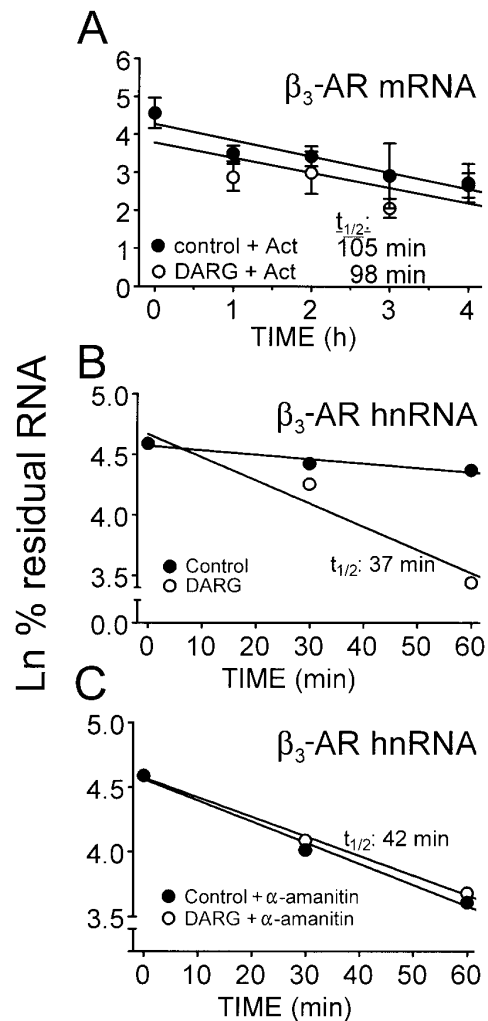


FIG. 6. Effect of TZDs on the stability of mature or precursor β_3 -AR mRNA in HIB-1B cells. In all cases, RNA levels were expressed as percent of basal and plotted as natural log. Cells were allowed to differentiate as described in RESEARCH DESIGN AND METHODS and were then treated as described below. RNA was extracted at the indicated times and analyzed as described in RESEARCH DESIGN AND METHODS. Each point represents the mean \pm SE of triplicates. Missing error bars are smaller than the dots indicating data points. **A:** At time zero, cells were treated with 5 μ g/ml actinomycin (Act) D \pm 30 μ mol/l darglitazone (DARG). Regression lines were significant at $P < 0.001$, but slopes were not significantly different. **B and C:** At time zero, cells were treated with 30 μ mol/l darglitazone or DMSO (**B**) plus 2 μ g/ml α -amanitin (**C**).

by NE was very similar immediately and 24 h after removing darglitazone, ~ 4 atmol/ μ g RNA, but this represented 62 and 32% decreases in the NE-induced UCP-1 expression, respectively. Note that the stimulation by NE + propranolol was greater at all times than that by CL, which may reflect a contribution of the α_1 -AR pathway. Altogether, the data are consistent with time-dependent recruitment of β_3 -AR after the removal of TZDs, paralleling the increase in β_3 -AR mRNA.

DISCUSSION

We have shown here that the TZDs darglitazone and troglitazone reduce the levels of β_3 -AR mRNA in adipose cell lines with a potency similar to that with which they bind to PPAR- γ . This effect of TZDs indeed necessitates the presence of PPAR- γ 2 and

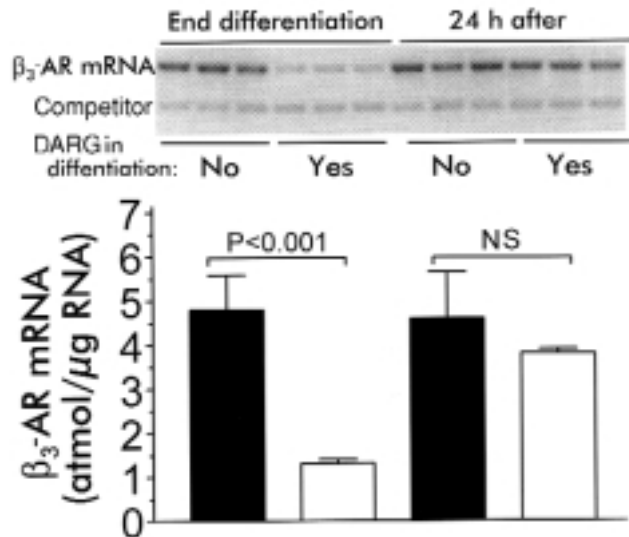


FIG. 7. Effect of darglitazone during HIB-1B cells differentiation on β_3 -AR mRNA. HIB-1B cells were differentiated as described in RESEARCH DESIGN AND METHODS in the presence or absence of 30 μ mol/l darglitazone (DARG). Triplicate wells were analyzed for β_3 -AR mRNA at the end of this period or 24 h after removing darglitazone or its vehicle. The upper panel shows the RT-PCR products and the lower panel shows the mean \pm SE of their videodensitometric analysis.

is caused by inhibition of transcription. This inhibitory effect of TZDs is, initially at least, nearly complete, and the rapid fall of the β_3 -AR mRNA is made possible by the intrinsically rapid turnover of this mRNA, but TZDs do not affect the half-life of either mature β_3 -AR mRNA or its precursors. Furthermore, the changes in mRNA level are paralleled by changes in β_3 -AR-mediated responses. Since the β_3 -AR is involved in fat mobilization and oxidation, these observations add yet another mechanism contributing to a lipogenic effect of TZDs.

The rapid reduction in β_3 -AR mRNA induced by TZDs, evident within the hour of their addition, could have been mediated by an acceleration of the mRNA degradation. El Hadri et al. (33) reported that thyroid hormone prolongs the half-life of β_3 -AR mRNA in 3T3-F442A cells, providing a precedent for regulation at this level. We report here that the half-life of this mRNA in HIB-1B cells is as fast as that in 3T3-F442A cells, but we find that TZDs do not accelerate the rate of disappearance of this β_3 -AR mRNA, nor do they affected the disappearance of the β_3 -AR mRNA precursors. Furthermore, in the presence of α -amanitin, these precursors disappear at the same rate as after TZDs, providing a strong argument for a rapid and initially complete repression of β_3 -AR mRNA transcription by these drugs. Additional support for repression of transcription came from transient transfection experiments in which TZDs inhibited reporter gene expression driven by the 5' flanking sequence of the mouse β_3 -AR gene, as further discussed below. The possibility that the repression of the β_3 -AR gene be mediated indirectly, by a gene product whose expression is stimulated by TZDs via PPAR- γ 2, seems very unlikely because within minutes of addition of TZD to the cells, we detected a reduction in β_3 -AR mRNA precursors and because the time course is identical to that of α -amanitin. Thus, we are confident that the effect of TZD reported here represents PPAR- γ 2-mediated repression of gene expression.

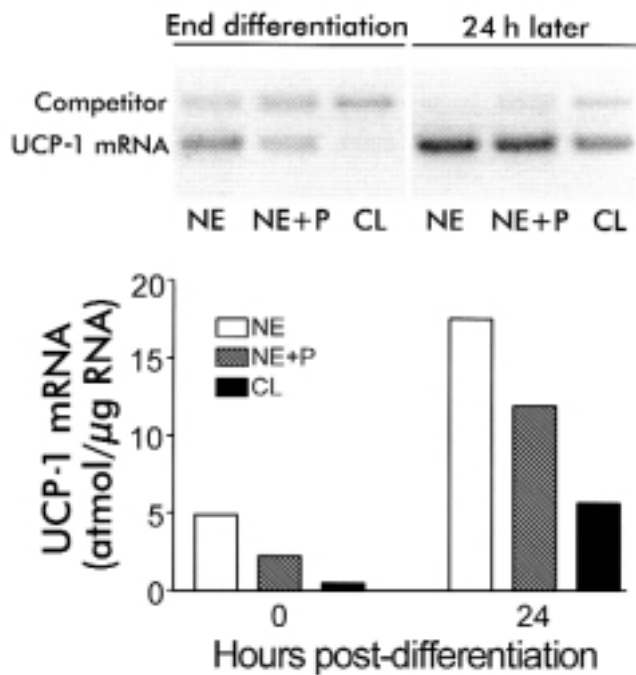


FIG. 8. Functional correlates of the effect of darglitazone on β_3 -AR mRNA in HIB-1B cells. UCP-1 mRNA levels in HIB-1B cells differentiated in the presence of 30 $\mu\text{mol/l}$ darglitazone, at the end of the differentiation period and 24 h after the removal of the darglitazone, as described in RESEARCH DESIGN AND METHODS. Triplicate wells of these cells were stimulated with 10 $\mu\text{mol/l}$ NE \pm 0.5 $\mu\text{mol/l}$ propranolol or the specific β_3 -AR agonist CL for 4 h, after which the RNA was extracted, pooled, and analyzed as described in RESEARCH DESIGN AND METHODS. The upper panel shows the PCR products of the RT, and the lower panel the calculated UCP-1 mRNA abundance. Note the limited stimulation by NE and CL, as well as the modest inhibition by propranolol of the effect of NE immediately after the darglitazone exposure, compared with the same responses 24 h after removing the TZD. At this time, propranolol reduced the effect of NE only by 32% and the effect of CL increased 12 times (from 0.43 to 5.2 atmol/ μg RNA).

In spite of the evidence favoring a complete initial repression of transcription, β_3 -AR mRNA does not decrease following a single exponential with half-life of \sim 100 min, but after a rapid initial decline disappearance rate decreases, and by 24 h of exposure to TZDs, mRNA levels are 20–25% of the baseline. Several explanations are possible. Degradation of the drug by the cells does not seem likely, since doses several times higher than the maximal did not change this outcome. Another possibility is the existence of a comparatively small pool of β_3 -AR mRNA, \sim 20% of the total, that is more stable. An alternate β_3 -AR mRNA splice variant has been identified in mouse BAT and WAT (24) and also in 3T3-F442A cells (34). Our assay does not distinguish these variants, and even though Granneman and Lahners (34) did not find that they are differentially regulated by cAMP, the possibility that one of them has a longer life span in HIB-1B cells remains to be tested. Another possibility is that the PPAR- γ 2 effect is attenuated with time after its activation. This may occur by phosphorylation of a consensus mitogen-activated protein (MAP) kinase target site present in the PPAR- γ that when phosphorylated, causes a loss or attenuation of activity (36) and when disabled by spontaneous mutations, is associated with a constitutively active PPAR- γ and massive obesity (37). One could, therefore, hypothesize that continued exposure to

TZDs results in attenuation of the receptor via its phosphorylation by MAP kinase.

Even though some effects of TZDs could be independent of PPAR- γ activation (38), several lines of evidence favor specifically a PPAR- γ 2 as mediator of the repression of β_3 -AR expression. First, we observed that the ID_{50} for the darglitazone effect is similar to the reported K_d for binding of the most potent TZDs to PPAR- γ (13,39) and that binding of TZDs to other PPAR receptors occurs with much lower affinity. Second, the effect was only evident on adipose cell lines containing PPAR- γ 2, and not in SK-N-MC cells lacking these receptors. Third, the expression of our 1.4 β_3 -Luc reporter gene transiently transfected in these cells was not reduced by TZDs unless we cotransfected PPAR- γ 2, and the inhibition increased in a dose-dependent manner with the amount of PPAR- γ 2 cDNA introduced in the cells.

The observation that the PPAR- γ 1 present in SK-N-MC cells did not suffice to mediate the response is in apparent conflict with the concept that TZDs are active on both PPAR- γ isoforms. The difference between the two PPAR- γ isoforms consists of an additional 30 amino acids in the NH_2 -terminal of PPAR- γ 2, and it is possible that this difference enables this receptor to interact with cofactors (corepressors in this case) with which PPAR- γ 1 cannot interact. There is precedent for such a mechanism, since in some systems PPAR- γ 2 has more ligand-independent transactivation capacity and is associated with more responsiveness to insulin (40). Differences in function and interaction with cofactors due to distinct NH_2 -terminals has also been reported for other nuclear receptors, thyroid hormone receptors β_1 and β_2 (41,42). It is thus plausible that the apparent specificity of PPAR- γ 2 to mediate β_3 -AR repression is related to an isoform-specific ligand-dependent interaction with a factor necessary for the repression of this gene.

The response of UCP-1 mRNA to NE and CL increased with time after the removal of darglitazone, shortly after the increase in β_3 -AR mRNA, and we provided evidence that such an increase in responsiveness reflected a β_3 -AR-mediated event. First, CL is a highly specific β_3 -AR ligand (43), and second, when we stimulated UCP-1 mRNA with NE, the increase in response was evident even after using propranolol in a concentration that would block β_1 -AR and β_2 -AR but not β_3 -AR. However, it should be noted that the response to NE + propranolol was greater than that to CL. Whereas part of this difference could be due to CL concentrations that were not maximal, it is also possible that there is a contribution of the α_1 -AR, which is activated by NE but not by CL. The fact that the time-dependent increase in response to NE + propranolol was less than that to CL (\sim 5-fold vs. \sim 12-fold) argues in favor of the α_1 -AR component, which has been demonstrated by others to enhance responses to cAMP (44). All things considered, then, our data strongly suggest that the changes in β_3 -AR mRNA induced by TZDs are shortly followed by corresponding changes in the contribution of this pathway to adrenergic stimulation.

The rapid turnover of β_3 -AR mRNA and the seemingly prompt response of the receptor protein point to a rapid regulation of β_3 -AR levels and function and constitute further evidence in favor of an important role for these receptors. This concept is further supported by the variety of factors that regulate the expression of β_3 -AR. Thus, its expression is inhibited by glucocorticoids (45) and insulin (46), whereas thyroid

hormone vigorously stimulates its expression in WAT while it as vigorously reduces the mRNA levels in BAT (35). It is interesting that TZDs, which sensitize cells to the action of insulin, also repress expression of the β_3 -AR gene.

PPAR- γ is known to mediate the stimulation of adipogenic genes. On the other hand, TZDs stimulate the expression of UCP-2 (21,47) and UCP-3 ([22] and E.B., J.E.S., unpublished observations), which could be interpreted as a coupling of energy-dissipating mechanisms—presumably mediated by UCPs—and lipogenic and adipogenic stimulation. This view would go along with previous observations by us and others (18,20,48) that TZDs induce the differentiation of BAT as well as WAT. However, as we have shown here, the differentiation induced by TZDs is associated with a reduction in one of the receptors involved in the adrenergic stimulation of the key molecule for energy dissipation in BAT, UCP-1, and TZDs do not directly stimulate UCP-1 expression (20); in addition, to the best of our knowledge, the only other gene reportedly repressed by TZDs is the leptin gene (39,49,50). Taken together, these observations suggest that the coupling alluded to, if it exists at all, is limited to increasing only the potential for energy dissipation, but that the realization of this potential depends on other factors that should overcome the downregulation of the β_3 -AR. Overall, the predominant effect of TZDs appears to be adipogenic, which goes along with the clinical observation that patients on these drugs gain weight and that TZDs reduce leptin expression (39,49–51).

In summary, we have shown that TZDs are potent inhibitors of β_3 -AR expression in adipose cell lines, an effect that is mediated by PPAR- γ 2, probably at the level of the β_3 -AR gene to repress transcription. In the context of other observations, our results support the concept that TZDs globally promote fat accumulation. Even though such effects are coupled to an increase in the energy dissipating potential, the realization of this potential may be kept under check via controlling the expression of leptin and an AR important in lipid mobilization and activation of thermogenesis.

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