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Regulation of Uncoupling Protein-2 mRNA in L6 Myotubules

I: Thiazolidinediones Stimulate Uncoupling Protein-2 Gene Expression by a Mechanism Requiring Ongoing Protein Synthesis and an Active Mitogen-Activated Protein Kinase

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The mitochondrial uncoupling protein-2 (UCP2) can uncouple phosphorylation to subserve several functions. It has been reported that the insulin sensitizers, thiazolidinediones (TZDs), increase UCP2 mRNA levels and, more recently, that TZDs stimulate UCP2 reporter genes but that the sequences involved do not bind peroxisome proliferator-activated receptor γ (PPARγ). We report here that TZDs stimulated UCP2 gene (*ucp2*) transcription in L6 myotubules involving an indirect mechanism. L6 cells contained comparatively small amounts of PPARγ mRNA but clearly detectable amounts of PPARγ2 protein. UCP2 mRNA levels were increased in a time- and concentration-dependent manner by TZDs. UCP2 mRNA had slow turnover ($t_{1/2} \approx 38$ h), and this was not affected by TZDs. Bisphenol A diglycidyl ether, a PPARγ antagonist, concentration dependently inhibited the TZD-induced increase in UCP2 mRNA. Blockade of protein synthesis with cycloheximide as well as abrogation of mitogen-activated protein kinase (MAPK) activity with PD98059 or U0126 also prevented the TZD-induced increase in UCP2 mRNA. As with autologous UCP2 mRNA, TZDs stimulated reporter gene expression directed by *ucp2* sequences in transiently transfected L6 cells. The effect was enhanced by cotransfection of PPARγ + retinoid X receptor γ and prevented by MEK blockade. TZDs, however, did not increase the activation of MAPK, nor did its activation by other means (change of medium, insulin-like growth factor-1, insulin) increase UCP2 mRNA, indicating that phosphorylation is not limiting. These results suggest that TZDs indirectly stimulate *ucp2* transcription by inducing—via PPARγ—limiting amounts of a protein, which must be phosphorylated by MAPK to stimulate the gene.

Key Words: Uncoupling protein-2; thiazolidinediones; mitogen-activated protein kinase; peroxisome proliferator-activated receptor γ; L6 cells; skeletal muscle.

Introduction

Uncoupling protein-2 (UCP2) and UCP3 are novel proteins with 57–59% homology to the brown adipose tissue UCP (now UCP1) *(1)*. UCP2 and UCP3 have the potential to uncouple phosphorylation, but their physiologic function has not yet been defined *(1,2)*. Recent observations made in transgenic mice with deletion of the corresponding genes do not seem to support a critical role in energy balance *(3–5)*. The ubiquitous presence of UCP2 suggests that it could subserve different functions in a tissue- or cell-specific manner. Thus, UCP2 could limit the production of reactive oxygen species, modulating the function of macrophages and protecting some other cells from oxidative stress *(5)*, whereas in pancreatic β-cells it could play an inhibitory role in insulin secretion *(6)*. A role in skeletal muscle remains speculative.

Thiazolidinediones (TZDs) are drugs that sensitize tissues to the action of insulin and have the potential to become central in the treatment of some patients with type II diabetes *(7,8)*. They bind to the peroxisome proliferator-activated receptor γ (PPARγ) and are believed to exert their effects largely by modulating gene expression via this receptor. There are two major variants of PPARγ, PPARγ1 and PPARγ2, the latter containing 30 additional amino acids in the N-terminus resulting from the use of an alternate promoter *(9–11)*. The two isoforms have different tissue distribution and may have different functions. PPARγ 2 is more prominently expressed in adipose cells whereas PPARγ1 is more ubiquitously found. PPARγ2 is stimulated by feeding and inhibited by fasting and is believed to be involved in the regulation of intermediary metabolism of lipids and carbohydrates.

As PPARγ ligands, TZDs induce adipogenesis and lipogenesis by enhancing the expression of genes involved in these processes (see refs. *7,12,* and *13*), but not much is known about PPARγ-mediated effects in skeletal muscle. This tissue is the major determinant of overall insulin sensitivity *(8)*, but the mechanisms whereby TZDs increase insulin sensitivity remain conjectural. Although signals from the adipose tissue, such as the novel hormone resistin, have the potential to explain the reduction in insulin resistance by TZDs *(14)*, there still remain many questions and direct actions in the muscle cannot be excluded (see ref. *15* for a review). L6 cells are a rat skeletal muscle cell line that has

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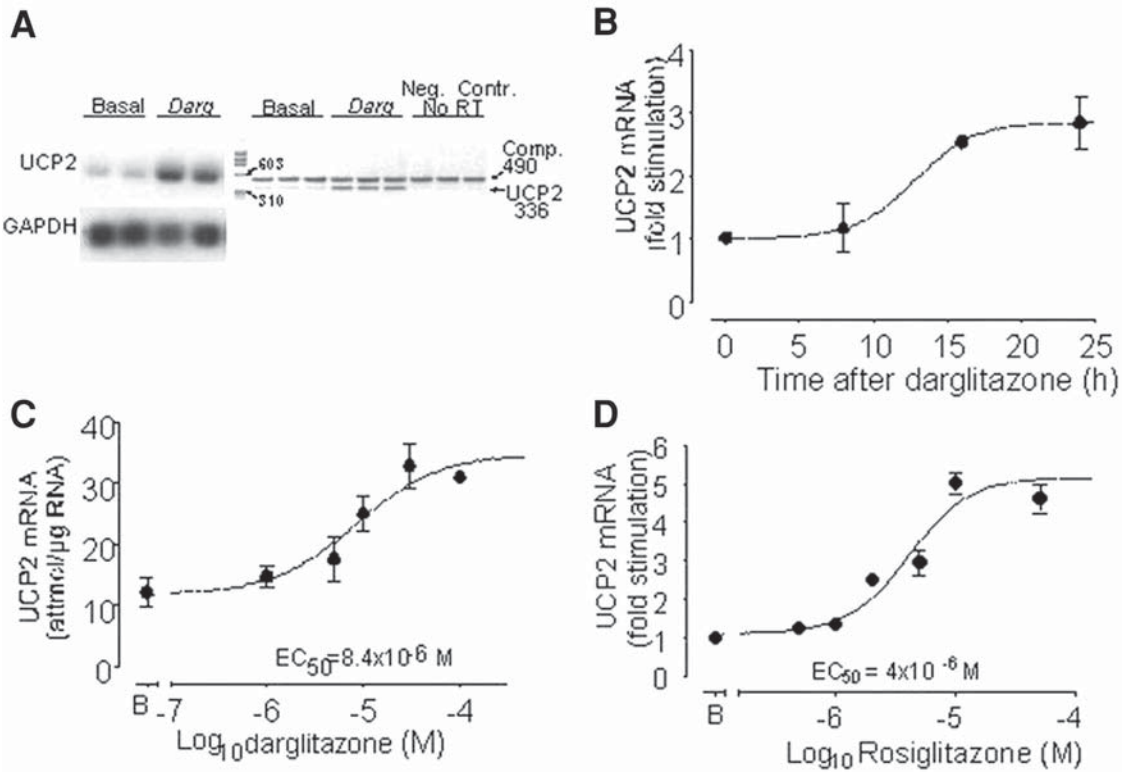

Fig. 1. Characterization of UCP2 mRNA response to darglitazone and rosiglitazone. (**A)** Northern blot and RT-PCR showing response of UCP2 mRNA to 24 h of exposure to 30 μ*M* darglitazone. In both cases, stimulation was about sixfold. RT-PCR shows the size of competitor and amplified UCP2 cDNA. The rightmost triplicates are negative controls wherein RT was omitted (No RT) (**B)** Time course of UCP2 mRNA response to 30 μ*M* darglitazone. (**C)** Darglitazone dose-response curve, after a 24-h exposure to the drug. (**D)** Equivalent dose-response curve for rosiglitazone. The lowest effective rosiglitazone concentration was 2 μ*M*, while the corresponding darglitazone concentration was 10 μ*M*. These curves represent the average of three separate experiments. See text for further details.

been extensively used to study various aspects of glucose metabolism in muscle, particularly mechanisms underlying insulin action and sensitivity *(16–18)*. Moreover, it has recently been reported that uncoupling phosphorylation in these cells with dinitrophenol is associated with increased glucose transport *(19)*.

Several laboratories, including ours, have reported that TZDs stimulate UCP2 gene (*ucp2)* expression in cultured cells and in vivo *(20–24)*. This effect was attributed to a direct stimulation of the gene mediated by PPARγ, but this has not been directly tested. Although Zierath et al. *(25)* have demonstrated the presence of PPARγ in rodent skeletal muscle, Nagase et al. *(26)* recently reported the absence of PPARγ mRNA in L6 myotubules, wherein TZDs clearly stimulate the expression of *ucp2 (21)*. More recently, however, a proximal enhancer element (–86/–44) was reported in the mouse *ucp2* that was necessary for expression in adipose and muscle cells, as well as for enhancement of reporter gene activity by PPARγ and TZDs *(27)*. Interestingly, this sequence contained no PPARγ response element (PPRE) nor did it bind PPARγ, altogether suggesting an indirect mechanism, mediated by a putative, PPARγ-induced transcription factor. We report here that TZDs stimulate the transcription of the autologous UCP2 gene in L6 cells via a mechanism requiring ongoing protein synthesis and mitogen-activated protein kinase (MAPK). Moreover, we show that L6 cells do express functional PPARγ2 protein, and the results are consistent with this receptor being involved in the mediation of the TZD effect.

Results

TZDs Stimulate UCP2 Gene Transcription by a Mechanism Requiring Ongoing Protein Synthesis

Figure 1 illustrates the most salient aspects of the response of UCP2 mRNA to the TZDs. Figure 1A shows a typical experiment in which cells were stimulated for 24 h with 30 μ*M* darglitazone. RNA was analyzed by both Northern blotting and reverse transcriptase polymerase chain reaction (RT-PCR). Note in the latter the clear separation of competitor DNA (490 bp) and amplified UCP2 cDNA (336 bp) and the absence of UCP2 signal when RT was omitted. With both analyses, the increase was about sixfold. Other aspects are illustrated in Fig. 1B–D. The response of UCP2 mRNA to darglitazone was slow, approaching a plateau around 16 h, as shown in Fig. 1B. The stimulation of UCP2 mRNA

was concentration dependent, as depicted in Fig. 1C (darglitazone) and Fig. 1D (rosiglitazone). These curves represent average results of three experiments with at least duplicates for each concentration (n = 6–9/concentration). Stimulation lasted 20–24 h. On average, lowest effective concentrations of rosiglitazone and darglitazone were 2 and 10 μ*M*, respectively, although in individual experiments lower concentrations elicited significant responses. Half-maximal concentrations were 4 and 8 μ*M*, respectively. Results with pioglitazone (one experiment) were nearly identical to those obtained with rosiglitazone. Unless stated otherwise, subsequent experiments were performed with maximal concentrations of TZDs.

Predictably, the blockade of transcription with actinomycin D (5 μg/mL) or α-amanitin (10 μg/mL) prevented the TZD-induced increase in UCP2 mRNA (Fig. 2A), but the decrease in UCP2 mRNA 24 h after darglitazone plus either transcriptional inhibitor was comparatively minor, suggesting that TZDs might stabilize UCP2 mRNA. This was not the case, as shown in Fig. 2B. The small reduction in Fig. 2A was owing to the slow turnover of the mRNA ($t_{1/2}$ = 38–42 h), and this was not significantly affected by the presence of darglitazone. Identical results were obtained when transcription was blocked with actinomycin D (not shown). We also tested whether ongoing protein synthesis was necessary for the UCP2 mRNA response to TZDs. Cycloheximide added together with darglitazone during the 24-h stimulation completely prevented the effect of the TZDs (results not shown). To determine at what stage during the stimulation ongoing protein synthesis was necessary, in subsequent experiments we added cycloheximide for 6 h at different times following the addition of darglitazone. Abrogation of the effect of darglitazone was maximal when the translation inhibitor was added during the first 6 h but was attenuated when added after and nil when added during the last 6–8 h of exposure to darglitazone (Fig. 2C). Note that separate controls were run for these timed additions to exclude artifacts derived from the manipulation of the cells. Altogether, these results indicate that TZDs increase UCP2 mRNA by stimulating transcription of the gene, and that ongoing protein synthesis is necessary.

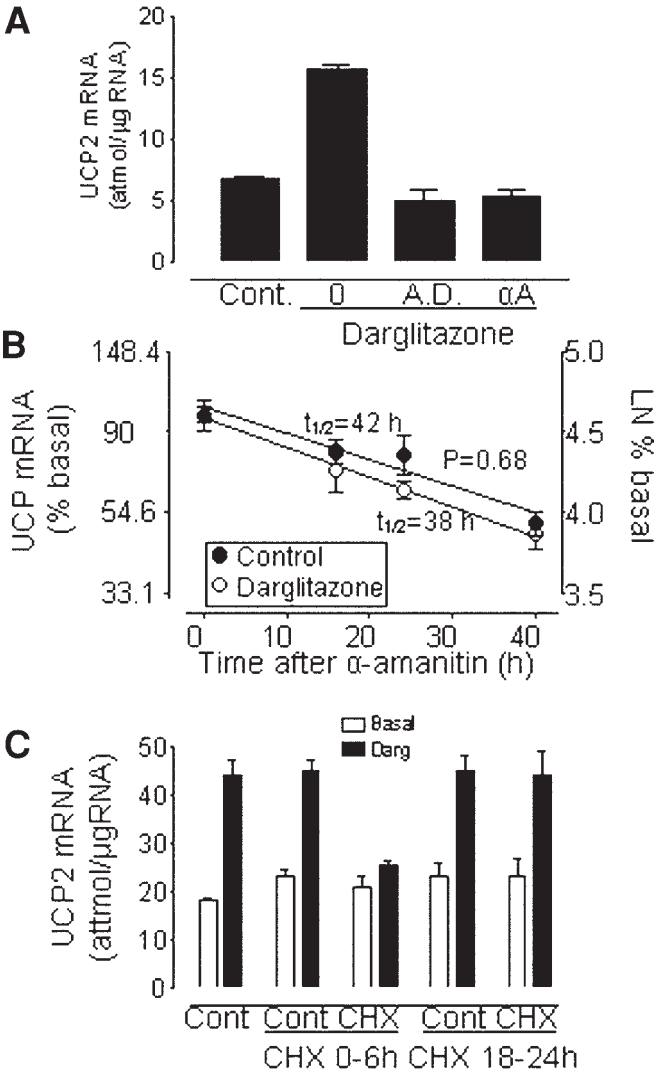

Fig. 2. Effects of inhibitors of transcription and translation on UCP2 mRNA stimulation by TZDs. (**A)** Effect of actinomycin D (A.D.; 5 μg/mL) and α-amanitin (αA; 10 μg/mL) on stimulation of UCP2 mRNA by 30 μ*M* darglitazone; inhibitors were added 30 min before darglitazone and treatments lasted 24 h. (**B)** Disappearance of UCP2 mRNA after addition of 10 μg/mL of α-amanitin in presence or absence of 30 μ*M* darglitazone; $t_{1/2} \approx 40$ h was not affected by the TZD. (**C)** Effect cycloheximide (CHX; 5 μg/mL) added either during first or last 6 h of treatment with 30 μ*M* darglitazone. See text for methodological details.

L6 Cells Contain PPARγ mRNA and Protein

TZDs are high-affinity ligands for PPARγ and are believed to exert most of their effects through this receptor *(28)*. PPARγ mRNA levels are, however, low in muscle *(13,29)*, and Nagase et al. *(26)* reported no evidence of PPARγ mRNA by RT-PCR in the rat L6 cells. Like them, we had no difficulty identifying the presence of PPARβ (PPARδ) mRNA, and found no PPARα mRNA, but using the rat PPARγ primers described in Materials and Methods, which span a larger product, we found a small but distinct amount of PPARγ mRNA by RT-PCR (Fig. 3A). To estimate the amount of this mRNA, we amplified graded amounts of mPPARγ cDNA in the PCR, from 0.01 to 100 attmol along with the RT-obtained cDNA from L6 or HIB-1B cell RNA. The intensity of the PCR products from L6 RNA, representing approx 110 ng of reverse-transcribed RNA, is equivalent to about 0.01 attmol of cDNA or 0.2 attmol of mRNA/μg of total L6 cell RNA. By contrast, HIB cells have at least 100 times more. The existence of the protein was investigated with the commercial antibodies described in Materials and Methods, using Jurkat cells and HIB-1B cells as positive controls. The former contains predominantly PPARγ 1 *(30,31)*, whereas the latter expresses both PPARγ isoforms. The calculated molecular weight based on two sets of size markers flanking the

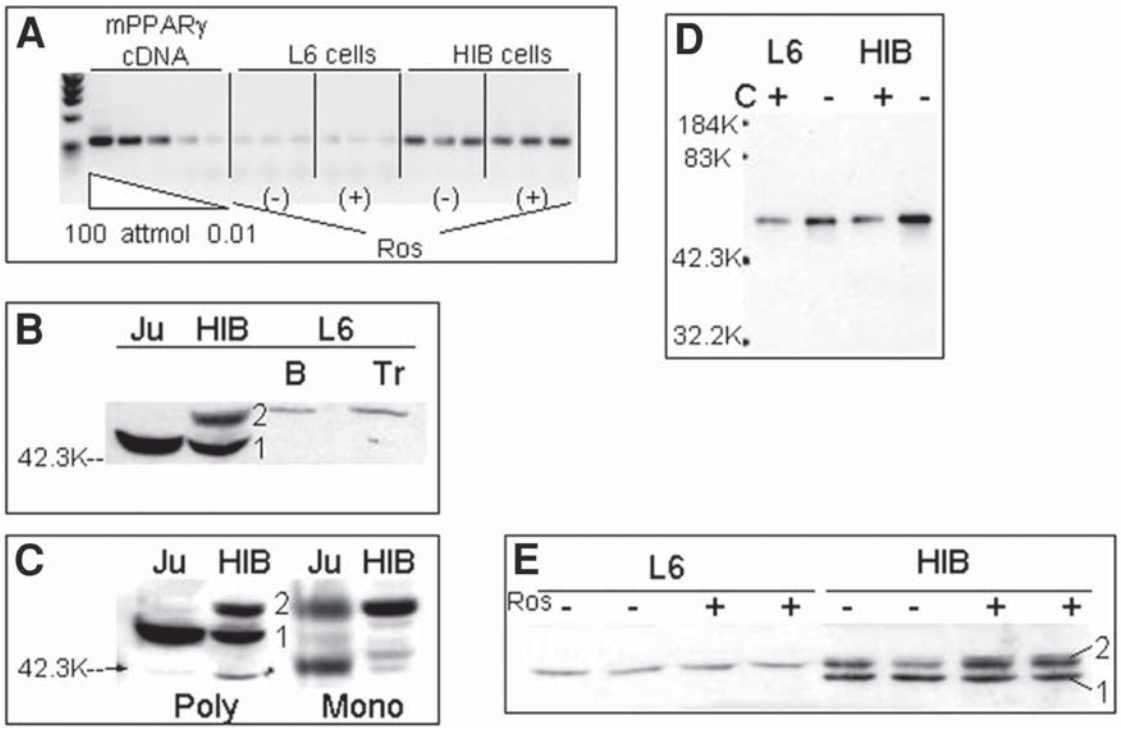

Fig. 3. Expression of PPARγ in L6 myotubules. See Materials and Methods for primers, antibody sources, and procedures. (**A)** RT-PCR of L6 and HIB-1B cell RNA. Each lane represents approx 110 ng of cell RNA reverse transcribed and then amplified as described in Materials and Methods. For quantitative estimation, graded amounts of mPPARγ cDNA, in multiples of 10, were added to the PCR. (**B)** Western blot of nuclear extracts from Jurkat, HIB-1B, and L6 cells using a commercial polyclonal antibody (1/500 dilution). Each lane represents a pool of triplicate 35-mm dishes. Half the L6 cells were transfected 24 h earlier with 1 μg of mPPARγ 2 cDNA in pSV-SPORT1. 1 and 2 = the signals corresponding to PPARγ 1 and PPARγ 2, respectively. (**C)** Comparison of polyclonal (1/250) and monoclonal (1/200) antibodies using Jurkat and HIB-1B cell nuclear extracts. Note that the strong band corresponding to PPARγ1 is barely visible (Jurkat) or not at all visible with the MAb. (**D)** Nuclear extracts of L6 and HIB-1B cells (≈300 μg of protein) were immunoprecipitated with a 1/100 dilution of MAb ± 40 μg/mL of competing ("C") immunizing peptide. (**E)** Western blot of L6 and HIB-1B cell nuclear extracts using the polyclonal antibody. 1 and 2 = the signals corresponding to PPARγ1 and PPARγ2. Each lane represents 50 μg of nuclear extract protein from a 60-mm dish. Half of the cells were exposed to 20 μ*M* rosiglitazone (Ros).

samples were ≈57,500 and ≈54,000, respectively, exactly the size of PPARγ2 and PPARγ1. There was a clearly detectable amount of PPARγ2 protein in L6 cell nuclear extracts that comigrated with the band corresponding to PPARγ2 in HIB-1B cells (Fig. 3B). The identity of the protein was confirmed by the enhancement of the band in cells transiently transfected with mouse PPARγ2 cDNA (B vs Tr in Fig. 3B). To obtain further support of the nature of the putative PPARγ2 band, we immunoprecipitated nuclear extracts of L6 cells using a monoclonal antibody (MAb) and then analyzed the immunoprecipitate by Western blotting, using the polyclonal antibody as probe. Even though nominally the MAb binds both PPARγ isoforms, it recognizes much better PPARγ2. Note in Fig. 3C that the intense PPARγ1 band of Jurkat and HIB-1B cells seen with the polyclonal antibody is barely if at all visible with the MAb. As shown in Fig. 3D, a single band corresponding to PPARγ2 was visualized in the material precipitated by the MAb. The addition of immunizing peptide to the immunoprecipitation significantly reduced the intensity of the signal. As shown in Fig. 3E, the addition of rosiglitazone did not significantly increase the signal in L6 cells. Thus, both PPARγ2 mRNA and protein are expressed in L6 cells, albeit in quantities substantially lower than in adipose cell lines. Notably, TZDs increased neither PPARγ mRNA nor PPARγ2 protein (Fig. 3A,E).

Evidence That PPARγ Is Functional in L6 Myotubules and May Mediate Effects of TZDs

For further evidence that PPARγ was present and functional in L6 myotubules, we examined the response of acyl CoA synthase (ACoAS) to TZDs and 15-deoxy-Δ-12,14-J2 (PgJ2). ACoAS is a muscle enzyme known to be stimulated by PPARγ ligands and contains a well-defined PPRE *(32)*. We also performed transient transfection assays using a construct containing the promoter and critical enhancer of the mouse *ucp2*, known to be responsive to PPARγ *(27)*, and a construct containing the promoter and PPRE of ACoAS. In addition, we tested the effect of the PPARγ antagonist bisphenol A diglycidyl ether (BADGE) *(33)* on the TZD stimulation of UCP2 mRNA, as well as the effect of overexpressing PPARγ ± retinoid X receptor (RXR) on *ucp2* reporter activity in transient transfections.

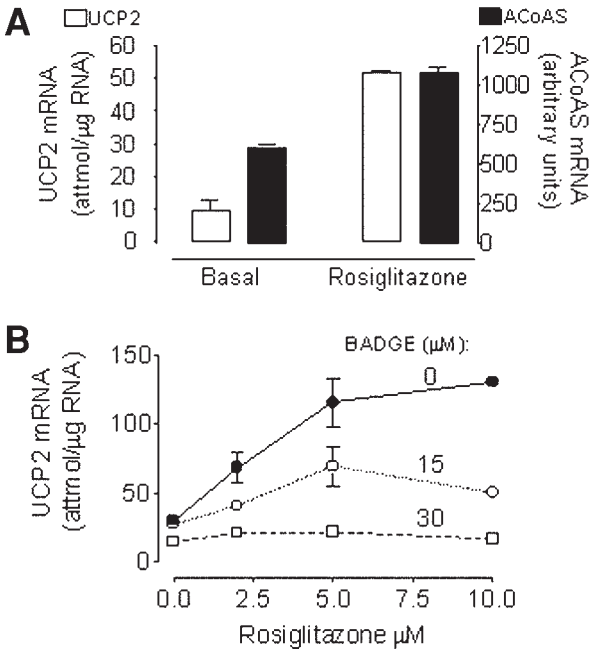

Fig. 4. Effect of rosiglitazone and PPARγ inhibitor BADGE on UCP2 and ACoAS mRNAs in L6 myotubules. **(A)** Effect of 20 µ*M* rosiglitazone on UCP2 and AcoAS mRNAs. UCP2 mRNA was quantified by competitive RT-PCR, as described in Materials and Methods, whereas ACoAS mRNA was estimated by direct densitometry of the RT-PCR products. **(B)** Effect of 15 or 30 µ*M* BADGE on stimulation of UCP2 mRNA by various concentrations of rosiglitazone. The effect of both concentrations of BADGE was highly significant ($p < 0.0001$). The effect of 30 µ*M* BADGE was significantly greater than that of 15 µ*M* ($p < 0.0001$). BADGE had no significant effect in the absence of rosiglitazone. See text for details.

As shown in Fig. 4A, rosiglitazone significantly increased the level of both UCP2 and ACoAS mRNA, although the latter was not as responsive. Figure 4B shows the effect of various concentrations of rosiglitazone (2, 5, and 10 µ*M*) in the absence or presence of BADGE, at 15 or 30 µ*M*. BADGE had no significant effect in the absence of rosiglitazone, whereas it significantly inhibited the effect of the latter at both concentrations (two-way analysis of variance [ANOVA]: $F = 36.2$ for 15 µ*M* and 163 for 30 µ*M* when compared with no BADGE; $p < 0.0001$). At 15 µ*M* BADGE, the effect of rosiglitazone was partially inhibited and abolished at 30 µ*M*, a highly significant difference between the two BADGE concentrations ($F = 58.6$; $p < 0.0001$ by two-way ANOVA).

Transient transfection results are depicted in Fig. 5. Mouse *ucp2* sequences have strong promoter activity in L6 cells, driving luciferase gene expression at least 50 times more than the promoterless pGL3 (Fig. 5A). For convenience, subsequent experiments were performed with the construct encompassing from –851 to +926 of the m*ucp2*. As shown in Fig. 5B,C, this construct had 10 times more promoter activity than ACoAS-luciferase, but both were clearly stimulated by rosiglitazone and PgJ2. These results indicate that L6 cells have all the factors necessary for the expression of these two genes and for their stimulation by PPARγ ligands, arguing for the presence of functional PPARγ in these cells. In search of further support for the involvement of PPARγ on the stimulation of *ucp2*, we examined the effect of cotransfecting PPARγ ± RXRγ in the response of *ucp2*-Luc to rosiglitazone (RXRγ is RXR isoform preferentially expressed in muscle *[34]*). As shown in Fig. 5, the cotransfection of PPARγ was associated with a doubling of the stimulation by rosiglitazone alone, and this was further doubled by the cotransfection of RXRγ. This receptor alone, however, did not enhance the effect of rosiglitazone.

MAPK Activity is Necessary for Stimulation of ucp2 by TZDs

Both PPARγ1 and PPARγ2 contain a MAPK consensus phosphorylation site in their amino terminus. Observations largely made in adipose cell lines indicate that the phosphorylation of this site by MAPK causes a marked reduction or loss of activity and decreased affinity for ligands, while the abrogation of MAPK activity is usually associated with increased receptor activity *(35,36)*. Unexpectedly, the stimulation of UCP2 mRNA by darglitazone in L6 cells was reduced by the MEK inhibitor PD98059 in a dose-dependent manner (Fig. 6A). When averaging two such experiments, EC_{50} (50% reduction of increment over baseline induced by darglitazone) was ≈35 µ*M*. PD98059 and another MEK inhibitor, U0126, caused a marked inhibition or abolishment of the response to troglitazone and rosiglitazone as well (Fig. 6B). Moreover, the transcriptional stimulation of the transfected *ucp2*-Luc construct by TZDs was prevented by the presence of PD98059 (Fig. 6C). As occurred with cycloheximide, the effect of these inhibitors of MAPK activation was maximal when present at the initiation of stimulation of the TZDs, but it was attenuated when given 6 h after the addition of the TZDs, as illustrated in Fig. 6D. The p38 kinase inhibitor SB-203580 had no effect (results not shown), arguing for the specific need for erk1/2 activity for TZD stimulation of *ucp2*.

These results indicate that MAPK activity is necessary for the stimulation of *ucp2* transcription by TZDs. TZDs, however, did not stimulate the phosphorylation of erk1 and erk2. As shown, in Fig. 7A, darglitazone did not significantly increase the phosphorylation of these kinases, in contrast to the well-known stimulation caused by the medium change; if anything, exposure to darglitazone was associated with a slight reduction in erk1/2 level of phosphorylation, 16–24 h after addition (Fig. 7B). That the medium stimulation of erk1 and erk2 phosphorylation is mediated by MEK activation is demonstrated in Fig. 7C. Medium containing the indicated concentrations of PD98059 was replaced and cells were harvested 1 hour later. There was a concentration-dependent inhibition of the medium change–induced erk1 and erk2 phosphorylation, with 75 and 95% inhibition

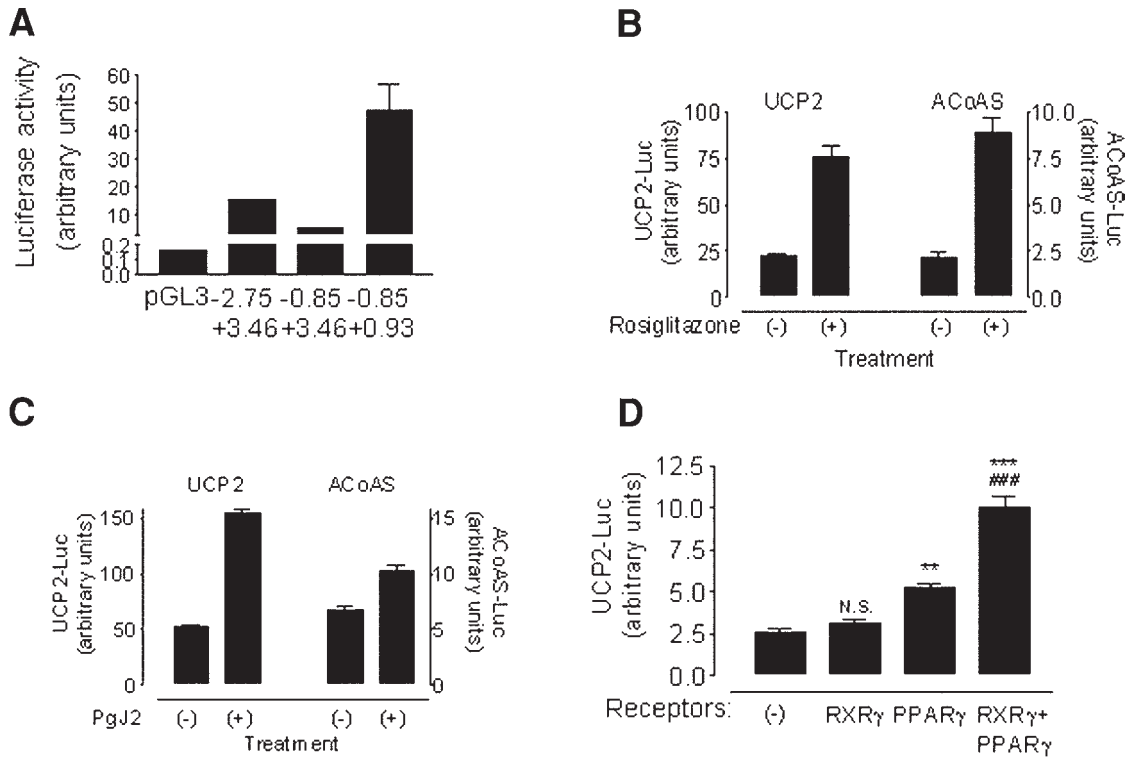

Fig. 5. Effect of rosiglitazone or prostaglandin PgJ2 on reporter gene activity driven by *ucp2* or ACoAS gene sequences. (**A)** Promoter activity of convenient restriction enzyme fragments of *ucp2*. Note that the *y*-axis is broken. The segment –851/+926 (–0.85/+0.93) was used in subsequent experiments. (**B)** Effect of 25 μ*M* rosiglitazone on promoter activity of *ucp2* and ACoAS genes. Note the 10X difference between the y-axes for *ucp2* and AcoAS. (**C)** Effect of 10 μ*M* PgJ2 on promoter activity of the above reporters. (**D)** Effect of cotransfection of RXRγ or PPARγ ± RXRγ cDNAs in expression vectors on the stimulation of *ucp2* promoter activity by 20 μ*M* rosiglitazone. No receptor cDNA was cotransfected in the control, denoted as (–). $^{**}p < 0.01$; $^{***}p < 0.001$ vs control; $^{\#\#\#}p < 0.001$ vs PPARγ.

at 40 and 80 μ*M*, respectively. On the other hand, the increased phosphorylation of erk1 and erk2 induced by medium change (Fig. 7D) or, for that matter, by insulin-like growth factor-1 (IGF-1) (Fig. 7E) or insulin (data not shown) was not associated with an increase in the levels of UCP2 mRNA. Thus, the sole activation of this signaling pathway is not associated with stimulation of this gene, indicating that this activity is necessary but not sufficient to effect the stimulation of *ucp2* by TZDs.

Discussion

Several laboratories, including ours, have reported that TZDs increase the expression of *ucp2* in skeletal muscle, in vivo and in vitro, in white and brown adipose cells, as well as skeletal muscle cell lines such as L6 *(20,21,23,24)*. The stimulation has been naturally ascribed to PPARγ activation by TZDs because these drugs bind those receptors with high affinity and mediate the transactivation of reporter genes directed by various PPREs in transient transfection assays (see ref. *13* for a review). The report by Nagase et al. *(26)* of the absence of PPARγ mRNA in L6 cells, in which TZDs clearly increase the level of UCP2 mRNA *(21)*, prompted us to reconsider this view. In addition, non-PPARγ-mediated effects of TZDs have been reported in muscle *(37)* and nonmuscle cells *(38–40)*. Moreover, Medvedev et al. *(27)* recently reported that PPARγ could increase the promoter activity of mouse UCP2 gene via a discrete proximal 40-bp enhancer (–86/–44), which nonetheless does not bind this receptor or contain a PPRE. On the other hand, L6 cells are a popular model to study muscle glucose metabolism *(16–18)*, and it has been reported that uncouplers of phosphorylation can enhance glucose uptake in these cells *(19)*. For all these reasons, then, it was of interest to investigate the mechanism of UCP2 stimulation by TZDs in L6 cells. As we show here, these cells contain functional PPARγ2 and TZDs stimulate *ucp2* transcription, but seemingly by an indirect mechanism involving the induction of a protein that needs to be phosphorylated by MAPK. Although further work is needed to identify the putative intermediary, the results illustrate a novel mechanism of action of TZDs in a skeletal muscle cell line.

All TZDs used in the present experiments increase UCP2 mRNA levels in a quantitatively and qualitatively similar

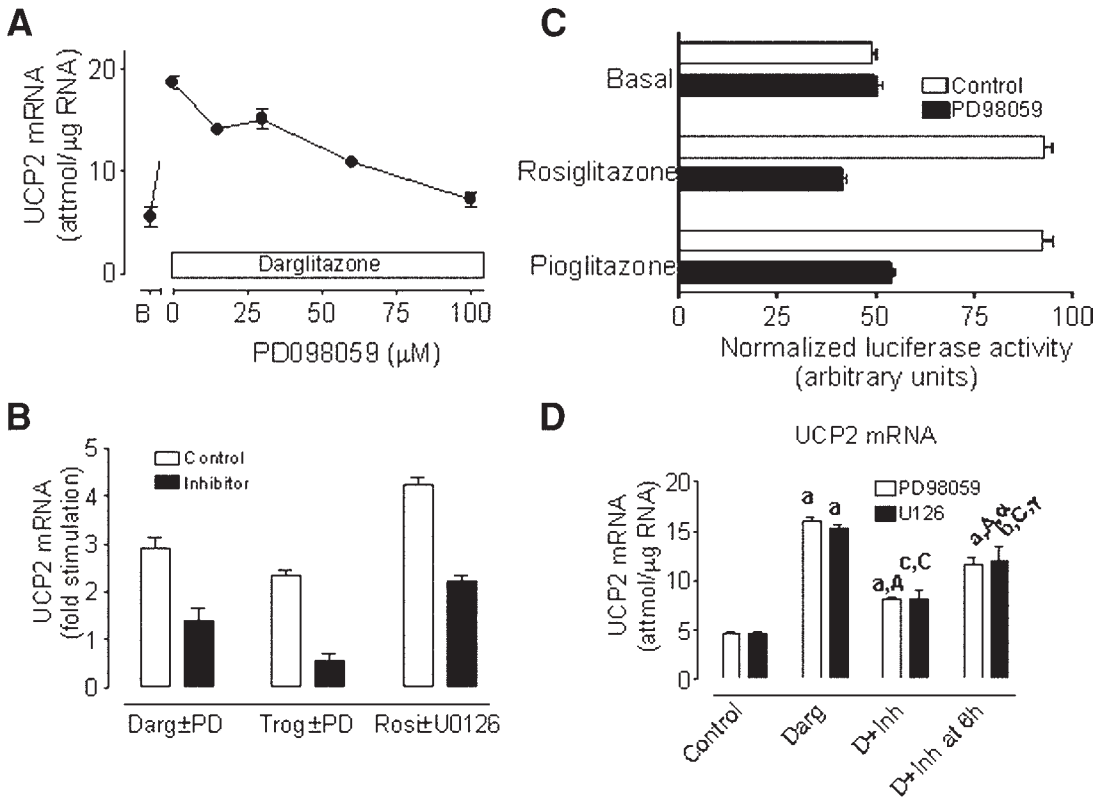

Fig. 6. Effect of inhibitors of MAPK activation on stimulation of UCP2 expression by TZDs. (**A)** Concentration-dependent inhibition of stimulation of UCP2 mRNA by darglitazone. PD098059 was added in the indicated concentrations 30 min before 30 μ*M* darglitazone, and UCP2 mRNA was measured 24 h later. (**B)** Effect of 80 μ*M* PD98059 (PD) or 10 μ*M* U0126 on stimulation of UCP2 mRNA by darglitazone (30 μ*M*), troglitazone (10 μ*M*), or rosiglitazone (20 μ*M*). (**C)** Effect of 80 μ*M* PD098059 on rosiglitazone (20 μ*M*) or pioglitazone (30 μ*M*) stimulation of *ucp2*-directed (+851/+926) reporter gene activity. (**D)** Effect of time of addition of 80 mM PD098059 or 10 μ*M* U0126 on inhibition of darglitazone (30 μ*M*) stimulation of UCP2 mRNA. When added right before darglitazone (D+inh), the MAPK inhibitors nearly abolished the stimulation, whereas when added 6 h after darglitazone (D+inh at 6 h), the inhibition was significantly less, and nil when MAPK inhibitors were added during the last 8 h of darglitazone stimulation (not shown). a, b, and c, $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively, to control; A and C: $p < 0.001$, and $p < 0.05$, respectively, to darglitazone alone; α and γ, $p < 0.001$, and $p < 0.05$, respectively, to inhibitors added right before darglitazone (D+inh).

manner. The increase reaches a plateau 2.5- to 6-fold the baseline levels in approx 24 h. These response characteristics of the mRNA reflect in part its slow turnover, with a $t_{1/2}$ of about 40 h (to the best of our knowledge, the first report on UCP2 mRNA half-life). The increase in mRNA is the result of transcriptional stimulation, since the TZDs do not affect its stability and can stimulate reporter gene expression directed by *ucp2* sequences.

Contrary to the findings of Nagase et al. *(26)*, we found small but distinct amounts of PPARγ mRNA in L6 cells, and, most important, we clearly demonstrated the presence of PPARγ2 protein, which albeit less abundant than in brown adipocytes is more abundant than the mRNA level would suggest (Fig. 3B–E). It is likely that the failure to demonstrate PPARγ mRNA by Nagase et al. *(26)* was owing to the comparatively low level of this messenger (about 1/100 of that present in brown adipocytes) and to the small size of PCR product generated with their primers. Note that PPARγ protein has also been demonstrated in skeletal muscle nuclei *(25)*, indicating that the presence of this receptor is normal in muscle and that L6 myotubules keep this characteristic.

Several lines of evidence indicate that PPARγ2 may mediate TZD stimulation of *ucp2* in L6 myotubules. First, TZDs and another nonrelated PPARγ ligand, PgJ2, can stimulate the ACoAS gene directed by a well-known target gene for PPARγ ligands, with a well-defined PPRE *(41)*. Both the autologous ACoAS mRNA and transiently transfected ACoAS reporter, containing solely the PPRE and the promoter, were stimulated by rosiglitazone and PgJ2. Second, the stimulation of *ucp2* by TZDs was inhibited in a concentration-dependent manner by the PPARγ antagonist BADGE. This substance antagonizes a number of effects of TZDs and other PPARγ ligands *(33,42)*. At half-maximal concentration (15 μ*M*), BADGE partially inhibited, and at maximal concentrations (30 m*M*) *(42)*, abolished the rosiglitazone-induced increase in UCP2 mRNA (Fig.4B). Finally, the stimulation

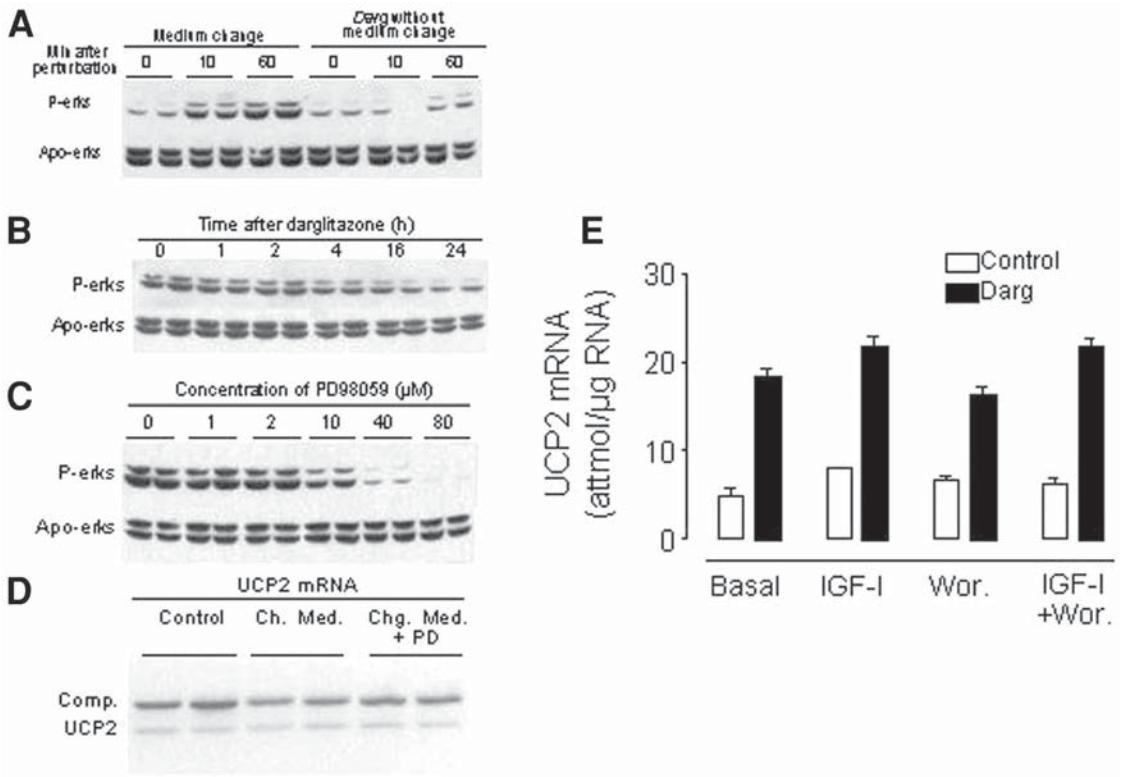

Fig. 7. MAPK activation and UCP2 mRNA stimulation by TZDs. (**A)** Short-term effect on the level of phosphorylation of erk1 and erk2 of changing medium or adding 30 μ*M* darglitazone without changing medium. (**B)** Long-term effect on level of phosphorylation of erk 1 and erk 2 of 30 μ*M* darglitazone. (**C)** Effect of PD098059 on medium change–induced phosphorylation of erk1 and erk2. Medium containing no PD98059 or the indicated concentrations of it was replaced and cells were harvested 1 h later for erk phosphorylation analysis. (**D)** Lack of effect of changing medium on UCP2 mRNA. (**E)** Lack of effect of 10 n*M* IGF-1 ± 100 n*M* wortmannin on darglitazone (30 μ*M*) stimulation of UCP2 mRNA.

of *ucp2*-luciferase reporter by rosiglitazone was significantly amplified by cotransfection of PPARγ2 expressing vector and further increased by the additional cotransfection of the dimeric partner RXRγ.

Is it possible that *ucp2* stimulation by TZDs can be mediated by other PPARs? Although TZDs can bind to PPARα in high concentrations, we did not detect traces of this receptor mRNA, in agreement with previous reports *(26)*, and WY-14643, a specific PPARα ligand, had no effect on *ucp2* expression (data not shown). PPARβ mRNA, on the other hand, is clearly expressed in L6 cells *(26)*, which we have confirmed, and this receptor conceivably might mediate the effect of TZDs. However, attempts to demonstrate binding of TZDs or PgJ2 to this receptor or mediation by PPARβ of transactivation by these substances in transient transfection assays have failed *(13,43)*. It could also be argued that the TZD concentrations needed to demonstrate significant stimulation of UCP2, which in the present studies is in the low micromolar range, are high for a PPARγ-mediated effect. The K_D of these drugs for PPARγ in binding assays is ≈50 n*M (44)*, and many effects can be demonstrated in the 20 to 500 n*M* range (see ref. *13* and references therein). This is not however, a strong argument against a PPARγ-mediated effect. Such an affinity has been demonstrated in cell-free assays, with chimeras consisting of glutathione S-transferase fused to the ligand-binding domain of PPARγ *(44)*, and does not necessarily represent the affinity of the whole receptor within the cell nucleus. Furthermore, the majority of studies showing effects in the nanomolar range have been performed in adipose cell lines, which abundantly express PPARγ. Indeed, a review of numerous studies performed in skeletal muscle or cell lines thereof indicates that maximal effects require TZD concentrations well into the 10 to 100 μ*M* range, as reported here (see, e.g., refs. *45–47* and references therein). This includes such a relevant effect as stimulation of glucose transport in L6 cells *(48)*. Such a higher TZD concentration requirement may reflect the lower concentration of PPARγ in muscle or the need for a higher extracellular/nuclear gradient to reach a critical concentration in the cell nucleus. Moreover, plasma concentrations reached after average single doses of medically approved TZDs in humans are also in the low micromolar range (see, e.g., refs. *49* and *50* and references therein). Thus, the effective concentrations found in the present study are comparable with

those in other studies involving muscle cells and are in a physiologic and medically relevant range.

Even though the results are consistent with the participation of PPARγ in the stimulation of *ucp2* by TZDs, the need of ongoing protein synthesis suggests an indirect mechanism. Since cycloheximide has to be given early during exposure to TZDs to prevent the effect, as is also the case with inhibitors of MAPK activation, the results suggest that a rapidly turned over protein, probably phosphorylated by MAPK, is necessary for the stimulation of *ucp2* transcription. Our findings are in agreement with a recent report by Medvedev et al. *(27)*. They describe an essential enhancer necessary for the gene's expression and for PPARγ and TZD stimulation of transcription. This 40-bp enhancer (–86/–44), however, does not bind PPARγ nor does it contain a sequence remotely related to a PPRE *(27)*. Altogether, these observations suggest that TZDs induce a protein that transactivates *ucp2*. This protein, and not MAPK activity, would be limiting for TZD stimulation of *ucp2*, since the sole stimulation of this kinase does not cause an increase in *ucp2* expression.

It has been shown that the phosphorylation of PPARγ by MAPK causes a reduction in the activity of this receptor and the affinity for its ligands *(35,51,52)*. In humans, an inactivating mutation of the consensus MAPK phosphorylation site is associated with increased adipogenesis, lipogenesis, and, ultimately, massive obesity *(53)*. This suggests that the receptor phosphorylation normally exerts a tonic inhibition of the receptor, preventing uncontrolled activation of genes stimulated via PPARγ. However, all these findings pertain to white adipose tissue. Here, we report that inhibition of MAPK is associated with a reduction in TZD stimulation in L6 myotubules. It is conceivable that phosphorylation of PPARγ by MAPK is a mechanism to control activity of the receptor where it is more abundant (e.g., white adipocytes). To our knowledge, there are no reports on PPARγ phosphorylation status in skeletal muscle or on the enhancement of the transactivation capacity of PPARγ in this tissue or muscle cells by reducing its phosphorylation level. On the other hand, it is not unprecedented that inhibition of MAPK attenuates some effects of TZDs. In transiently transfected Chinese hamster ovary cells, the stimulation of a P2 reporter by insulin and TZDs is blocked by inhibition of MAPK *(54)*.

In summary, we have demonstrated here that L6 myotubules contain PPARγ2, which is functionally active, and that TZDs increase the transcription of *ucp2* probably via this receptor. Moreover, we have demonstrated a novel mechanism of action of TZDs in which they induce a protein, present in limiting amounts, that needs the presence of MAPK activity to stimulate the transcription of the gene. Such protein is possibly a transcription factor, perhaps binding to the E-boxes needed for TZD stimulation of *ucp2 (27)*. Upstream transcription factors bind to this kind of sequence but other, yet unidentified factors may as well, as has been the case for the stimulation of the lipogenic transcription factor S14 *(55)*. The pursuit of the mechanisms involved in TZD stimulation of *ucp2* may reveal a factor (or factors) mediating effects of TZDs in a concerted manner in muscle.

Materials and Methods

Cell Culture and Cell Manipulations

L6 cells, a rat skeletal muscle cell line, was a gift from Dr. P. C. Holland, Montreal Neurological Institute, Montreal, QC, Canada. Cells were grown to confluence in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum, as is standard, and were subsequently differentiated by a 3-d exposure to Dulbecco's minimal essential medium with 2% horse serum. At this time, the cells form myotubules. During the third day, they were treated with the TZDs darglitazone, troglitazone, rosiglitazone, or pioglitazone for the indicated times and concentrations. Initially, most of the experiments were performed with darglitazone, and other TZDs were added as became available. Darglitazone was a gift from Pfizer (Groton, CT; Dr. E. Pagani), troglitazone from Parke-Davis (Ann Arbor, MI; B. S. Jared), pioglitazone from Takeda (Osaka, Japan), and rosiglitazone from SmithKline Beecham (London, UK). The former three were dissolved in dimethyl sulfoxide and the latter in ethanol, and they were added to the medium in 500- to 1000-fold the final concentration so that the maximal concentration of vehicle in the medium was 0.2%. The appropriate volume of vehicle was routinely added to control cells. Other drugs used included the prostaglandin, PgJ2, a PPARγ agonist; the PPARγ antagonist BADGE; the MAPK inhibitors PD98059 and U0126; the p38 kinase inhibitor SB-203580; and other substances such as wortmannin, insulin, and IGF-1, all of which were obtained from Sigma or Biomol. Times of exposure, concentrations, and conditions are described with the individual experiments.

RNA Analysis

At the end of treatments, medium was aspirated, cells were washed with phosphate-buffered saline (PBS), and RNA was extracted using standard methods *(56)*. UCP2 mRNA was analyzed by an RT-competitive PCR assay setup in the laboratory as described elsewhere *(57)*. Primers for rodent (mouse and rat) UCP2 cDNA were: 5'-GCTAGTG CGCACCGCAGCCAGCGCCCA for the sense, and 5'-TT CGACAGTGCTCTGGTATCTCCGACC for the antisense, corresponding respectively to nucleotides 752–778 and 468–442 of the mouse cDNA sequence, accession no. U69135. The primers to create the competitor DNA contained these two sequences upstream of 5'-CAAGTTTCGTGAGCTG ATTG and 5'-TTGAGTCCATGGGGAGCTTT, respectively. These two latter sequences span 436 nucleotides of the v-*erb*B cDNA provided in the PCR-MIMIC kit from Clontech, which, with the sequences of UCP2 cDNA generated a competitor of 490 bp, 1.46 times larger than the UCP2 cDNA PCR product. In initial experiments, relative changes

in mRNA levels were confirmed by Northern blot analysis, performed as described previously *(21)*, using a mouse UCP2 cDNA cloned by PCR along with a glyceraldehyde phosphate dehydrogenase cDNA probe (from American Type Culture Collection) to control for equivalence of mRNA loading.

Primers utilized to detect mRNA for the various PPAR isoforms in L6 cells were those described by Nagase et al. *(26)*. In view of the failure of these investigators to detect PPARγ mRNA, we created our own primers designed to obtain a larger product (229 bp) from rat PPARγ mRNA (accession no. NM-013124). These primers target the sequence common to PPARγ1 and PPARγ2 and were as follows: sense, 5-GCCTTCAAACTCCCTCATGG; antisense, 5-GTGAG ACATCCCCACAGCAA. The primers for rat acyl-CoA synthetase (accession no. D90109) were as follows: sense, 5-GT GAAAGGGGCAAATGTGTT; antisense, 5-CTTCGCT CCGCAGGTAGATA.

Northern blots and RT-PCR products were analyzed videodensitometrically, and mRNA was expressed as either arbitrary densitometric units or attomoles per microgram of total RNA in the case of UCP2. The quantity of UCP2 mRNA was derived by videodensitometric analysis of the competitive PCR products using appropriate amounts of competitor DNA. Products were separated in agarose gels, stained with ethidium bromide, and photographed. Densitometric readings (Scion Image, Scion, Frederick, MD) were corrected by the difference in size between the competitor and target DNA, and the mass of UCP2 mRNA was calculated as previously described *(57)*. Uniformity of RNA aliquots for RT was checked by electrophoresis of parallel aliquots of total RNA and PCR of β-actin cDNA in the RT product using primers described elsewhere *(58)*.

Western Blot Analyses

The presence of PPARγ1 and PPARγ2 was investigated in cell lysates and nuclear extracts of L6 cells by Western blot analysis and immunoprecipitation, using polyclonal and monoclonal antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody (sc-7196) was raised against a recombinant protein spanning amino acids 6–105 of PPARγ1 or 36–135 of PPARγ2 and, consequently, recognizes both isoforms of the PPARγ. The MAb (sc-7273) was raised using the carboxyl end of PPARγ. Such antibody should recognize both PPARγ1 and PPARγ2, but it is clearly more sensitive to PPARγ2 (see Results). Neither antibody crossreacts with PPARα or PPARβ. The presence of erk1 and erk2 and their phosphorylation state were also tested by Western blot analysis using specific antibodies from New England Biolabs (Beverly, MA) for both the phosphorylated and nonphosphorylated proteins.

For total cell lysate preparation, cells were washed in PBS; trypsinized; washed once more in suspension; and lysed in RIPA buffer containing 20 m*M* Tris, 150 m*M* NaCl, 0.5% deoxycholate, 0.2% Nonidet, 1 m*M* EDTA, 1 m*M* sodium orthovanadate, and protease inhibitors (500 μ*M* phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL of aprotinin, and 20 μg/mL of leupeptin). Cell lysate was cleared by centrifugation. For nuclear extract preparation, the washed cell pellet was resuspended and allowed to swell on ice for 10 min in cold, hypotonic lysing buffer (10 m*M* HEPES-KOH, pH 7.9; 1.5 m*M* $MgCl_2$; 10 m*M* KCl; 0.5 m*M* dithiothreitol; 0.2 m*M* PMSF). Swollen cells were broken by vigorous vortexing, and the lysate was centrifuged at 1000*g* for 10 min to collect the nuclei. These were washed once more in the same buffer, and then extracted with RIPA buffer for 30 min in ice, with frequent vortexing. The insoluble chromatin was pelleted by centrifuging at 14,000*g* for 10 min at 4°C. The nuclear extract (supernatant) was collected and stored frozen at −80°C until use. Proteins were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (8% polyacrylamide) and electrotransferred to nitrocellulose membranes. These were blocked with 5% skimmed milk, 0.1% Tween-20 and exposed to the appropriate dilutions of the corresponding antibodies, usually in the range recommended by the provider.

Transient Transfections

Transient transfections using reporter genes driven by *ucp2* or ACoAS gene sequences were performed in L6 cells. The reporter gene was the firefly luciferase cloned in the promoter less vector pGL3 (Promega, Madison, WI) where *ucp2* and ACoAS sequences were cloned directionally utilizing convenient restriction sites. The entire 5'-flanking region of the mouse *ucp2*, spanning from −7236 down to +3463, was cloned by PCR from genomic mouse DNA using several sets of primers based on published sequences, accession nos. AF115319 and AB018416 *(59)*. For the purpose of the analysis presented here and based on recently published analysis of the mouse *ucp2 (27)*, we used a convenient restriction fragment encompassing −851/+926. The nucleotide +926 is located 69 nucleotides into the second exon, which is nontranslated. This fragment retains full promoter activity and encompasses the −86/−44 enhancer described elsewhere *(27)*.

The sequence encompassing the promoter and PPRE of the rat ACoAS gene (−166/+10; accession no. D38589) was obtained by PCR from genomic rat DNA with the following primers: Sense, 5'-GTCTGATAGGTACCTTTAGCC CAG, to which nucleotides were added to create a KpnI site; antisense, 5'-GCTGACTGCTAGCTGACACACCA, which encompasses an *Nhe*I site. These sites were utilized for directional cloning in the reporter gene vector. The identity of *ucp2* and ACoAS gene PCR products was confirmed by restriction enzyme mapping and sequencing.

L6 cells were transfected after being differentiated as indicated earlier, using the calcium phosphate method essentially as reported *(60)*. The DNA transfected consisted of

up to 6 µg of test DNA, 1 µg of receptor DNA (PPARγ2, cloned in pSV-SPORT1, and RXRγ, cloned in pSG5), and 0.1–0.2 µg of a thymidine kinase-renilla luciferase expression vector as internal control for transfection efficiency. The amount of test DNA was adjusted for size to have the same number of copies. The total amount of DNA was made up to 10 µg with irrelevant plasmid DNA. Treatments of transfected cells, described where appropriate, lasted 24 h. Results are expressed in arbitrary luminescence units of firefly luciferase normalized by renilla luciferase. Since the luminescence generated by both luciferases normally varies from experiment to experiment, the ratio of both varies widely among experiments.

Data and Statistical Analyses

Data are reported as mean ± SEM. Unless noted otherwise, each treatment was done in triplicate. All experiments were repeated at least once to ensure reproducibility. Responses to continued variables (stimulus concentrations, time) were fitted to curves using the software GraphPad Prism 3.0 (GraphPad, San Diego, CA). Multiple treatments were analyzed by one or two-way ANOVA followed by tests for multiple comparisons with a control group (Dunnet) or among the various treatments (Neuman-Keuls). Statistical significance is shown in the figures only when relevant to an argument. When the error bar is not evident in the figures it is because its size is smaller than the corresponding symbol.

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References

1. Ricquier, D. and Bouillaud, F. (2000). *Biochem. J.* **345,** 161–179.
2. Adams, S. H. (2000). *J. Nutr.* **130,** 711–714.
3. Gong, D. W., Monemdjou, S., Gavrilova, O., et al. (2000). *J. Biol. Chem.* **275,** 16251–16257.
4. Vidal-Puig, A. J., Grujic, D., Zhang, C. Y., et al. (2000). *J. Biol. Chem.* **275,** 16258–16266.
5. Arsenijevic, D., Onuma, H., Pecqueur, C., et al. (2000). *Nat. Genet.* **26,** 435–439.
6. Chan, C. B., MacDonald, P. E., Saleh, M. C., Johns, D. C., Marban, E., and Wheeler, M. B. (1999). *Diabetes* **48,** 1482–1486.
7. Martin, G., Schoonjans, K., Staels, B., and Auwerx, J. (1998). *Atherosclerosis* **137(Suppl.),** S75–S80.
8. Donnelly, R. and Qu, X. Q. (1998). *Clin. Exp. Pharmacol. Physiol.* **25,** 79–87.
9. Elbrecht, A., Chen, Y. L., Cullinan, C. A., et al. (1996). *Biochem. Biophys. Res. Commun.* **224,** 431–437.
10. Yanase, T., Yashiro, T., Takitani, K., et al. (1997). *Biochem. Biophys. Res. Commun.* **233,** 320–324.
11. Fajas, L., Auboeuf, D., Raspe, E., et al. (1997). *J. Biol. Chem.* **272,** 18779–18789.
12. Reginato, M. J. and Lazar, M. A. (1999). *Trends Endocrinol. Metab.* **10,** 9–13.
13. Desvergne, B. and Wahli, W. (1999). *Endocr. Rev.* **20,** 649–688.
14. Steppan, C. M., Bailey, S. T., Bhat, S., et al. (2001). *Nature* **409,** 307–312.
15. Vidal-Puig, A. and O'Rahilly, S. (2001). *Clin. Endocrinol. (Oxf.)* **55,** 437–438.
16. Ballard, F. J. (1994). *J. Nutr.* **124,** 1540S–1545S.
17. D'Amore, T. and Lo, T. C. (1986). *Biochem. Cell Biol.* **64,** 1081–1091.
18. Klip, A. and Marette, A. (1992). *J. Cell Biochem.* **48,** 51–60.
19. Khayat, Z. A., Tsakiridis, T., Ueyama, A., Somwar, R., Ebina, Y., and Klip, A. (1998). *Am. Physiol.* **275,** C1487–C1497.
20. Aubert, J., Champigny, O., Saintmarc, P., et al. (1997). *Biochem. Biophys. Res. Commun.* **38,** 606–611.
21. Camirand, A., Marie, V., Rabelo, R., and Silva, J. E. (1998). *Endocrinology* **139,** 428–431.
22. Shimabukuro, M., Zhou, Y. T., Lee, Y., and Unger, R. H. (1997). *Biochem. Biophys. Res. Commun.* **237,** 359–361.
23. Strobel, A., Siquier, K., Zilberfarb, V., Strosberg, A. D., and Issad, T. (1999). *Diabetologia* **42,** 527–533.
24. Viguerie-Bascands, N., Saulnier-Blache, J. S., Dandine, M., Dauzats, M., Daviaud, D., and Langin, D. (1999). *Biochem. Biophys. Res. Commun.* **256,** 138–141.
25. Zierath, J. R., Ryder, J. W., Doebber, T., et al. (1998). *Endocrinology* **139,** 5034–5041.
26. Nagase, I., Yoshida, S., Canas, X., et al. (1999). *FEBS Lett.* **461,** 319–322.
27. Medvedev, A. V., Snedden, S. K., Raimbault, S., Ricquier, D., and Collins, S. (2001). *J. Biol. Chem.* **276,** 10817–10823.
28. Schoonjans, K. and Auwerx, J. (2000). *Lancet* **355,** 1008–1010.
29. Braissant, O., Foufelle, F., Scotto, C., Dauca, M., and Wahli, W. (1996). *Endocrinology* **137,** 354–366.
30. Goke, R., Goke, A., Goke, B., and Chen, Y. (2000). *Cell Immunol.* **201,** 77–82.
31. Yang, X. Y., Wang, L. H., Chen, T., et al. (2000). *J. Biol. Chem.* **275,** 4541–4544.
32.. Martin, G., Schoonjans, K., Lefebvre, A. M., Staels, B., and Auwerx, J. (1997). *J. Biol. Chem.* **272,** 28210–28217.
33. Wright, H. M., Clish, C. B., Mikami, T., et al. (2000). *J. Biol. Chem.* **275,** 1873–1877.
34. Liu, Q. and Linney, E. (1993). *Mol. Endocrinol.* **7,** 651–658.
35. Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996). *Science* **274,** 2100–2103.
36. Shao, D., Rangwala, S. M., Bailey, S. T., Krakow, S. L., Reginato, M. J., and Lazar, M. A. (1998). *Nature* **396,** 377–380.
37. Brunmair, B., Gras, F., Neschen, S., et al. (2001). *Diabetes* **50,** 2309–2315.
38. Wang, M., Wise, S. C., Leff, T., and Su, T. Z. (1999). *Diabetes* **48,** 254–260.
39. Chawla, A., Barak, Y., Nagy, L., Liao, D., Tontonoz, P., and Evans, R. M. (2001). *Nat. Med.* **7,** 48–52.
40. Palakurthi, S. S., Aktas, H., Grubissich, L. M., Mortensen, R. M., and Halperin, J. A. (2001). *Cancer Res.* **61,** 6213–6218.
41. Marcus, S. L., Miyata, K. S., Zhang, B., Subramani, S., Rachubinski, R. A., and Capone, J. P. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 5723–5727.
42. Bishop-Bailey, D., Hla, T., and Warner, T. D. (2000). *Br. J. Pharmacol.* **131,** 651–654.
43. Burris, T. P., Pelton, P. D., Zhou, L., Osborne, M. C., Cryan, E., and Demarest, K. T. (1999). *Mol. Endocrinol.* **13,** 410–417.
44. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995). *J. Biol. Chem.* **270,** 12953–12956.
45. Cha, B. S., Ciaraldi, T. P., Carter, L., et al. (2001). *Diabetologia* **44,** 444–452.
46. El-Kebbi, I. M., Roser, S., and Pollet, R. J. (1994). *Metab. Clin. Exp.* **43,** 953–958.

47. Hattori, Y., Akimoto, K., and Kasai, K. (2000). *Biochem. Biophys. Res. Commun.* **273,** 1144–1149.
48. Taha, C., Tsakiridis, T., McCall, A., and Klip, A. (1997). *Am. J. Physiol.* **273,** E68–E76.
49. Miller, A. K., Inglis, A. M., Culkin, K. T., Jorkasky, D. K., and Freed, M. I. (2001). *Eur. J. Clin. Pharmacol.* **57,** 105–109.
50. Prueksaritanont, T., Vega, J. M., Zhao, J., et al. (2001). *J. Clin. Pharmacol.* **41,** 573–581.
51. Adams, M., Reginato, M. J., Shao, D., Lazar, M. A., and Chatterjee, V. K. (1997). *J. Biol. Chem.* **272,** 5128–5132.
52. Camp, H. S. and Tafuri, S. R. (1997). *J. Biol. Chem.* **272,** 10811–10816.
53. Ristow, M., Müller-Wieland, D., Pfeiffer, A., Krone, W., and Kahn, C. R. (1998). *N. Engl. J. Med.* **339,** 953–959.
54. Zhang, B., Berger, J., Zhou, G. C., et al. (1996). *J. Biol. Chem.* **271,** 31771–31774.
55. Koo, S. H. and Towle, H. C. (2000). *J. Biol. Chem.* **275,** 5200–5207.
56. Chomczynski, P. and Sacchi, N. (1987). *Anal. Biochem.* **162,** 156–159.
57. Boivin, M., Camirand, A., Carli, F., Hoffer, L. J., and Silva, J. E. (2000). *J. Clin. Endocrinol. Metab.* **85,** 1975–1983.
58. Zhou, Y. T., Shimabukuro, M., Koyama, K., et al. (1997). *Proc. Natl. Acad. Sci. USA* **94,** 6386–6390.
59. Yoshitomi, H., Yamazaki, K., and Tanaka, I. (1999). *Biochem. J.* **340,** 397–404.
60. Rabelo, R., Schifman, A., Rubio, A., Sheng, X., and Silva, J. E. (1995). *Endocrinology* **136,** 1003–1013.