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Regulation of Uncoupling Protein-2 mRNA in L6 Myotubules

II: Thyroid Hormone Amplifies Stimulation of Uncoupling Protein-2 Gene by Thiazolidinediones and Other Peroxisome Proliferator-Activated Receptor Ligands in L6 Myotubules: Evidence for a Priming Effect

Irma López-Solache, Véronique Marie, Anne Camirand, and J. Enrique Silva

Department of Medicine, Division of Endocrinology, Jewish General Hospital, Lady Davis Institute, McGill University, Montreal, Quebec, Canada

The stimulation of the uncoupling protein-2 gene *(ucp2)* by thyroid hormone (triiodothyronine [T_3]) in vivo is variable, suggesting complex interactions and even the possibility of indirect effects. We investigated the effect of T_3 on *ucp2* expression in L6 myotubules. Alone, T_3 did not significantly stimulate *ucp2* expression in L6 cells, but it amplified the stimulation by thiazolidinediones (TZDs). L6 cells expressed both α1 and β1 thyroid hormone receptors and the data were consistent with the effect being mediated by these receptors. T_3 also enhanced the stimulation of *ucp2* by the nonselective peroxisome proliferator-activated receptor (PPAR) ligands bezafibrate and carbacyclin, but not that by oleic acid or norepinephrine. L6 cells expressed PPARβ and PPARγ, but not PPARα. As short as a 1-h preexposure of L6 cells to T_3 was sufficient to amplify the effect of PPAR ligands. Neither transcription nor translation was needed for this effect of T_3. T_3 did not affect the $t_{1/2}$ of UCP2 mRNA. The histone deacetylases inhibitor trichostatin A (TSA) stimulated the expression of *ucp2* but did not add to the effect of T_3 nor did this hormone enhance the effect of TSA. These results suggest that T_3 selectively enhances the transcriptional stimulation of *ucp2* by TZDs and nonselective PPAR ligands by priming the gene to a transactivating signal(s) generated by such ligands.

Key Words: Thiazolidinediones; upcoupling protein-2; *ucp2*; L6 cells; triiodothyronine; skeletal muscle.

Introduction

Uncoupling protein-2 (UCP2) bears 57–59% homology with brown adipose tissue uncoupling protein (now UCP1) and, as UCP1, is located in the inner membrane of the mitochondria, where it can uncouple adenosine 5'-diphosphate phosphorylation. In contrast to UCP1, UCP2 has a wide tissue distribution and is likely to subserve different functions in a tissue-specific manner (see ref. *1* for recent review). Thus, in macrophages it may modulate the production of reactive oxygen species *(2)*, while in the β-cells of pancreatic islets it may modulate the amount of adenosine triphosphate produced and thereby control the response of these cells to glucose *(3)*. UCP2's function in other tissues, notably in skeletal muscle, remains to be defined. Both the mRNA and protein are expressed in human skeletal muscle and its content increases with body fat *(4)*, but the mRNA is also increased in fasting *(5)*. Interestingly, the basal expression is quite variable in muscle of lean, healthy humans *(6)*, suggesting a multifactorial, complex regulation.

Thyroid hormones play critical roles in differentiation, growth, and metabolism. The effects of these hormones are mediated by specific nuclear receptors (T_3R) that bind to a discrete sequence of target genes from where they recruit, in a ligand-dependent manner, cofactors that ultimately affect their transcription (reviewed in ref. *7*). Triiodothyronine (T_3) clearly stimulates the expression of UCP3 in rodents *(8,9)*. There are conflicting reports, however, regarding the stimulation of UCP2 gene (*ucp2*) expression by thyroid hormone in vivo, with some indicating that it does stimulate *(10–13)*, and others that it does not *(8)*. These findings suggest complex interactions with the participation of other factors. Even less is known about the mechanism whereby thyroid hormone would increase the expression of *ucp2*. The effect could be indirect, e.g., increasing free fatty acid concentration *(14)*, or it could be that T_3 amplifies the effect of other stimuli, as occurs with the brown adipose tissue UCP1 *(15)*. To discern these possibilities necessitates the use of simpler models in which the variables could be better controlled. Given the importance of skeletal muscle in energy balance and glucose homeostasis and the popularity of L6 cells as a model system to study these processes, we chose to study the stimulation of *ucp2* by T_3 in these cells. L6 cells have been reported to have T_3R and respond to T_3. Both T_3 binding and responses are increased when they differentiate as myotubules *(16)*.

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Author to whom all correspondence and reprint requests should be addressed: Dr. J. Enrique Silva, Jewish General Hospital, Division of Endocrinology, Room E104, 3755 Cote-Ste-Catherine Road, Montreal, Quebec, H3T 1E2, Canada. E-mail: enrique.silva@staff.mcgill.ca

We and others have reported that the insulin sensitizer drugs, thiazolidinediones (TZDs), stimulate the expression of *ucp2* in muscle and adipose tissue *(17–20)*, and notably in L6 cells *(17)*. TZDs are drugs with effects other than their insulin-sensitizing capacity. They have been reported to reduce endothelin production by endothelial cells *(21)* and to ameliorate the proliferative response of vascular smooth muscle to a number of stimuli *(22,23)*. This finding and the observation that UCP2 may reduce oxidative stress add a new interesting dimension to the therapeutic potential of these drugs. We reported in our preceding study *(24)* that TZDs indirectly stimulate *ucp2* transcription in L6 cells by inducing a protein that mediates the enhancement of transcription. Here, we report that this process is further amplified by T_3 acting at a genomic level.

Results

T_3 Amplifies Stimulation of UCP2 mRNA by TZDs but Does Not Increase Level of That Message

The most salient characteristics of the effect of T_3 on the stimulation of the *ucp2* gene by TZDs are depicted in Fig. 1. A representative experiment (Fig. 1A) shows that darglitazone (30 μ*M*) increases the level of UCP2 mRNA by a factor of 4 to 5. T_3, 50 n*M*, given alone, had no significant effect on the mRNA level; however, when given with darglitazone, the effect of this drug was further increased by a factor of about 2, bringing the total stimulation to seven- eightfold. Figure 1B shows the effects of darglitazone ± T_3 over 24 h. In this experiment, the stimulation by darglitazone alone was about 2.5-fold; T_3 alone did not cause stimulation but at every time point significantly enhanced the effect of darglitazone, bringing the overall increase in UCP2 mRNA to greater than sixfold. As shown in Fig. 1C, amplification by T_3 of the stimulation of UCP2 mRNA by darglitazone was maximal (about 2.5-fold) by 8 h after the additions.

T_3R Expression in L6 Cells and Muscle

To document the expression of T_3R in our L6 myotubules, we assayed the RNA for the presence of α_1T_3R and β_1T_3R mRNA. These were also measured in mouse skeletal muscle for comparison. As shown in Fig. 2, L6 myotubules express both receptors. For a quantitative estimate, we amplified the product of reverse transcription (equivalent to ≈110 ng of total RNA per sample) along with the corresponding receptor cDNAs cloned in pSG5, in 10-fold increments, from 0.01 to 100 attmols. Figure 2B shows the results of this quantification expressed on the basis of 1 μg of total RNA. There was a similar amount of α_1T_3R mRNA in L6 and mouse muscle, but the amount of β_1T_3R mRNA was less in L6 cells.

Both LevorotatoryT_4 and T_3 Amplify Effect of TZDs in Physiologic Range of Concentrations

In the experiment depicted in Fig. 3A, cells were stimulated with darglitazone for 24 h in the absence or presence of T_3 in concentrations ranging from 0.1 to 100 n*M*. In the absence of T_3, darglitazone increased the level of UCP2 mRNA by a factor of approx 2.5. T_3 amplified this response

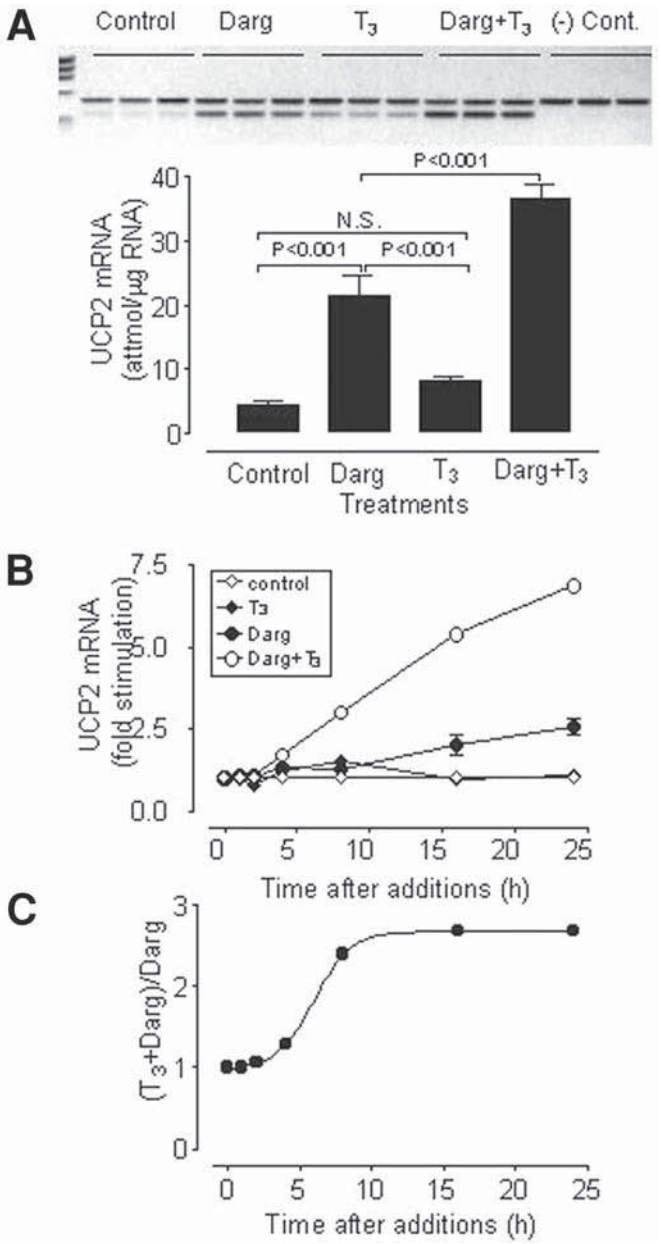

Fig. 1. Effects and interactions of darglitazone and T_3 on UCP2 mRNA levels of L6 myotubules. **(A)** Representative result after exposing cells to 30 μ*M* darglitazone, 50 n*M* T_3, or both for 24 h. The upper part shows the polymerase chain reaction (PCR) products obtained from reverse-transcribed RNA. In the negative controls ([–] Cont.), RT was omitted during the reverse transcription of pooled RNA samples; these lanes show only one band, corresponding to the competitor DNA. The lower part shows the RNA calculated from the densitometric analysis of the above electrophoresis, as described in Materials and Methods. **(B)** Time course of UCP2 mRNA accumulation over 24 h of exposure to darglitazone, T_3, or both, in the same concentrations as in (A**)**. **(C)** Time course of amplification by T_3. The value obtained at each time point with T_3 + darglitazone in (B) was divided by the corresponding value obtained with darglitazone alone.

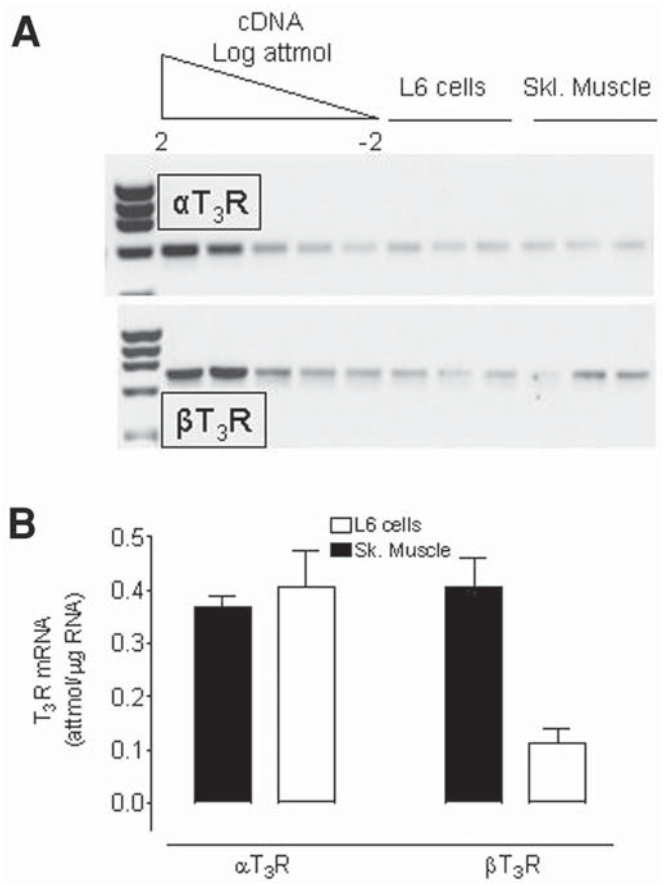

Fig. 2. Expression of T_3R mRNA in L6 and mouse skeletal muscle. (**A)** Each lane represents approx 110 ng of cell RNA reverse transcribed and then PCR amplified as described in Materials and Methods. For quantitative estimation, graded amounts of T_3R's cDNA, from 0.01 to 100 attmols, in 10X increments was added to the PCR. (**B)** Results of densitometric analysis of data in (A).

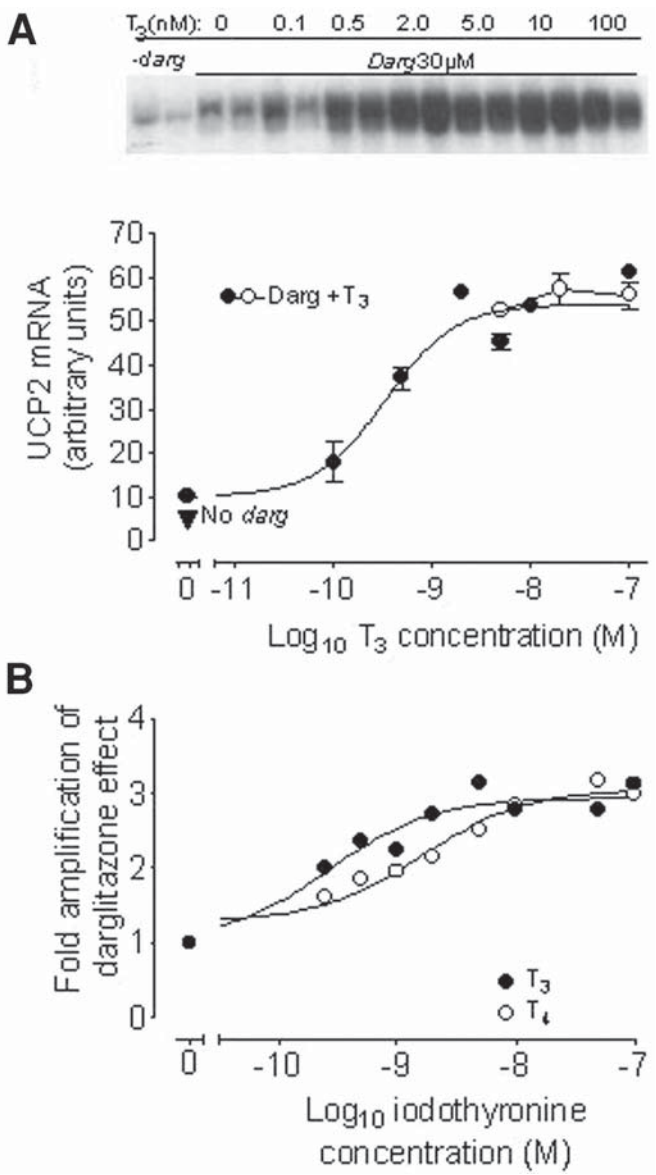

Fig. 3. T_3 and T_4 concentration dependency of amplification of darglitazone stimulation of UCP2 mRNA in L6 myotubules. **(A)** T_3 concentration-response curves. In these early experiments, RNA was analyzed by Northern blot, shown at the top. Cells were treated with only medium (▼) or darglitazone with the indicated concentrations of T_3 for 24 h. The open circles correspond to a separate experiment. **(B)** Cells were treated with darglitazone as in (A) plus either T_3 or T_4 in the indicated concentrations.

in a concentration-dependent manner with an EC_{50} = 0.35 n*M* and a maximal response seen with ≈2 n*M* T_3. An experiment with 5, 20, and 100 n*M* T_3 (open circles in Fig. 3A) was subsequently done and confirmed that concentrations of T_3 > 2 n*M* elicited a maximal response. As shown in Fig. 3B, the maximal amplification of the effect of darglitazone, approximately three times in this experiment, was the same with levorotatory thyroxine (T_4) and T_3, but the latter was about seven times more active than T_4 (EC_{50} of 0.26 vs 1.8 n*M*). Neither propylthiouracil nor iopanoic acid affected significantly the effect of T_4 (data not shown), indicating that T_4 was not being converted to T_3 and that L6 cells probably do not have significant levels of iodothyronine deiodinase activity.

In the experiments depicted in Fig. 4, cells were treated with increasing concentrations of the indicated TZDs, with or without a maximal concentration of T_3 (50 n*M*). In both the absence and presence of T_3, the three TZDs tested increased the UCP2 mRNA levels in a concentration-dependent manner. It is evident that T_3 amplifies the responses to all three TZDs, even at concentrations where the effect of the TZDs alone was minor, not statistically significant. T_3 also amplified the effect of troglitazone (data not shown).

T_3 Also Amplifies Stimulatory Effects of Other Peroxisome Proliferator-Activator Receptor Agonists on UCP2 mRNA

To obtain further insight into the mechanism of T_3 action, we investigated whether it amplified the effect of other stimuli of UCP2 mRNA. The nonspecific peroxisome proliferator-activator receptor (PPAR) ligands bezafibrate and carbacyclin can also increase the levels of UCP2 mRNA in L6

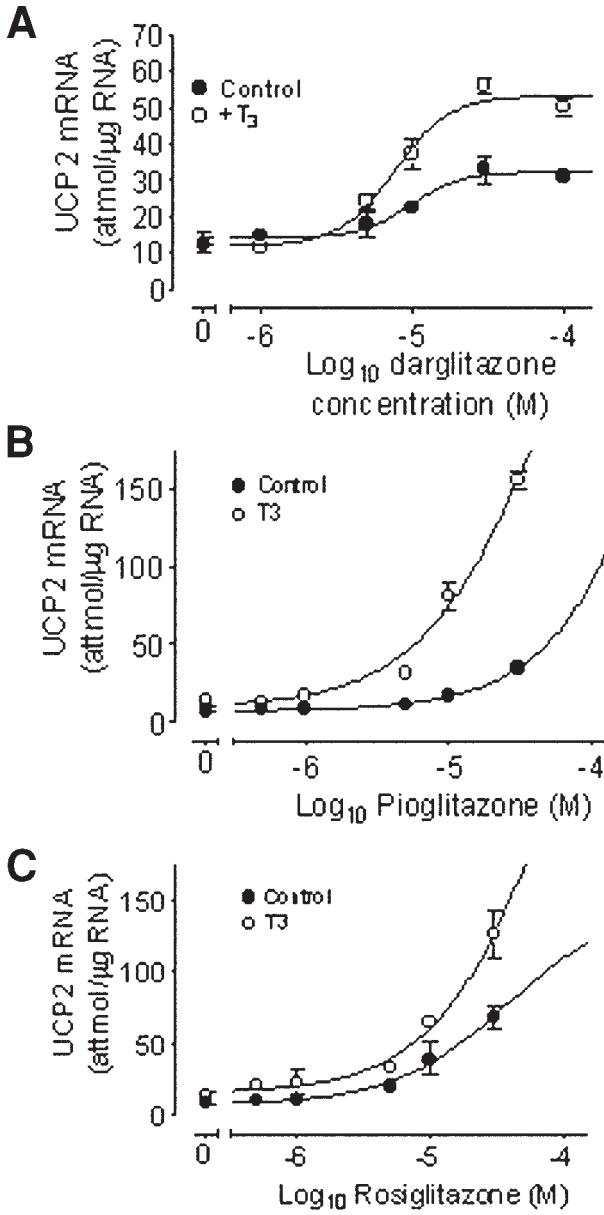

Fig. 4. Effect of T_3 on increase in UCP2 mRNA induced by increasing concentrations of darglitazone, pioglitazone, or rosiglitazone. Cells were treated for 24 h with the indicated concentrations of these TZDs ± 50 n*M* T_3.

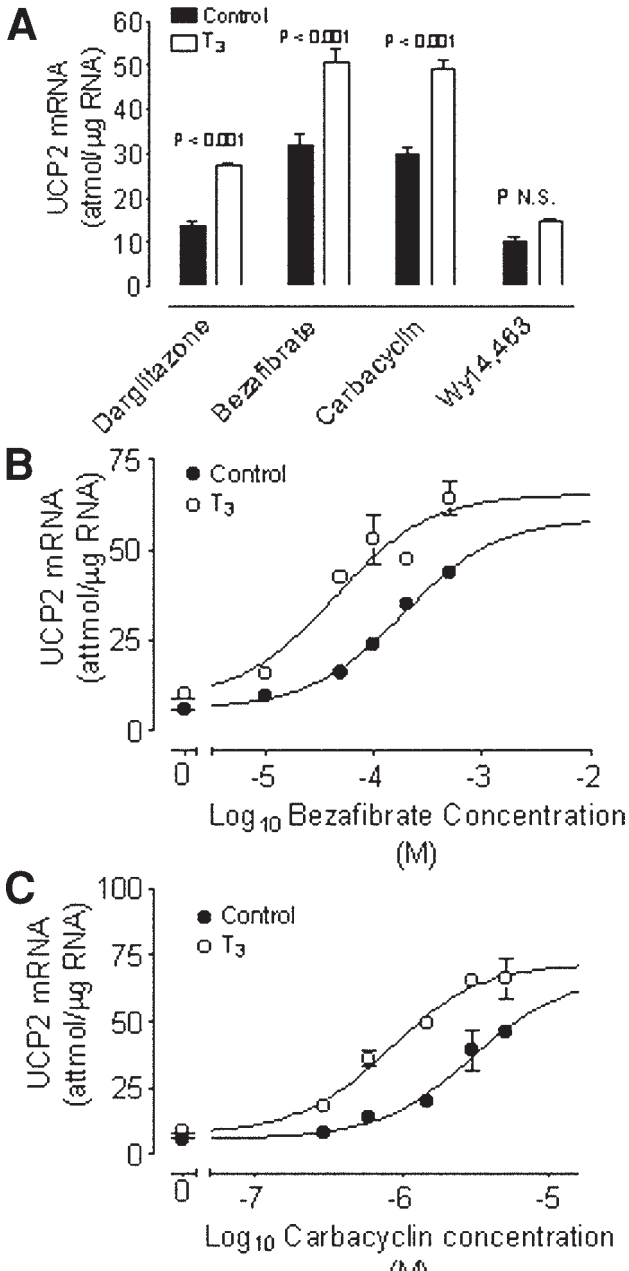

Fig. 5. Effect of T_3 on increase in UCP2 mRNA induced by various PPAR ligands. **(A)** Cells were treated with the indicated PPAR ligands for 24 h in the absence or presence of 50 n*M* T_3. Concentrations were 30 μ*M* darglitazone, 100 μ*M* bezafibrate, 2 μ*M* carbacyclin, and 20 μ*M* Wy14,463. **(B,C)** Cells were treated for 24 h with the indicated concentrations of bezafibrate and carbacyclin, respectively, in the absence or presence of 50 n*M* T_3.

myotubules, whereas the selective PPARα agonist Wy14,463 cannot, in agreement with the lack of PPARα in these cells *(25)*, a finding that we have confirmed. The results depicted in Fig. 5 illustrate the effect of T_3 over the level obtained by the corresponding PPAR ligand alone. T_3 significantly amplified the effect of darglitazone (30 μ*M*), bezafibrate (500 μ*M*), and carbacyclin (5 μ*M*), but not Wy14,643 (50 μ*M*), on UCP2 mRNA (Fig. 5A). The effect of bezafibrate and carbacyclin was concentration dependent, and T_3 significantly enhanced this effect at each of the concentrations tested (Fig. 5B,C). On the other hand, the increase in UCP2 mRNA levels induced by norepinephrine (4.28 ± 0.22-fold) or oleic acid (4.33 ± 0.29-fold) in L6 cells was not significantly amplified by T_3, indicating that the amplification on *ucp2* stimulation by T_3 is stimulus selective.

Short Exposure to T_3 is Sufficient for Amplification of Stimulation of ucp2 by TZDs

As indicated in Fig. 1C, the maximal amplification of the effect of darglitazone by T_3 was attained within 8 h, suggesting that most of the action of T_3 took place earlier. The

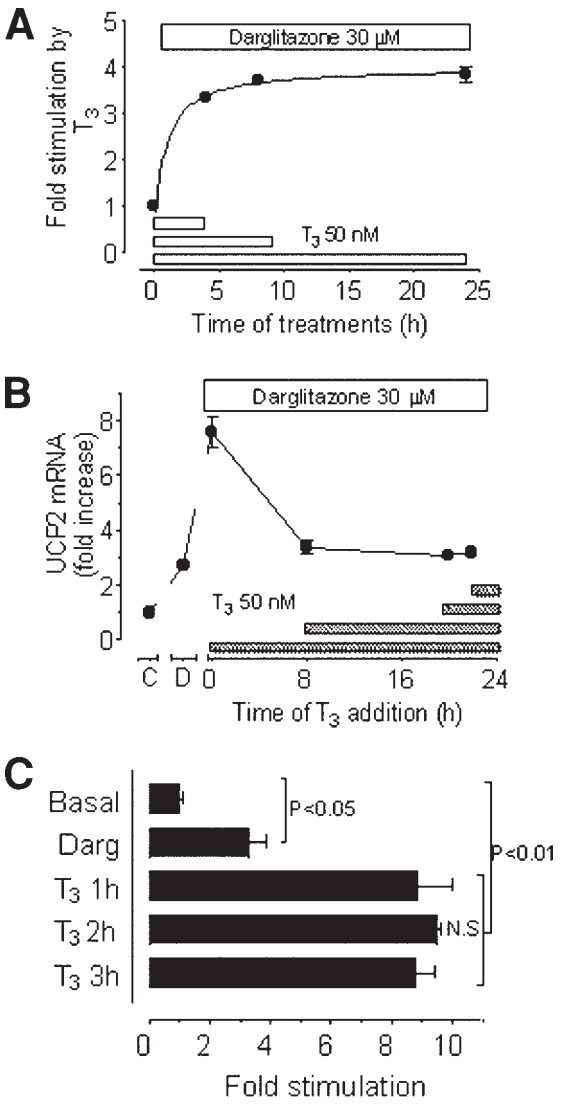

Fig. 6. Effect of timed additions of T_3 on stimulation of UCP2 by darglitazone. In all cases cells were treated for a total of 24 h with 30 μ*M* darglitazone. (**A**) In separate triplicate dishes with their corresponding controls, cells were treated with T_3 or its vehicle. T_3 was removed as indicated, at 4 or 8 h, or left throughout the stimulation with darglitazone. See text for methodological details. (**B**) Cells were treated with darglitazone alone or plus 50 n*M* T_3, which was added 0, 8, 20, or 22 h after initiating the exposure to darglitazone. See text for methodological details. (**C**) Cells were treated with T_3 for 1, 2, or 3 h or for 3 h with plain medium before the initiation of darglitazone treatment. After washing extensively and incubating for 1 h with medium containing charcoal-stripped serum, fresh medium with 30 μ*M* darglitazone or its vehicle, but no T_3, was replaced and cells were incubated for 24 h before harvesting the RNA.

experiments summarized in Fig. 6 were performed to define more precisely the timing and the length of exposure to T_3 necessary for the amplification of TZD stimulation of *ucp2*. In the experiment in Fig. 6A, cells were exposed to 30 μ*M* darglitazone for 24 h, while triplicate cultures were exposed in addition to T_3 for 4, 8, or 24 h. At the end of these periods, medium was removed, and the cells were washed with phosphate-buffered saline (PBS) at 37°C and then with medium containing charcoal-stripped serum to remove T_3. Finally, darglitazone-containing medium was replaced and the cells were incubated to complete 24-h exposure to this drug. Corresponding sets of triplicates not treated with T_3 were submitted to the same manipulations. As shown in Fig. 6A, by 4 h the effect was complete. In a subsequent experiment (Fig. 6B), sets of triplicate cultures were treated with darglitazone for 24 h and T_3 was added 0, 8, 20, or 22 h after the addition of darglitazone. Only when present within the first 8 h of exposure to darglitazone did T_3 amplify the effect of this drug. These observations suggest that cells have to be exposed to T_3 early during the treatment with TZDs, but that the exposure to T_3 does not need to be more than 4 h. Such findings prompted us to test whether preexposure to T_3 would have the same effect. As shown in Fig. 6C, this seems to be the case. In this experiment, cells were pretreated with T_3 for 1, 2, or 3 h, washed as just described, and left for 1 h in medium containing charcoal-stripped serum at 37°C, following which they were washed again with prewarmed PBS and treated with 30 μ*M* darglitazone for 24 h. Control cells were submitted to the same manipulations but without T_3 and then equally exposed to darglitazone or its vehicle. It is evident that as little as 1 h of exposure to T_3 prior to treatment with darglitazone was sufficient to cause full amplification of the effect of darglitazone, with no further increase if the exposure to T_3 was prolonged.

Inhibitors of Transcription or Translation During Exposure to T_3 Do Not Prevent UCP2 Stimulation

The observation that after a timely and short treatment with T_3 its presence is no longer necessary to accomplish the effect suggests the possibility that during this time T_3 induced a protein that indirectly mediated its effect. Alternatively, T_3 could induce a change in *ucp2* gene to facilitate the action of other transcription stimulators, such as TZDs. To discern between these possibilities, cells were treated with inhibitors of transcription or translation during the exposure to T_3. In the experiment summarized in Fig. 7A, cells were exposed to α-amanitin during the 2-h treatment with T_3 and subsequently both were removed as described above, following which fresh medium containing darglitazone was replaced. Control incubations were maintained with α-amanitin or actinomycin D to show that the concentration of α-amanitin was effective in preventing transcription. The concomitant exposure of the cells to T_3 and α-amanitin did not abort the enhancement by T_3 of the subsequent darglitazone-induced increase in UCP2 mRNA. Stimulation by darglitazone ± T_3 was abolished when cells were maintained for 24 h with α-amanitin or actinomycin D, indicating that α-amanitin effectively prevented transcription at the concentration used. Note, on the other hand, that in these two groups of cells the level of UCP2 mRNA was modestly reduced, in agreement with its long ≈40-h $t_{1/2}$ *(24)*, and that the presence of T_3 made no difference, indicating

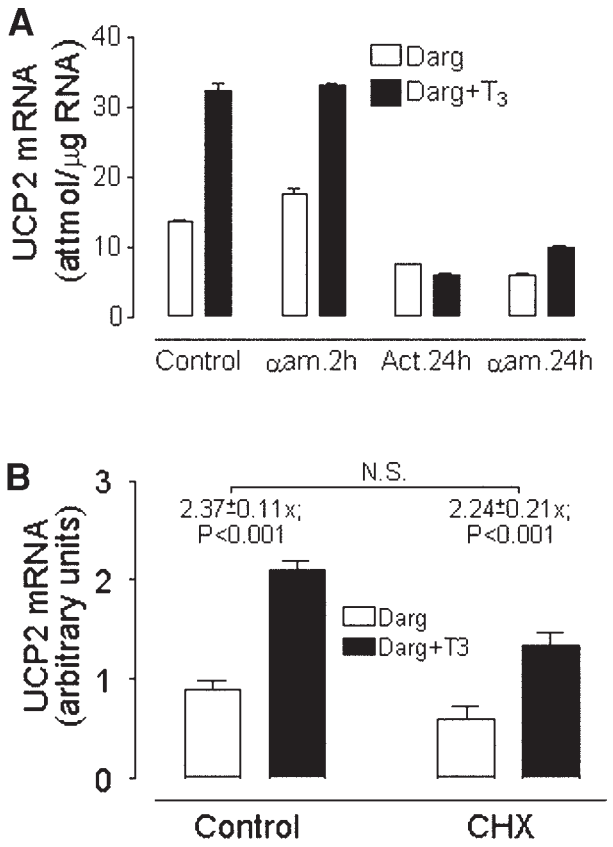

Fig. 7. Lack of effect of inhibitors of transcription or translation on amplification by T_3 of the darglitazone-induced increase in UCP2 mRNA in L6 myotubules. (**A**) Cells were incubated with plain medium or medium containing 10 μg/mL of α-amanitin or 5 μg/mL of actinomycin D and 50 nM T_3 or its vehicle. After 2 h, the medium was aspirated, the cells were extensively washed and incubated for 1 h in medium containing charcoal-stripped serum and no specific treatments, and the cells were washed again and fresh medium containing 30 μ*M* arglitazone was added to all cells. Sets of triplicates were treated with actinomycin D or α-amanitin in the concentrations indicated above during this period. See text for methodological details. (**B**) Cells were incubated for 6 hours with 30 μ*M* darglitazone + 50 n*M* T_3 ± 10 μg/mL cycloheximide. See text for rationale.

the this hormone does not work by prolonging the $t_{1/2}$ of the message. These findings argue against the induction by T_3 of a gene whose product would subsequently mediate the effect on UCP2 mRNA. Since we have found that TZDs increase UCP2 mRNA by increasing transcription *(24)*, these results reinforce the idea that T_3 enhances the transcriptional response to TZDs.

In another experiment, cells were exposed to cycloheximide during the treatment with T_3. Based on the results shown in Fig. 1B, we chose to treat cells for only 6 h with darglitazone plus T_3, because 6 h is sufficient to see the amplification of the TZD effect by T_3 and because further exposure to cycloheximide is too toxic for the cells. As shown in Fig. 7B, the increase in UCP2 mRNA in cells treated with darglitazone and T_3 was modest but significant ($p < 0.001$), not different from that shown in Fig. 1B, and the magnitude of the stimulation was the same regardless of the presence of cycloheximide (2.37- vs 2.24-fold, N.S.). Thus, inhibition of translation does not prevent the effect of T_3, strongly supporting the idea that the mechanism does not involve the *de novo* synthesis of a protein or the participation of a rapidly turned over protein.

Inhibitors of Histone Deacetylases Further Increase Stimulation of UCP2 by TZDs but Do Not Enhance Effect of T_3

The aforementioned results suggest that T_3 somehow primes the cells for the transcriptional effect of TZDs. This could be accomplished by changing the chromatin around the TZDs' site of action in the gene. To investigate the role of histone acetylation on the stimulation of *ucp2* by TZDs and the amplification of this effect by T_3, cells were treated with TZDs ± trichostatin A (TSA), a nonspecific inhibitor of histone deacetylases, and increasing concentrations of T_3. As shown in Fig. 8A, TSA enhanced the stimulation of *ucp2* by darglitazone to the same level, regardless of the T_3 treatment. Furthermore, the maximal response to T_3 was equal to that to TSA. Figure 8B shows essentially identical findings when the experiment was done with pioglitazone. In this case, the highest T_3 concentration tested (50 n*M*) was over the range that elicited a maximal response (see Fig. 3), yet the response of UCP2 mRNA was the same, with or without TSA.

Discussion

The results that we have presented indicate that while the stimulation of *ucp2* by thyroid hormone alone in L6 myotubules is insignificant, the hormone amplifies the stimulating effect of TZDs and other nonselective PPAR ligands such as bezafibrate and carbacyclin, but not the effect of oleic acid or norepinephrine. This effect of T_3 is thus selective for stimuli that may act through the activation of signaling pathways emanated from PPARγ and/or PPARβ. In our preceding study *(24)*, we showed that TZDs stimulate *ucp2* transcription indirectly, via a signal that requires ongoing protein synthesis and an active mitogen-activated protein kinase. Along with recent results by other laboratories *(26)*, our data support the idea that PPAR does not directly act on the gene but, rather, is the action of the liganded receptor elsewhere that generates a signal that interacts with a critical sequence of *ucp2*. Since amplification of TZD stimulation of *ucp2* by T_3 does not need ongoing protein synthesis or transcription, it is unlikely that this hormone enhances the synthesis of the putative protein(s) mediating the TZD effect. The independence of the T_3 effect from transcription or translation is also consistent with the lack of effect of T_3 ± TZDs on either PPARγ mRNA or protein (data not shown). It appears, then, that T_3 interacts at the genomic level, probably via its receptor, with signals emanated from the

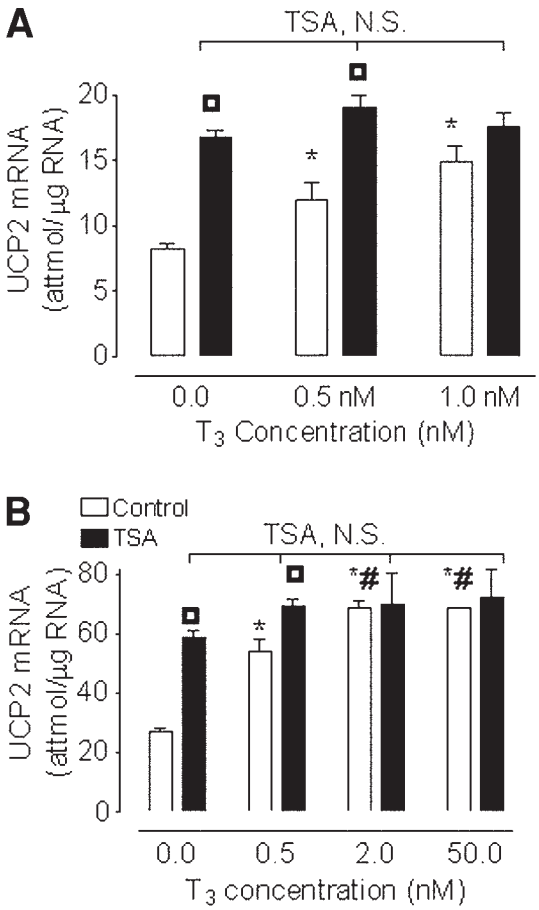

Fig. 8. Effect of inhibitor of histone deacetylase activity, TSA, on UCP2 mRNA level and its responses to TZDs ± T_3 in L6 cells. (**A**) Cells were incubated with 30 μ*M* darglitazone for 24 h ± 0.67 μ*M* TSA and T_3 in the indicated concentrations. The response to TSA was not significantly affected by the presence of T_3; * significant effect of T_3 ($p < 0.05$); □significant effect of TSA ($p < 0.01$). The effect of TSA was not affected by the presence of T_3. (**B**) Cells were incubated with 10 μ*M* pioglitazone for 24 h ± 0.67 μ*M* TSA and T_3 in the indicated concentrations. The effect of TSA was not significantly influenced by the presence of T_3; *significant effect of T_3 ($p < 0.01$); □significant effect of TSA ($p < 0.01$); #significantly greater than 0.5 n*M* T_3.

action of TZDs and other PPAR ligands elsewhere in L6 cells.

Several lines of evidence support the idea that this action of T_3 is initiated on its nuclear receptor (T_3R). First, there is the presence of high-affinity, saturable T_3 binding to L6 nuclear extracts and stimulation by T_3 of other processes such as sarcoplasmic Ca^{2+} uptake *(16)*. RNA analysis in the present studies shows that these cells express both α_1T_3R and β_1T_3R mRNAs. Second, the effect of T_3 is rapid and does not involve synthesis of a mediator. Third, the maximal effect of both T_3 and T_4 is the same but the potency of T_4 is seven times less than that of T_3 (Fig. 3B), which is within the reported affinity of T_4 relative to that of T_3 for the T_3R. The EC_{50} for T_3 is in the physiologic range, $\approx 3 \times 10^{-10}$ *M*, which in the presence of 2% thyroid hormone–stripped horse serum generates a free concentration in the euthyroid range and corresponds approximately to the K_d of the receptor for the hormone.

It has become apparent that the mechanisms whereby T_3 regulates gene transcription are complex and varied. Corepressors and coactivators are recruited to the thyroid hormone response element (TRE) in a T_3-dependent manner. Some of these cofactors have, or recruit molecules with, histone transacetylating or histone deacetylating activity, which are believed to modify the structure of the chromatin, thus modulating the access of critical sequences to various other transacting factors. Whether T_3 will directly affect transcription depends on the recruitment of additional cofactors that interact with the complex of transcription factors bound to the gene promoter (reviewed in refs. *27* and *28*). The experiments reported here show that TSA amplified the effect of TZDs on UCP2 mRNA but to a level no higher than that attained with maximal concentrations of T_3, while T_3 did not enhance the stimulation by TSA; that is, at maximal concentrations the effects of TSA and T_3 were not additive. Consequently, we hypothesize that T_3 amplifies the effect of TZDs by recruiting to a critical part of *ucp2* factors that result in a reduction in histone deacetylation, thus facilitating the access of transcription factors to target DNA sequences.

The observation that thyroid hormone does not significantly activate a given gene *per se*, while it amplifies the effect of other stimuli on the transcription of the gene, is not unprecedented. We made this observation with the UCP1 gene (*ucp1*) in brown adipose tissue. Thus, if the participation of endogenous norepinephrine was excluded, T_3 had a minor effect on *ucp1* transcription and mRNA levels, yet it amplified the effect of norepinephrine by a factor of at least 10 *(29)*. The fact that *ucp2* and *ucp1* may derive from a common ancestor makes this parallel even more interesting and intriguing. It is possible, as occurs with *ucp1 (30,31)*, that the TRE(s) of *ucp2* is a relatively weak TRE, not sufficient to mediate T_3 stimulation but sufficient to amplify the effect of other stimuli, in this case PPAR ligands. Another example is the protein S14 gene. The expression of this gene is stimulated by a signal coming from glucose intermediary metabolism and this is greatly amplified by T_3, whereas if cells are glucose deprived, T_3 only modestly stimulates this gene expression *(32–34)*.

This mode of action of T_3 on *ucp2* expression in L6 cells might explain the variable and conflicting in vivo observations. As mentioned earlier, the effect of T_3 on *ucp2* expression in muscle is reportedly quite variable *(10–13)*, with reports that thyroid hormone does not stimulate its expression (see, e.g., ref. *8*). We have found that the expression of *ucp2* in skeletal muscle of healthy, lean humans is highly variable *(6)*, a finding also consistent with many factors participating in the regulation of its expression. It is therefore conceivable that the variable responses to T_3 reported in animals may depend on which of such factors prevails in

the different experimental conditions. A corollary of the present observations is that the thyroid status of the cells may be a variable in the response of *ucp2* to various endogenous and exogenous stimuli, such as fatty acids, fibrates, or TZDs.

The synergism between TZDs and T_3 is also very interesting because it hints to unsuspected possibilities regarding the function of UCP2 and potential therapeutic effects of TZDs. Although the deletion of *ucp2* is not associated with evidence of insulin resistance *(3)*, other studies suggest that uncouplers of phosphorylation result in increased glucose uptake by L6 cells *(35)*. On the other hand, UCP2 seems to provide a mechanism to protect the cells against oxidative stress *(36)*. The fact that thyroid hormone can affect *ucp2* expression in the physiologic range of concentrations suggests that this antioxidant function or another function of UCP2 may be necessary to accommodate the cell to the stimulation of oxidations induced by thyroid hormone, depending on nutritional conditions or type of cell fuel available. Altogether, these considerations make the present observations interesting and worth pursuing.

Materials and Methods

Cell Culture and Cell Manipulations

L6, a rat skeletal muscle cell line, was a gift from Dr. P. C. Holland, Montreal Neurological Institute, Montreal, and Quebec, Canada. L6 myoblasts were grown to confluence as described in our preceding study *(24)* and were subsequently differentiated by a 3-d exposure to Dulbecco's minimal essential medium with 2% horse serum. From the second day, serum stripped of thyroid hormones by a resin *(37)* was replaced in the medium at the same concentration. By the end of the third day cells form myotubules. At this time, they were treated with TZDs and other substances, ± T_3, in concentrations and timing described with each of the experiments. TZDs were the same as in our preceding study *(24)*. Darglitazone, troglitazone, and pioglitazone were dissolved in dimethylsulfoxide and rosiglitazone in ethanol. These TZDs 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 nonselective PPAR ligands bezafibrate and carbacyclin, the PPARα-selective ligand WY14,643 (highly selective PPARα), TSA, oleic acid, thyroxine, propylthiouracil, and iopanoic acid, 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, the medium was aspirated, the cells were washed with PBS, and RNA was extracted using standard methods *(38)*. RNA was spectrophotometrically quantified and its quality assessed by gel electrophoresis. In initial experiments, UCP2 mRNA was analyzed by Northern blot as described previously, using a mouse UCP2 cDNA probe cloned by PCR *(17)*. A glyceraldehyde phosphate dehydrogenase cDNA probe (from American Type Culture Collection) was used to control for equivalence of mRNA loading. Subsequently, we designed a reverse transcriptase (RT)–competitive PCR assay, as described elsewhere for human UCP2 *(6)*, but using primers suitable for rodents (mouse and rat) UCP2 cDNA, as described in the preceding paper *(24)*.

The primers for T_3R mRNA detection were designed to target specifically α_1T_3R or β_1T_3R mRNA, of rat or mouse (accession no. M18028 and J03819, respectively). Primers for α_1T_3R generated a 585-bp product and were as follows: sense, 5-CATTGGCCAGTCACCTATTG; antisense, 5-ACT TTCATGTGGAGGAAGCG. Primers for β_1T_3R generated a 740-bp product and were as follows: sense, 5-ATCCTCA CACCTCATCCAGG; antisense, 5-ATCTGGTCTTCACAG GGCAG.

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. For this, the quantity of UCP2 mRNA was derived by videodensitometric analysis of the PCR products as described elsewhere *(24)*. 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 *(39)*.

Data and Statistical Analyses

Data are reported as mean ± SEM. Unless noted otherwise, each treatment was given in triplicate. When the error bar is not evident in the figures it is because the size of the error is smaller than the corresponding symbol. 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 analysis of variance followed by tests for multiple comparisons with a control group (Dunnet) or among the various treatments (Neuman-Keuls).

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