PGC-1α regulates the isoform mRNA ratio of the alternatively spliced thyroid hormone receptor α transcript

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Abstract

Transcripts derived from the thyroid hormone receptor α (TRα) gene are alternatively spliced resulting in a functional receptor TRα1 and a non-T3-binding variant TRα2 that can exert a dominant negative effect on the transactivation functions of other TRs. There is evidence that the ratio of TRα isoform transcripts can be modulated and here, we investigate whether the PPARγ co-activator α (PGC-1α) has an effect on this splicing process. PGC-1α was discovered not only as a transcriptional co-activator, but also has certain motifs characteristic of splicing factors. We demonstrate that PGC-1α alters the ratio of endogenously expressed TRα isoform transcripts in HepG2 cells, by decreasing TRα1 mRNA levels twofold. This change in isoform ratio is accompanied by a decrease in 5′-deiodinase expression, whereas no differences were found in TRβ1 expression. Deletion of the RNA-processing domain of PGC-1α abrogated the effect on the TRα splicing, whereas expression of only the RNA-processing domain favored TRα1 expression. PGC-1α showed a similar effect on the splicing of a TRα minigene containing only the last four exons and introns of the TRα gene. These data suggest that PGC-1α is involved in the RNA processing of TRα transcripts.

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Introduction

Thyroid hormone receptor α (TRα) has an important role in the regulation of physiological functions, such as the control of body temperature and heart rate (Wikstrom et al. 1998). The TRα gene encodes several variants due to alternative promoter usage and the existence of an alternative splice site in exon 9 (Laudet et al. 1991). TRα1 is a functional receptor and TRα2 is a non-T3-binding variant that results from the use of the alternative splice site. TRα2 can still bind to DNA and may have a dominant negative effect on the transactivation of the other TRs. The balance between TRα1 and TRα2 (TRα1:TRα2 ratio) is likely to be important for the sensitivity to thyroid hormone. Mice that lack TRα2, and as a result overexpress TRα1, show certain features of hyperthyroidism, such as increased heart rate, weight loss, and elevated body temperature, as well as some features of hypothyroidism, such as low serum thyroid hormone levels with an inappropriately normal thyroid-stimulating hormone concentration (Salto et al. 2001). The mixed expression of hyper- and hypothyroidism likely results from tissue-specific changes in thyroid hormone-responsiveness, which may reflect the different ratios of TRα1:TRα2 in different tissues of wild-type mice, and consequently tissue-specific increases in TRα1 signaling in the TRα2−/− mouse.

Similarly, mice that lack all TRα isoforms show increased sensitivity to thyroid hormone, which can be contributed to the abrogation of the silencing effect of TRα2 in tissues expressing the TRβ isoform (Macchia et al. 2001).

Several studies from our lab have provided evidence that the TRα1:TRα2 ratio is not constant and can be modulated by changes in the metabolic (or physiologic) state. For instance, when rats are subjected to fasting, the TRα2 mRNA levels in liver increase threefold, whereas no change in TRα1 is observed, resulting in a decrease in the TRα1:TRα2 ratio (Bakker et al. 1998). Similarly, HepG2 cells that are treated with pharmacological levels of T3 show a decrease in their endogenous TRα1:TRα2 ratio (Timmer et al. 2003). No correlation with the expression levels of known splicing factors has been observed and the mechanism behind the possible regulation of the splicing process remains unknown. Interestingly, both fasting and T3 induce the expression of the PPARγ co-activator α (PGC-1α) in liver (Herzig et al. 2001, Yoon et al. 2001, Weitzel et al. 2003). PGC-1α was originally identified not only as a transcriptional co-activator interacting with the nuclear receptor (NR) PPARγ but also enhances the activity of many other NRs, including TRα (Puigserver et al. 1998, Knutti et al. 2000). In addition to enhancing transcription, PGC-1α is involved in RNA processing. It has been shown to alter the splicing of a minigene, depends on...
the presence of a binding site for an NR and on the putative RNA-processing motifs present in the C-terminus of PGC-1α (Monsalve et al. 2000). These motifs consist of an RNA recognition motif, which is homologous to domains found in known splicing factors, such as the serine-, arginine-rich (SR), and heterogeneous nuclear ribonucleoproteins (hnRNP) protein families, and two regions rich in serine–arginine pairs, which are characteristic for SR proteins. These findings suggest that PGC-1α can provide a molecular link between transcription and RNA processing. However, there is no evidence yet that PGC-1α regulates splicing of genes in their natural context.

We hypothesized that PGC-1α plays a role in the splicing process of TRz transcripts. To this end, we studied the effect of PGC-1α on the endogenously expressed TRz transcripts in HepG2 cells, as well as on a TRz minigene.

Materials and methods

Materials

T₃ was obtained from Sigma Chemical Co. and dissolved in 5 mM NaOH to a concentration of 1 mg/ml. It was further diluted in Eagle’s minimum essential medium (E/MEM) (Biowhittaker, Verviers, Belgium) and stored at −20°C.

Plasmids

TRz was expressed from a minigene pCMV-erbAm that spans exons 7–10 of the rat TRz gene under the control of a CMV promoter and was a kind gift of Dr Munroe (Hastings et al. 2000). The expression plasmid for full-length human PGC-1α containing a hemagglutinin (HA) epitope-encoding sequence (pcDNA3 pBS/HA-hPGC-1α) has been described (Knutti et al. 2000). The plasmid pBS/HA-hPGC-1α was used as a template for the generation of all PGC-1α deletion variants. To make SR/E/RRM (565–754), primers C8/1713-NdeI (TAC GCC GGT CAT ATG CGG TCT CGT TCA AGG TCC), and C8/2443-a (GAC TGA CTC GAG TTA CTT GCG TCC ACA AAA GTA C) were used to PCR amplify a DNA fragment encoding amino acids 565–754 of PGC-1α. The PCR-product was digested with NdeI and XhoI and used to replace the NdeI/XhoI fragment of pBS/HA-hPGC-1α variant ΔSR/E/RRM, and pcDNA3/HA-PGC-1α. A pcDNA3 plasmid (Invitrogen) was used as empty control vector.

Cell culture and transient transfections

The human hepatoma cell line, HepG2, was obtained from the ATCC (#HB 8065, American Type Culture Collection, Rockville, MD, USA). Cells were cultured in Eagle’s medium supplemented with 10 U/ml penicillin/streptomycin/fungizone (p/s/f) and 5% fetal calf serum (all from Biowhittaker, Verviers, Belgium). Cells were seeded in six-well plates 18 h before transfection and reached approximately 60% confluence at the time of transfection. Per treatment (PGC-1α forms and/or T₃) we performed six independent transfections. We used FuGENE (Roche Diagnostics) as transfection reagent at a 3:2 ratio. Standard amount of reporter and expression plasmids per transfection assay were: 1 µg pCMV-erbAm and 2 µg PGC-1α-wild type (WT) or 0–2 µg PGC-1α deletion variants or pcDNA3 (with a total amount of 3 µg DNA per cell). When cells were incubated with T₃, medium was changed after 24 h to incubation medium with 5% fetal calf serum (FCS) and 10⁻⁷ M T₃ or the diluant NaOH in E/MEM.

RNA isolation and RT-PCR

After 48 h transfection, cells were lysed in 200 µl lysis buffer and RNA was isolated using the MagNaPure LC RNA Isolation Kit II (culture cells) in the MagNaPure LC Instrument (Roche Molecular Biochemicals). RNA was reverse transcribed into single-stranded cDNA using the First Strand cDNA synthesis kit with random primers (Roche Molecular Biochemicals).

Real-time PCR

Real-time PCR reactions were performed in a LightCycler (Roche Molecular Biochemicals). TRz1 and TRz2 were simultaneously detected in the same sample using sequence-specific hybridization probes and a LightCycler-FastStart DNA Master Hybridization Probes kit. Probes, primers, and program were as previously...
described (Bakker 2001). PGC-1α, 5′-deiodinase, GAPDH and total TRα were measured in a total reaction volume of 20 μl with 2μl cDNA using the LightCycler-DNA Master SYBR Green kit. The sequences of the primers of PGC-1α were as follows: 5'-GCA CCG AAA TTC TCC CTT GTA-3' (exon 9) and 5'-TTT GCT TGG CCC TCT CAG AC-3' (exon 10). The 5′-deiodinase mRNA was detected using the primer sequences 5′-AGC CAC GAC AAC TGG ATA CC-3′ (forward) and 5′-ACT CCC AAA TGT TGC ACC TC-3′ (reverse) and the TRβ1 mRNA using forward, 5′-AAG TGC CCA GAC CTT CCA AA-3′ and reverse 5′-AAA GAA ACC CTT GCA GCC TTG-3′. Primers for GAPDH were as described (Schreiber et al. 2003). For each mRNA assayed, a sequence-specific standard was generated and analyzed in the range of 0·1–1000 fg/20 μl in parallel to the samples. The crossing points of the standards with the noise band, which is set at the beginning of the log-linear phase, were plotted against the logarithm of the concentration and fitted to a standard curve. The concentration of cDNA of each gene was then calculated from its own standard curve and normalized to the amount of GAPDH in the sample. Minus-RT samples were tested in each experiment for GAPDH amplification to exclude genomic contamination. The individual efficiencies of the samples calculated using the LinReg program (Ramakers et al. 2003) were within 0·05 from the median efficiency of the run.

Protein extraction and analysis
The cells were washed with cold PBS, scraped in 2 ml PBS and centrifuged at 4 °C for 5 min at 3000 r.p.m. The pellet was resuspended in 100 μl 2× loading buffer (2% SDS, 10% glycerol, 100 mM DTT, 60 mM Tris (pH 6·8) bromophenol blue) and pre-heated at 80 °C. Subsequently, 8 μl lysate was loaded onto a 15% SDS-PAGE gel. After blotting onto a PVDF membrane (Millipore, Brussels, Belgium), PGC-1α and its variants were detected by blocking for 60 min in PBS containing 1% casein hydrolysate at 37 °C followed by an incubation of 60 min with a mouse monoclonal antibody against the HA epitope (clone 3F10) conjugated with peroxidase in PBS. After washing three times, LumiLight Plus substrate was added and signals were visualized using a LumilImager (all reagents, Roche Molecular Biochemicals).

Statistical analysis
The balance between the mRNA levels of the two splicing variants, TRz1 and TRz2, is expressed as the TRz1:TRz2 ratio. The mean TRz1:TRz2 ratio in the control cells is always set at 1 and the TRz1:TRz2 ratio in the treated groups is calculated relative to control. Statistical differences between groups were calculated using a t-test (only when n≥4).

Results

T₃ alters the ratio of endogenous TRα transcripts
As shown in Fig. 1, 10⁻⁷ M T₃ decreases the ratio of the endogenous TRα isoform transcripts. This is the result of a decrease in the level of TRz1 (from 0·65±0·09 to 0·41±0·04, relative to GAPDH, P<0·001), whereas no effect on the TRz2 levels was seen. However, T₃ has no significant effect on the TRz1:TRz2 ratio derived from a transfected TRz minigene (pCMV-ErbA), which contains only the last four exons and introns of the TRz gene.

PGC-1α modulates the ratio of the endogenous alternatively spliced TRα transcripts
We next investigated whether PGC-1α has an effect on the splicing direction of the endogenously expressed TRz transcripts in HepG2 cells. As shown in Fig. 2A, the TRz1:TRz2 ratio decreased in a dose-dependent manner in cells when PGC-1α was expressed. The decrease is mainly the result of a decrease in the TRz1 mRNA level (from 1·0±0·32 finally to 0·42±0·17, P<0·01). No change in the total level of TRz mRNA was observed and PGC-1α mRNA levels rose by 50-fold as a result of the transfection (data not shown). The PGC-1α-induced twofold decrease in the endogenous TRz1 expression levels is accompanied by a decrease in the expression of the 5′-deiodinase mRNA (Fig. 2B). No effect was seen on the expression of TRβ1 (Fig. 2C).

![Figure 1](https://example.com/figure1.png)

**Figure 1** The effect of T₃ on the TRz1:TRz2 ratio of the endogenously expressed TRz transcripts in HepG2 cells, and of TRz transcripts that are derived from the pCMV-ErbA minigene transfected in HepG2 cells. Cells (six wells/group) were incubated for 24 h with 10⁻⁷ M T₃ (white bars) or vehicle (black bars). Mean values ± s.d. (n=6) are depicted, and differences between groups are calculated with a t-test.
The effect of PGC-1α deletion variants on the TRα transcript ratio

To determine if the effect of PGC-1α on the endogenous TRα isoform mRNA ratio depends on its putative RNA-processing domains, we studied two PGC-1α deletion variants (Fig. 3A). One variant lacks the putative RNA-processing domain (ΔSR/E/RRM) and the other variant only expresses the putative RNA-processing domain (565–754). Expression of PGC-1α-WT resulted in a decrease ($P=0.03$) in the endogenous TRα1:TRα2 ratio when compared with the control cells (Fig. 3B). Transfection of PGC-1α ΔSR/E/RRM resulted in a TRα1:TRα2 ratio which was not statistically different from control. When cells were transfected with 565–754 a significant increase in the TRα1:TRα2 ratio ($P=0.01$) was observed compared with control. The increase was a result of an increase of TRα1 (1.2 ± 0.4 to 2.8 ± 0.6, $P<0.05$). There was no difference in the total TRα mRNA expression between groups (data not shown). Next, we studied the effect of PGC-1α-WT or deletion variants ΔSR/E/RRM and 565–754 on the TRα1:TRα2 mRNA ratio when transcripts derived from a TRα minigene. Again, the TRα1:TRα2 ratio decreased significantly as a result of co-transfection of PGC-1α-WT when compared to control ($P<0.01$, Fig. 3C). Co-transfection of ΔSR/E/RRM resulted in a TRα1:TRα2 ratio that was not significantly different from control. However, when 565–754 was co-transfected, a significant decrease compared to control was observed in the TRα1:TRα2 ratio. The decrease in the ratio is a result of a decrease in TRα1 (7.6 ± 0.8 to 4.9 ± 0.3, $P<0.01$) and a modest though not significant change in the TRα2 level was seen. Expression of PGC-1α or its variants did not result in a difference in the total TRα mRNA expression compared to control (data not shown). We confirmed the presence of PGC-1α mRNAs by western blotting with an anti-HA antibody. The triplet seen in the case of the SR/E/RRM region of PGC-1α is most probably the result of post-translational modifications.

Discussion

In a previous study, we have shown that pharmacological levels of T3 can change the splicing direction of TRα transcripts towards TRα2 (Timmer et al. 2003). Now we show that, in contrast to the effect on the endogenously expressed TRα transcripts, T3 does not exert a significant effect on the isoform mRNA levels derived from a TRα minigene. This minigene contains the last four exons and introns of the TRα gene and thus lacks the natural promoter sequence. This could indicate that the effect of T3 is promoter dependent. Although the TRα promoter does not appear to contain a known thyroid hormone response element (TRE), it...
is a target of the estrogen-related receptor α (ERRα; Laudet et al. 1993, Vanacker et al. 1998). Interestingly, the expression and activity of ERRα are regulated by the transcriptional co-activator PGC-1α, which in turn is induced by T₃ (Schreiber et al. 2003, Weitzel et al. 2003). Since PGC-1α contains putative RNA-processing motifs and has been shown to affect the splicing of a fibronectin minigene (Monsalve et al. 2000), we hypothesized that PGC-1α might have an effect on the ratio of TRα isoform transcripts. Indeed, in this study, we provide the first evidence that PGC-1α has an effect on the mRNA levels of isoforms that result from the alternative splicing of the endogenously expressed TRα gene, by favoring the production of TRα2 over TRα1 transcripts.

We have recently shown that the expression of the TR isoforms in liver is zonal with TRβ1 expressed in a narrow layer of cells around the central vein (Zandieh-Doulabi et al. 2002). Similarly, TRβ1 expression is located around the central vein but extends further towards the portal vein (Zandieh-Doulabi et al. 2005). In the first study, it was also shown that 5′-deiodinase is expressed in a zonal fashion overlapping with, but broader, than TRβ1 (Zandieh-Doulabi et al. 2002), suggesting a possible involvement of TRβ1 in 5′-deiodinase regulation as well. Indeed, data derived from mice lacking either TRβ or TRβ1 indicate that liver 5′-deiodinase expression, although mainly dependent on the predominant isoform TRβ, is also influenced by TRβ1 (Amma et al. 2001). We, therefore, selected 5′-deiodinase, which was recently demonstrated to show a marked responsiveness to T₃ (Zavacki et al. 2005), to see whether the effects of PGC-1α on TRα1 levels resulted in an effect on this gene. Our finding that the PGC-1α-induced lowering of TRα1 levels is associated with a decrease in 5′-deiodinase, supports the idea that TRα1 plays a role in 5′-deiodinase expression.

A PGC-1α construct that lacks the C-terminal domain containing the RNA-processing motifs has no effect, indicating that the C-terminal domain is necessary. This agrees with the findings of Monsalve et al. (2000), who have shown that the effect of PGC-1α on the splicing of a fibronectin minigene depends on the RNA-processing domain (Monsalve et al. 2000). Furthermore, Monsalve et al. (2000) have shown that the effect of PGC-1α on splicing is promoter dependent. We find an effect of PGC-1α not only on the TRα isoform ratio transcribed from the endogenous promoter, but also on the TRα isoform ratio expressed from the minigene. This may indicate that PGC-1α acts by loading on to the TRα promoter. Another possibility is that PGC-1α may bind to an alternative promoter, which is present in intron 7 of the TRα gene and included in the minigene. This promoter has a consensus binding site for the glucocorticoid receptor and thus a potential site for PGC-1α, and is responsible for the transcription of the N-terminally truncated TRα transcripts ΔTRα1 and ΔTRα2 that have been detected in several mouse tissues and embryonic stem (ES) cells (Chassande et al. 1997).
When we tested a construct expressing only the PGC-1α RNA-processing domain (565–754) opposite effects were observed depending on the experimental setup. The RNA-processing domain acted on the endogenously expressed TRα transcripts by increasing the levels of TRα1, whereas in the presence of the TRz minigene, it decreased the TRα1 levels. A possible explanation for this context-dependent effect could be the RRM motif and the arginine–serine regions located in the RNA-processing domain of PGC-1α which are contained in the 565–754 construct. These motifs probably mediate the physical association and localization with splicing factors in the so-called nuclear speckles. The PGC-1α arginine–serine region is reminiscent of that found in the SR proteins, which are required for general splicing, but which can also regulate alternative splicing by promoting the use of the proximal 5′-splice site (Ge & Manley 1990, Kainer et al. 1990). This effect on alternative splicing is counteracted by proteins of the hnRNP family, which promote the use of the distal 5′-splice site instead (Mayeda & Kainer 1992). In the case of the TRz transcript, use of the proximal 5′-splice site (which is in fact the poly-A addition site) would lead to TRz1, whereas the use of the distal 5′-splice site leads to TRz2. If the alternative splicing direction of TRz pre-mRNA indeed depends on an equilibrium between the levels of pre-mRNA and splicing proteins from the SR and hnRNP families, the direction could change when either one protein family or the pre-mRNA itself is more abundantly expressed. Therefore, in the case of the endogenous gene where there is not much pre-mRNA but a lot of (SR-like) PGC-1α, the proximal site is used (TRz1). On the other hand, when there is more pre-mRNA (transfected minigene), the protein/RNA ratio changes and the proximal site loses its supremacy resulting in less TRz1. This does not happen in the case of the wtPGC-1α; this could be because in this case a promoter-dependent process is involved as well, coupling transcription and RNA processing. Thus, the effects of PGC-1α on the endogenous TRz gene could result in recruitment of splice factors by a PGC-1α domain other than or additionally to the RRM.

In conclusion, PGC-1α has an effect on the formation of endogenous TRz transcripts resulting in a decreased TRz1:TRz2 ratio. In addition to the studies on the endogenously expressed TRzs, we have used a TRz minigene (which does not contain the natural TRz promoter) and shown that PGC-1α decreases the TRz1:TRz2 ratio of this minigene as well. Therefore, the TRz minigene is probably a suitable model to help to distinguish promoter-dependent from promoter-independent effects of PGC-1α on splicing.

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