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Negative regulation of superoxide dismutase-1 promoter by thyroid hormone

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Running Title: Regulation of the SOD-1 promoter by TR

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Abbreviations:

DBD, DNA binding domain; GRIP1, glucocorticoid receptor-interacting protein; LBD, ligand binding domain; N-CoR, nuclear receptor co-repressor; PMA, phorbol 12-myristate 13-acetate; SMRT, silencing mediator for retinoid and thyroid receptors; SRC-1, steroid receptor co-activator-1; SOD, superoxide dismutase; TR, thyroid hormone receptor; TRE, thyroid hormone responsive element; GAL-4 TR β 1, chimerical TR consisting of the TR β 1LBD fused to GAL-4 DBD; T₃, 3,5,3'-triiodothyronine

TR mutants – F451X, deletion of helix 12; G345R, mutation in the ligand binding; GS125, mutation in the DBD; I280K, mutation in the co-repressor binding site.

Abstract

The role of thyroid hormone (T_3) and the thyroid hormone receptor (TR) in regulating growth, development and metabolic homeostasis is well established. It is also emerging that T_3 is associated with oxidative stress through the regulation of the activity of superoxide dismutase-1 (SOD-1), a key enzyme in the metabolism of oxygen free radicals. We found that T_3 reverses the activation of the SOD-1 promoter caused by the free radical generators, paraquat and PMA, through the direct repression of the SOD-1 promoter by liganded TR. Conversely the SOD-1 promoter is significantly stimulated by unliganded TRs. This regulation requires the DNA-binding domain of the TR, which is recruited to an inhibitory element between -157 and +17 of the SOD-1 promoter. TR mutations, which abolish recruitment of co-activator proteins, block repression of the SOD-1 promoter. Conversely a mutation, which inhibits co-repressor binding to the TR, prevents activation. Together, our findings suggest a mechanism of negative regulation in which TR binds to the SOD-1 promoter but co-activator and co-repressor binding surfaces have an inverted function. This effect may be important in T_3 induction of oxidative stress in thyroid hormone excess.

Introduction

Thyroid hormones (TH) control growth, development and metabolism in virtually all mammalian tissues. Indeed, a primary role of T_3 is to regulate oxygen consumption and metabolic rate (Baxter and Webb, 2006; Yen, 2001). More recently it is emerging that TH is associated with the induction of oxidative stress in certain tissues. In fact, the hypermetabolic state in hyperthyroidism is associated with oxidative tissue injury, including alterations of heart electrical activity, muscle weakness and liver injury (Venditti and Meo, 2006).

Oxidative injury is normally limited through the activity of the superoxide dismutase (SODs) enzymes, which serve as the first line of defence against the damaging effects of superoxide radicals (O_2^-) by convert O_2^- to hydrogen peroxide. Of the different SOD enzymes, SOD-1 is the most abundant (90%) and is widely distributed (Johnson and Giulivi, 2005). *Drosophila* that lack SOD-1 shows a reduced life span (Phillips et al., 1989). Moreover, perturbations in SOD-1 activity have been associated with several diseases (Peled-Kamar et al., 1995; Stathopoulos et al., 2003).

The antioxidant defense system is influenced by the thyroid hormone status. For example, thyroxine (T_4) treatment decreases Cu/Zn SOD (SOD-1) activity in the liver of old rats (Saicic et al., 2005). Conversely, progressive hypothyroidism leads to an increase of superoxide dismutase activity in the brain of rats (Rahaman et al., 2001). Recently, cDNA microarray experiments to identify genes perturbed in hyperthyroid rat hearts revealed a number of genes, including SOD-1, that were down regulated by T_3 (De et al., 2004). Whilst the inverse association between SOD-1 and T_3 in several tissues was clear, the mechanism involved in this regulation remained poorly understood.

The genomic actions of thyroid hormone are mediated by TRs, which are ligand-regulated transcription factors belonging to the nuclear receptor superfamily (McKenna and O'Malley, 2002; Nettles and Greene, 2005). The molecular mechanism of positive transcriptional regulation by TR is well established. TRs interact directly with specific DNA sequences, known as thyroid hormone response elements (TREs) (Yen et al., 2006). Unliganded TRs recruit specific co-repressor proteins which, in turn, form part of a large co-repressor complex that contains histone deacetylases and represses transcription of nearby genes by condensing chromatin (Codina et al., 2005; Li et al., 2000; McKenna and O'Malley, 2002). Ligand binding induces changes in receptor conformation and dynamics (Nagy and Schwabe, 2004), that leads to the release of co-repressors and subsequent recruitment of p160 co-activators, such as glucocorticoid receptor-interacting protein (GRIP1) and steroid receptor co-activator-1 (SRC-1) (Ribeiro et al., 1998).

In contrast to positive regulation, the molecular mechanism of negative regulation by nuclear receptors is less well understood. Several hypotheses have been proposed to explain the action of TR on negative TREs (Lazar, 2003). One hypothesis is that the TR directly regulates transcription through direct binding to target promoters, either to unusual DNA response elements or via protein-protein interactions with other transcription factors associated to cognate response elements. Another hypothesis suggests that the role of TR is indirect through the squelching of co-regulators from other transcription factors.

In this study, we sought to understand the mechanism through which T_3 regulates the proximal region of the SOD-1 gene promoter. We showed that T_3 can reverse the activation of the SOD-1 promoter caused by free radical generators, such as paraquat and PMA. We observed that TR β 1 (and also TR α 1) activates promoter in the absence of ligand and T_3

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reversed this activation in a dose dependent manner. We found that the region of the SOD-1 promoter between -157 and the +17 was essential for TR β 1 regulation and this regulation requires the TR DNA binding domain for binding to the proximal region of the SOD-1 promoter. TRs mutant that are defective in co-repressor recruitment no longer activates the SOD-1 promoter. Conversely, a receptor that is defective in co-activator recruitment, but still able to interact with co-repressor, shows impaired down-regulation in response to T₃. We suggest TR may play a role in oxidative stress by directly binding to the SOD-1 promoter, but that TR co-regulator binding surfaces have an inverted function. This effect may be important in production of intracellular superoxide radicals in conditions of thyroid hormone excess.

Materials and Methods

Plasmids

The TR mutants F451X, G345R and GS125 TR β 1 were created using QuickChange site-directed mutagenesis kits (Stratagene) into the pCMX vector that encodes 461 amino acids of hTR β 1 sequence. The mutated sequence was verified by DNA sequencing using Sequenase Kits (Stratagene). The 5 deletions of SOD-1 promoter cloned upstream of the luciferase gene (Minc et al., 1999) were kindly provided by Dr. Christian Jaulin, from Centre de Recherche en Cancérologie (E229), Montpellier, France. Plasmids encoding hTR β 1 (Ribeiro et al., 2001) Gal-4 hTR β 1, GAL- responsive element-5 Luciferase, GST-GRIP1 (563-767) (Darimont et al., 1998), GST-SRC1a (381-882) (Feng et al., 1998), GST-SMRT (987-1491) (Webb et al., 2003) and TR mutant I280K (Marimuthu et al., 2002) were gifts from Dr. J. D. Baxter, from University of California, San Francisco.

Cell Culture and Transfection

U937, MG63 and rat HTC cells were maintained and subcultured in RPMI-1640 medium or Dulbecco's modified Eagle medium, containing 5% fetal calf serum/ 50 units/ml penicillin/ 50 μ g/ml streptomycin, at 37°C and 5% CO₂. Transfections procedures were described previously (Ribeiro et al., 2001) with some modifications. Briefly, MG63 and HTC cells were divided 48h prior to transfection to generate 40-60% confluence in 150-mm plates at the time of transfection. Cells were collected by centrifugation and then resuspended in transfection solution (1.5 X 10⁷ cells/0.5 ml) containing DMEM without phenol red (Gibco) and 250mM sucrose, and then co-transfected with 3 μ g of SOD-1 luciferase reporter gene, 500 ng of control β -galactosidase vector and 1.5-4.5 μ g of wtTR β 1 expression vector or its mutants.

Cells were transferred to a cuvette and then electroporated by using a Bio-Rad gene pulser under 290 mV and 960 μ F. After electroporation, cells were transferred to a fresh media and then plated in 12-well multiplates and treated with T_3 (10^{-7} M or different concentrations) or ethanol (control). After 24h, cells were collected by centrifugation, lysed by the addition of 150 μ l 1X lysis buffer (Promega), and assayed for luciferase and β -galactosidase activity (kit from Promega Corp.). Transfection data are mean \pm SEM of a minimum of triplicate samples, which was repeated 3-5 times. The empty vector pCMX was used as a control for the transfections without TR (Fig. 1B). Since we did not notice any difference between transfections with SOD-1 promoter alone and co-transfections with empty pCMX vector (data not shown), some assays were performed in absence of pCMX.

Gel Shift Assay

Binding of TR to DNA was assayed by mixing 20 fmols of 35 S labeled TR β 1 or GS125 TR β 1 produced in a reticulocyte lysate system, TNT T7 (Promega), in presence or absence of T_3 10^{-6} M, with 600 fmols of unlabeled different SOD-1, DR-4 (5'-AGTTC AGGTCA CAGG AGGTCA GAG-3') and inverted palindrome F2 (5'-TTC TGACCC CATTGG AGGTCA-3') oligonucleotides and 1 μ g of poly(dI-dC) (Amersham Pharmacia Biotech) in a 20 μ l reaction. The binding buffer contained 25mM HEPES, 50mM KCl, 1mM DTT, 10 μ M ZnSO₄, 0.1% NP-40 and 5% glycerol. After 30 min at room temperature, the mixture was loaded onto a 5% nondenaturing polyacrylamide gel that was previously run for 30 min at 200 V. To visualize the TR-DNA complexes, the gel was run at 4°C for 120 min at 240 V, in a running buffer containing 6.7 mM Tris (pH 7.5), 1 mM EDTA, and 3.3 mM sodium acetate. The gel was then fixed, treated with Amplify (Amersham Pharmacia Biotech), dried and exposed for autoradiography. TRs used in this assay were quantified through 125 I- T_3 binding assay.

Amounts used for gel shift assay were also confirmed through SDS-PAGE run of ³⁵S-TRs, where gels were fixed, dried and exposed for autoradiography. Bands visualized in X-ray films were quantified with a Kodak imager. SOD-1 oligonucleotides (Fig. 4A):

Seq1 (-87 to -46) GAGCGCGTGCGAGGCGATTGGT**TTGGGG**CCAGAGTGGGCGAG;

Seq1mut (-87 to -46)GAGCGCGTGCGAGGCGATTGGAT**GCAT**GCCAGAGTGGGCGAG;

Seq 2 (-51to-7) GGCGAGGCGCGGAGGTCTGGCCT**TATAA**AGTAGTCGCGGAGACGGG;

Seq 3 (-12 to + 29) GACGGGGTGCTGGTTT**TCGTC**GTAGTCTCCTGCAGCGTCTGG; Seq

4 (+23 to +69) TCTGGGGTTTCCGTTGCAGTCCTCGGAACCAGGACCTCGGCGTG; Seq

5 (+64 to +104) GGCGTGGCCTAGCGAGTTATGGCGACGAAGGCCGTGTGCG

GST pull -down assay

pCMX-TRβ1wt or pCMX-mutants vectors were used to produce radiolabeled full-length receptor in vitro, using the TNT-Coupled Reticulocyte Lysate System (Promega[®], Madison, WI) and [³⁵S] methionine. GST SRC1a (381-882), GST-GRIP1 (563-767), and GST-SMRT (987-1491) fusion proteins were prepared using conventional protocols (Pharmacia Biotech). Briefly, the plasmids were transformed into BL21, culture into 2xLB medium, pelleted and resuspended in TST 1X buffer (50mM Tris pH 7.5, 150 mM NaCl, 0.05 % Tween 20) with 1mM DTT, 0.5mM PMSF and protease inhibitor cocktail 1:1000 (Sigma[®]). Then, the solution was incubated with lysozyme and sonicated (3 cycles of 2.5 min amplitude 70%, 1 pulse/sec with a break of 5 min between each cycle).

The debris were pelleted and the supernatant was incubated for 2h with 500μl of glutathione-sepharose 4B beads equilibrated with TST 1x. GST fusions proteins beads were washed with with TSF 1x containing 0.05% Nonidet P-40 and resuspended in TST 1X with 1mM DTT, 0.5mM PMSF, protease inhibitor cocktail 1:1000 (Sigma[®]), 50% glycerol, and stored at -20°C.

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All procedure above was carried out in 4°C. For the binding assay, the glutathione bead suspension containing 4µg of GST fusion protein was incubated with 3µl of ³⁵S-labeled protein in 150µl of TST 1x buffer with 0.1% Nonidet P-40, 0.1% triton-X-100, 1mM DTT and 2µg/ml bovine serum albumin, in the presence of T₃ 10⁻⁶M or vehicle. Following 2h incubation at 4°C, the beads were washed with the same incubation buffer. The beads with associated proteins were analyzed in 10% SDS-PAGE and visualized by autoradiography.

Statistical Analysis

One-way ANOVA followed by Student-Newman-Keuls multiple comparison test was employed for assessment of significance (GraphPad Prism version 4.0a; GraphPad Software Inc.). Differences were considered to be significant at P < 0.05. NS is non significant.

Results and Discussion

The SOD-1 promoter is negatively regulated by TR β 1/T₃

To explore the regulation of the proximal SOD-1 gene, we used a reporter plasmid with the proximal promoter region -1499 to +17 of the SOD-1 fused to the luciferase gene (SOD-luc). Here, we observe that treatment with L-3,5,3'-triiodothyronine (T₃), the active thyroid hormone, reverses the effect of the paraquat through a direct or indirect repression of the SOD-1 promoter (Fig. 1A).

To determine whether the effect of T₃ on the SOD-1 promoter is mediated by the TR, we examined the effect of transfected TR β 1 on the SOD-1 promoter activity in U937 cells (Fig. 1B and 1C), osteosarcoma MG63 cells (data not shown) and rat HTC cells (Fig. 3B) in the presence or absence of T₃. We observed that unliganded TR β 1, also TR α 1 (data not shown), activates the SOD-1 promoter and that T₃ reverses this effect. TR β 1 activated the SOD-1 promoter in U937 cells by 2 to 3 fold in a concentration-dependent manner and T₃ treatment reversed this activation by 50-60% (Fig. 1B). T₃ repression was dose-dependent (Fig. 1C), with maximum inhibitory effect at 0.5 nM, typical for thyroid hormone responses.

The SOD-1 promoter behaves similarly to those of thyrotropin-releasing hormone (TRH) (Feng et al., 1994) and the pituitary thyroid-stimulating hormone (TSH) α and β - subunit genes (Bodenner et al., 1991; Chatterjee et al., 1989), which all contain nTREs.

A T₃-responsive sequence in SOD-1 promoter

To characterize the element responsible for unliganded TR β 1 activation and T₃ inhibition in the SOD-1 proximal promoter, we examined effects of T₃ upon different 5' deletions of the SOD-1 promoter. Constructs with a 5' boundary of pGLS -157 or longer were repressed by T₃ (Fig.

2A). The shorter construct pGLS -71/+17 also showed a significant response to T₃ treatment, but the constitutive activity of the promoter is so much lower one cannot be fully confident that the full T₃ response is preserved. Together these results suggest that a T₃-response element is located between the nucleotides -157 and + 17 region.

To confirm the opposing activities of free radical generator and T₃ on the -157/+17 promoter we tested the effect of PMA on SOD-1 Luc cotransfected with TRβ1. As expected, PMA activated this promoter and T₃ antagonized this effect (Fig. 2B).

The DNA binding domain (DBD) of TR is required to regulate the SOD-1 promoter

To understand whether the regulatory effect of the TR on the activity of the SOD-1 promoter required TRβ1 DNA-binding, we examined the activity of a chimeric TR lacking the DBD, but fused to the heterologous GAL-4 DBD (GAL-4 TRβ1) (Fig. 3A). This chimeric protein showed a lower activation of the SOD-1 promoter compared to wtTR and did not repress SOD-1 promoter in the presence of T₃. GAL-4 TRβ1 did activate GAL Luciferase reporter gene in presence of T₃ (data not shown) indicating that this protein was functionally active. In addition, we prepared and analyzed the activity a TRβ1 mutant, GS125, as previously described for TRβ2 (Shibusawa et al., 2003b). This mutant did not regulate the -157 SOD-1 promoter (Fig. 3B) but did showed the same binding affinity to T₃ as did wt TRβ1 / T₃ confirming that it is functional (data not shown).

Nuclear receptors regulate transcription by binding to specific DNA sequences in target genes but can also modulate gene expression by mechanisms independent of DNA binding. Analysis of the “knock in” mouse that harbours a TRβ mutant defective in DNA binding described by Shibusawa *et al.* (Shibusawa et al., 2003a) reveals that thyroid hormone fails to suppress TSH

gene transcription in these mice, supporting the conclusion that negative regulation of the TSH gene requires DNA binding by TR. Our data indicate that two TR mutants that cannot bind to canonical TREs, GAL-4 TR β 1 and GS125 TR β 1, both failed to repress SOD-1 promoter activity. The GS125 TR β 2 mutant, which binds to a TRE/GRE promoter but showed low affinity for positive and negative TREs, abolishes transactivation on three classic pTREs (DR+4, LAP, and PAL) and all negatively regulated promoters in the H-P-T axis (TRH, TSH β , and TSH α) (Shibusawa et al., 2003b). Thus, our results suggest that TR DNA binding activity is required for regulation of the SOD-1 promoter.

TR β 1 binds to SOD-1 promoter

To test the hypothesis that TR binds to the SOD-1 promoter, we performed gel shift assays with radiolabeled TR β 1 and different sequences from the SOD-1 promoter and the first exon of SOD-1 gene (Fig. 4A). As expected, the TR β 1 binds as a homodimer to two canonical positive TREs (F2 and DR4) in the absence of ligand and T₃ shifts the balance toward monomer binding (Fig 4A, lanes 3-6). Three regions of the SOD-1 sequence support weak TR β 1 binding. The sequence 1 of SOD-1 promoter (-87 to -46) binds monomeric TR and this binding was slightly increased in the presence of T₃ (Fig 4A, lanes 7 and 8). Sequences from the first exon of SOD-1 transcript (+23 to +69 and +64 to +104) support weak homodimer and monomer binding (Fig. 4A, lanes 13-16) with T₃ favouring TR monomer formation (Fig 4A, lanes 14 and 16). Interestingly we noticed that TR β 1 bound rather weakly to the SOD-1 promoter compared to DR4 or F2 elements.

The sequence -87 to -46 of SOD-1 promoter is close to the TATA box region and contains the sequence TTTGGG, which is also present in others previously characterized negatively

regulated genes (Kim et al., 2005). Mutation of this sequence (ATGCAT) abolished TR monomer binding (Fig.4 B, lanes 5-8). Moreover, the GS125 TR β 1 mutant, which cannot regulate SOD-1 activity, was also unable to bind to the DR-4 element or SOD-1 sequences (Fig.4B, lanes 3, 4 and Fig. 4C). Both lines of evidence suggest that TR regulates SOD-1 activity by binding to the sequence 1 nTRE.

These results are in accordance with other studies, which showed the presence of nTREs in the promoters very close to the TATA box (Belandia et al., 1998; Perez-Juste et al., 2000). Interestingly, TR also binds weakly to two different sequences in the first exon of SOD-1 gene; here, unliganded TR bound as homodimers and liganded TR as monomer units. Belandia B *et al.* has proposed that T₃ represses beta-amyloid precursor protein (APP) promoter activity by a mechanism that requires binding of TR to a specific sequence located in the first exon (Belandia et al., 1998).

The TR-DNA interaction observed in our study is weak compared to other positive TREs, F2 and DR4. Nevertheless, nTREs are generally comprised of weak TR binding sites. Kim SW *et al.* demonstrated that nuclear receptor co-repressor (NCoR) activates CD44 promoter by a weak unliganded TR-DNA interaction, 100 fold less than DR4. This weak TR-DNA binding was essential for CD44 regulation by T₃. Our results lend support to this finding, also showing a weak TR-SOD promoter interaction (Kim et al., 2005).

While our data indicate that TR monomer units are important for the repression mechanism of SOD-1 promoter by T₃, we cannot exclude that squelching of co-regulators may play a part in this regulation. Our results reveal GAL-4 TR does activate the SOD-1 promoter in the absence of hormone to a significant degree. Since the LBD of Gal-4 TR can bind to co-regulators, it is possible that the squelching mechanism could contribute to activation by unliganded TRs.

Furthermore, indirect regulation through other transcription factors may cooperate with liganded TR to negatively regulate the SOD-1 promoter, since the -157 to +17 region in this promoter shows binding sites for the transcription factors: Sp1, AP-1, Egr, NF- κ B and AhR. Of these, it has been well established that AP-1 can be subject to “trans-repression” by nuclear receptors. Therefore, we tested whether a mutation to the AP-1 site might reduce the activity of TR on this promoter (data not shown). Our results clearly showed this not to be the case.

Activation of the SOD-1 promoter by unliganded TR requires the co-repressor binding surface

To explore the role of TR co-regulator binding surfaces in SOD-1 promoter regulation we made use of mutations that have been previously characterised. We first confirmed that a natural mutation of the TR (F451X) in which helix 12 is absent, from patients with resistance to thyroid hormone (RTH), increases TR binding to co-repressor NCoR (Marimuthu et al., 2002) in GST pull-down assays. In this study we showed that T₃ decreases the binding of TR to the co-repressor SMRT (Fig. 5A, wtTR lane 5) and increases binding to the co-activators GRIP and SRC (Fig. 5A, wtTR lanes 7 and 9). Furthermore, F451X shows an enhanced constitutive binding to SMRT (Fig. 5A, F451X lanes 4 and 5) and decreased binding to both co-activators (Fig. 5A, F451X lanes 6-9). In transfection assays, F451X increased Luc expression from the SOD-1 promoter by 2.2 fold and T₃ could not reverse this activation (Fig. 5B).

We also analysed the actions of another RTH mutant (G345R), which binds co-repressor (Liu et al., 1998) but cannot bind ligand (Takeshita et al., 1996; Yen et al., 1995), on SOD-1 promoter activity. Like F451X, G345R activated the -157 SOD-1 promoter but failed to repress the SOD-1 promoter in presence of T₃ (Fig. 5C). Together, these results indicate that two TR β 1

mutants that bind co-repressors but not co-activators can enhance SOD-1 promoter activity.

Previous studies indicate that co-repressors may be involved in activation of genes negatively regulated by thyroid hormone, such as TSH β , TSH α and TRH (Berghagen et al., 2002; Tagami et al., 1999). Our results are in agreement with these findings. The natural mutant F451X, where the helix 12 of wtTR was deleted and the co-repressor-binding surface exposed, enhances co-repressor and inhibits co-activator binding, stimulates the SOD-1 promoter stronger than wtTR β 1 and T₃ did not reverse this effect. Likewise, another RTH mutant, that binds co-repressor but not ligand or co-activator, activated the SOD-1 promoter better than wtTR β 1, and as expected, failed to repress transcription in response to T₃

To investigate the role of the co-repressor binding surface in SOD-1 promoter regulation we made use of previously a characterized TR mutant that inhibit co-repressor binding (Marimuthu et al., 2002). One of the residues that forms the co-repressor-binding surface, I280, lies mostly underneath helix 12 and is solvent inaccessible in the liganded TR-LBD structure. The mutant I280K (manuscript in preparation) shows a decreased SMRT binding and also a weak binding to GRIP and SRC in presence of T₃. The unliganded TR I280K mutant did not activate the SOD-1 promoter as well as wtTR β 1 in transfections (Fig. 5D) and did not repress SOD-1 promoter activity in the presence of T₃. Together, our data indicate that the co-repressor binding surface is required for activation of the SOD-1 promoter by unliganded TRs, and that the co-activator binding surface is required for T₃-dependent repression. These results indicate that the role of TR co-repressor and co-activator binding surfaces is reversed at the SOD-1 promoter.

It is presently thought that nuclear hormone receptors promote dynamic recruitment of different co-regulator complexes to target promoters and that these effects are associated with an equally

dynamic binding of the nuclear receptor itself to the promoter (Perissi and Rosenfeld, 2005). In this context, further studies will be important to elucidate the dynamic mechanism of the recruitment of multiple complexes, such as histone deacetylases/ co-repressors/ TR, in order to alter the chromatin structure surrounding the promoter of SOD-1 gene. Nevertheless, our data support the hypothesis of an inverted role of co-regulators on negative TREs.

In conclusion, we have showed that the SOD-1 promoter is a novel target for TR action. Given that SOD-1 is a key enzyme against the damaging effects of superoxide radicals, this closely associate the thyroid hormone and the formation of oxygen radicals and other reactive species, which lead to oxidative stress. In addition, this study highlights the SOD-1 promoter as a useful tool for studying genes that are negatively regulated by thyroid hormone, providing new insights into the negative regulation by nuclear hormone receptors.

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Footnotes

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Legends

Fig. 1 Regulation of SOD1 by thyroid hormone receptor

(A) Human promonocytic U937 cells were cotransfected with 3 μ g of a reporter gene containing a construction pGLS -1499 of SOD-1 promoter encoding luciferase (SOD-1 Luc) and treated or not with 50 μ M paraquat; *P < 0.001 vs. no TR, **P < 0.001 vs. no T₃ (B) U937 cells were cotransfected with 1.5, 3.0 and 4.5 μ g of expression vector encoding wt hTR β 1 and 3 μ g of -1499 of SOD-1 Luc; * P < 0.001 vs. TR no T₃, **P < 0.001 vs. no TR no T₃, ***P < 0.01 vs. no TR no T₃, # P < 0.01, ## P < 0.001. (C) U937 cells were cotransfected with 1.5 μ g of wt hTR β 1 and 3 μ g of -1499 SOD-1 Luc and then treated with increasing amounts of T₃; * P < 0.001 vs. no T₃. Luciferase activity was expressed as percentage of -1499 SOD-1 Luc in the absence of T₃ and without or with cotransfected wt hTR β 1.

Fig. 2 TR β 1 activation and T₃ inhibition in different 5' deletions of the SOD-1 promoter linked to the luciferase gene

U937 cells were cotransfected with 1.5 μ g of expression vector encoding hTR β 1wt and 3 μ g of different pGLS constructs of SOD-1 promoter encoding luciferase; * P < 0.001 vs. no T₃, ** P < 0.05 vs. no T₃ (A) or with -157SOD1 Luc, treated or not with 100 ng/mg PMA; * P < 0.001 vs. no PMA no T₃, ** P < 0.001 vs PMA no T₃ (B). The data show a representative experiment, which was repeated 3-4 times.

Fig. 3 DBD is required to regulate SOD-1 promoter

(A) U937 cells were cotransfected with 3 μ g of -1499 SOD-1 Luc and 1.5 μ g of GAL-4 TR β 1 or wt hTR β 1; *P < 0.001 vs. no TR no T₃, **P < 0.001 vs. TR no T₃, *** P < 0.001. (B) HTC

cells were cotransfected with 3 μ g of pGLS -157 SOD-1 Luc and 1.5 μ g of TR β 1 or GS125 hTR β 1; * $P < 0.001$ vs. no TR no T_3 , ** $P < 0.001$ vs. TR no T_3 , *** $P < 0.001$. The data show a representative experiment, which was repeated 3-4 times.

Fig. 4 TR β 1 binds to different sequence of SOD-1 promoter

Gel shift assays contained 20 fmols of the *in vitro*-translated 35 S hTR β 1(A, B and C) or 35 S GS125 TR mutant (B and C) and 600 fmol of DR4 (A, lanes 5 and 6; B, lanes 1-4; C, lanes 1 and 2) or F2 (A, lanes 3 and 4) or different sequences of SOD-1 (A, lanes 7-16) or only the sequence 1 of SOD-1 mutated (B, lanes 5 and 6) or not mutated (B, lanes 7 and 8; C, lanes 5-6).

Fig. 5 TR β 1 mutations in co-activator and co-repressor binding surfaces

(A) Pull-down experiments examining the binding of labeled receptors to SMRT, GRIP and SRC protein fragment. Binding is expressed as the percent of input labeled receptor. Binding of [35 S] -labeled wt hTR β 1 or F451X to GST-SMRT, GST-SRC and GST-GRIP in presence or absence of 10^{-6} M T_3 .

U937 cells were cotransfected with 3 μ g of pGLS -157 SOD-1 Luc and 1.5 μ g of wt hTR β 1 or F451X (B), * $P < 0.001$ vs. no TR no T_3 , ** $P < 0.001$ vs. TR no T_3 , *** $P < 0.001$; G345R(C), * $P < 0.001$ vs. TR no T_3 ; or I280K (D), * $P < 0.001$ vs. no TR, ** $P < 0.001$ vs. TR no T_3 , *** $P < 0.01$. (B and C) Luciferase activity was expressed as percentage of -157 SOD-1 Luc in the absence of T_3 and without or with cotransfected wt hTR β 1. (D) The data show a representative experiment, which was repeated 3-5 times.

Figure 1

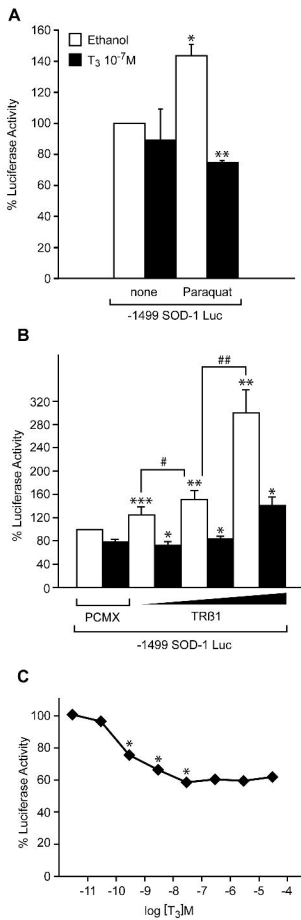
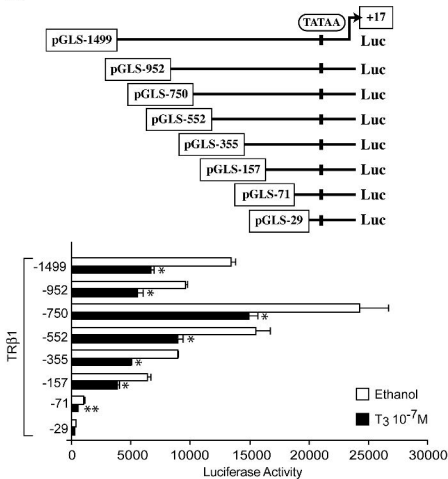


Figure 2

A



B

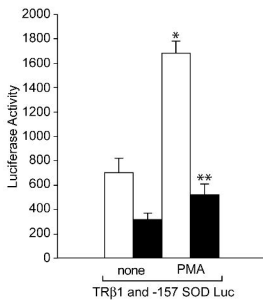


Figure 3

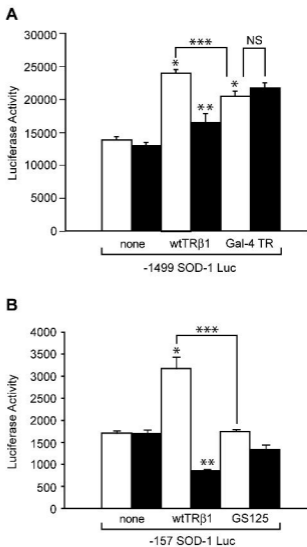
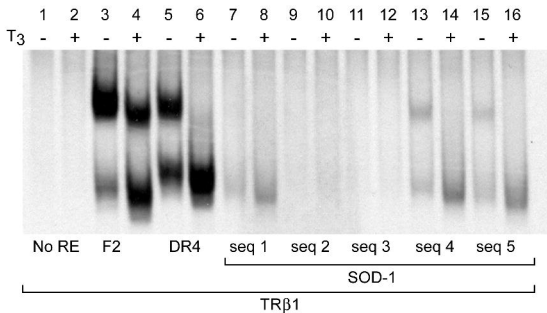


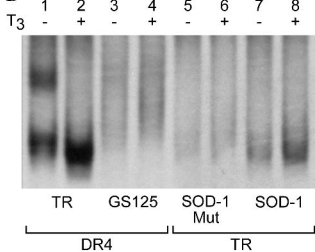
Figure 4

Seq 1 (-87 to -46): GAGCGCGTGCGAGGCGATTGG**TTTGGGG**CCAGAGTGGGCGAG
 Seq 1mut (-87 to -46): GAGCGCGTGCGAGGCGATTGG**ATGCAT**GCCAGAGTGGGCGAG
 Seq 2 (-51 to -7): GGCGAGGCGCGGAGGTCTGGCCT**TATAA**AGTAGTCGCGGAGACGGG
 Seq 3 (-12 to +29): GACGGGGTGCTGGTTTGCCTCGTAGTCTCCTGCAGCGTCTGG
 Seq 4 (+23 to +69): GTCTGGGGTTTCCGTTGCAGTCCTCGGAACCAAGGACCTCGGCGTG
 Seq 5 (+64 to +104): GGCGTGGCCTAGCGAGTTATGGCGACGAAGGCCGTGTGCG

A



B



C

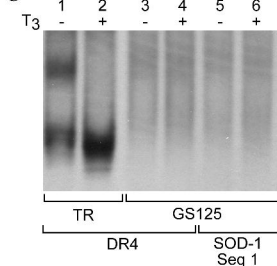


Figure 5

