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3,5-diiodo-L-thyronine upregulates rat-liver mitochondrial F_oF_1-ATP synthase by GA-binding protein/nuclear respiratory factor-2

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ABSTRACT

Besides triiodothyronine (T_3), 3,5-diiodo-L-thyronine (T_2) has been reported to affect mitochondrial bioenergetic parameters. T_2 effects have been considered as independent of protein synthesis. Here, we investigated the effect of *in vivo* chronic T_2 administration to hypothyroid rats on liver mitochondrial F_oF_1-ATP synthase activity and expression. T_2 increased state 4 and state 3 oxygen consumption and raised ATP synthesis and hydrolysis, which were reduced in hypothyroid rats. Immunoblotting analysis showed that T_2 up-regulated the expression of several subunits (α, β, F_oI-PVP and OSCP) of the ATP synthase. The observed increase of β-subunit mRNA accumulation suggested a T_2-mediated nuclear effect. Then, the molecular basis underlying T_2 effects was investigated. Our results support the notion that the β-subunit of ATP synthase is indirectly regulated by T_2 through, at least in part, the activation of the transcription factor GA-binding protein/nuclear respiratory factor-2. These findings provide new insights into the T_2 role on bioenergetic mechanisms.

1. Introduction

Thyroxine (T_4), the major iodothyronine secreted by the thyroid gland, is metabolized through outer ring deiodination by a deiodinase enzyme [1] to the 3,5,3′-triiodo-L-thyronine (T_3), which has several effects on growth, development and metabolism. Until recently, T_3 was generally assumed to be the most active iodothyronine, and T_4 its precursor. However, a growing body of evidence indicates that other iodothyronines, in particular 3,5-diiodo-L-thyronine (T_2), produced by peripheral deiodination of T_3, exhibit important biological effects [2–5]. Mitochondria, given their biochemical functions, are natural candidates as a target for the effects of thyroid hormones (THs) [6–9]. THs may influence mitochondrial activity either indirectly or directly by interaction with nuclear receptors and with the mitochondrial energy apparatus [10]. Evidence has been presented indicating that T_2, previously considered only as a T_3 catabolite, is able to mimic effects of T_3 on energy metabolism. Several reports showed that T_2 administration to rats resulted in significant changes in mitochondrial activity. T_2 action appeared to be more rapid than T_3 effect [3,5,7,11,12]. While T_3 is generally considered to play genomic actions [3], many laboratories have demonstrated T_2 metabolic effects independent of protein synthesis [4,5,7,13,14]. It has been shown that T_2 effects can be mediated by T_2 binding to a specific mitochondrial site [15]. Subunit Va of cytochrome oxidase (COX) has been identified as a binding site for T_2 [16].

F_oF_1-ATP synthase, the mitochondrial complex V responsible for ATP synthesis, is composed of three parts: the catalytic sector F_1, consisting of five subunits (3α; 3β; γ; δ; ε), the H^+ translocating sector F_o and a stalk connecting F_1 with F_o, both consisting of a variable number of subunits [17,18]. In the F_oF_1–ATP synthase complex, in addition to the F_1-γ and the F_oI-PVP(b) subunits [17], also the oligomycin-sensitivity-conferring protein (OSCP) is involved in the coupling of the F_1 catalytic activity to transmembrane proton translocation by F_o [19]. It has been shown that the F_oF_1-ATP synthase represents one of the sites of T_3 action [20,21].

However, studies on T_2 effects on this complex are lacking. The present study represents the first evidence that chronic T_2 administration to hypothyroid rats raises F_oF_1-ATP synthase activity in both ATP synthesis and hydrolysis direction. Interestingly, an increase of both mRNA and protein levels of F_oF_1-ATP synthase subunits were observed, thus suggesting a T_2-induced nuclear effect. To study the

Abbreviations: ANOVA, analyses of variance; BSA, bovine serum albumin; ChIP, chromatin immunoprecipitation; ESMP, EDTA-submitochondrial particles; FT_3, free 3,5,3′-triiodo-L-thyronine; FT_4, free thyroxine; GH, growth hormone; IOP, iopanoic acid; NRF-2α, α-subunit of GA-binding protein/nuclear respiratory factor-2; OSCP, oligomycin-sensitivity conferring proteins; PTU, 6-n-propyl-2-thiouracil; RCI, respiratory control index; RPA, RNase protection assay; T_2, 3,5-diiodo-L-thyronine; THs, thyroid hormones; TR(α1α2, β1, β2), different thyroid hormone receptor

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molecular basis of these observations, the effect of T_2 administration on the expression of GA-binding protein/nuclear respiratory factor-2 α-subunit (NRF-2α), one of the nuclear respiratory factors suggested to coordinate expression of the nuclear and mitochondrial genomes [22], was analysed.

2. Materials and methods

2.1. Chemicals

Oligomycin, 6-n-propyl-2-thiouracil (PTU), iopanoic acid (IOP), T_2 (more than 99% pure) and all other reagents were from Sigma-Aldrich. RPAIII kit was from Ambion. SYBR Green was from FluoCycle, Euroclone.

2.2. Animal treatment

Male Wistar rats (200–250 g) were housed in individual cages in a temperature-(22 ± 1 °C) and light-(light on 08:00 h–20:00 h) controlled room. T_2 and IOP were dissolved in 0.05 M NaOH and diluted with 0.09% NaCl. The rats were divided into three groups. Group 1, representing euthyroid control rats, was treated with only vehicle for 4 weeks; group 2, rats were made hypothyroid by continuous administration of PTU (0.1% w/v, in drinking tap water) for 4 weeks together with a weekly i.p. injection of IOP (6 mg/100 g b.w.); group 3, rats were made hypothyroid as group 2 but received during the last week a daily i.p. injection of T_2 (15 μg/100 g b.w.) (hypothyroid + T_2). No significant change in food intake, liver and body weight was observed in T_2-treated rats relative to hypothyroid rats (data not shown).

The experimental design was in accordance with local and national guidelines regarding animal experiments.

To determine serum levels of THs, blood was collected from rats and free T_3 (FT_3) and free T_4 (FT_4) were determined as in [23].

2.3. Isolation of mitochondria and preparation of sub-mitochondrial particles

Rat-liver mitochondria were prepared following standard procedures.

Inside-out sub-mitochondrial particles were prepared in the presence of EDTA (ESMP) by exposure of mitochondria to ultrasonic energy [24].

Protein concentration was determined by using the Bradford BioRad protein assay and bovine serum albumin (BSA) as a standard.

2.4. Mitochondrial respiration

Mitochondrial respiration (0.3 mg of mitochondrial protein/mL) was measured in a medium consisting of 220 mM sucrose, 20 mM KCl, 2.5 mM KH_2PO_4, 1 mM EDTA, 20 mM Hepes, 5 mM $MgCl_2$, 2 μg/mL rotenone, 0.1% BSA (fraction V, fatty acid-free from Sigma) and 5 mM K-succinate, pH 7.4, by a Clark oxygen electrode at 25 °C. This temperature, even if non-physiological for mammals, shows a reduced contribution of proton leak to basal respiration and is closed to thermoneutrality for rats [25,26]. State 3 respiration was initiated by the addition of 0.3 mM MgADP and followed until total consumption of ADP when state 4 respiration was measured. Then, 4 mM CCCP was added to measure uncoupled respiration. State 3, state 4 and uncoupled respiration, respiratory control index (RCI) and ADP/O ratio were calculated as in [27].

2.5. ATP synthase and hydrolase activity

The rate of mitochondrial ATP synthesis was determined on freshly isolated mitochondria [21]. Oligomycin-sensitive ATP hydrolase activity of ESMP was measured with an ATP-regenerating system [24].

2.6. Probe design

ATP synthase β subunit cDNA was amplified by reverse transcriptase PCR using rat-liver total RNA as template and the following primers: forward 5′-CTGCAGAATTCAGAGATGAGTGTTGAACAGG-3′ and reverse 5′-AAGCTTTACAGAATAACCACCATGGG-3′ (GenBank™ accession number NM_134364), containing respectively an EcoRI and HindIII sites (underlined), added for subcloning purposes. The amplified product (174 bp) was subcloned, sequenced and used in the *in vitro* transcription reactions.

2.7. RNase protection assay (RPA)

mRNA level was investigated by RPA (RPAIII™, Ambion). To this end, antisense RNA was synthesized by an *in vitro* transcription reaction [28]. Total RNA (10 μg), extracted from rat liver [23], was hybridized with 2×10^5 cpm (~2 fmol, 1.7×10^9 cpm/μg) of ^{32}P-labeled specific antisense probe in 20 μL of hybridization reaction at 50 °C for 16 h. For the normalization, a 2×10^3 cpm of antisense ^{32}P-labeled 28S rRNA probe was added in each hybridization reaction. Pilot experiments with constant amount of probes but different concentrations of RNA confirmed that the concentration of probes was in molar excess. As a control for testing the RNase activity, the probe was also hybridized with 10 μg of yeast RNA (not shown). After digestion with RNase A/T1, the protected fragments were separated onto 6% denaturing polyacrylamide gel. Gels were dried, exposed to Hyperfilm for autoradiography and the intensity of the bands was evaluated by densitometry with Molecular Analyst software (BioRad).

2.8. Quantitative RT-PCR analysis

Reverse transcriptase reaction (20 μL) was carried out using 5 μg of total RNA, 100 ng of random hexamers and 200 U SuperScript™ III RNase H-Reverse transcriptase. Quantitative gene expression analysis was performed using SYBR Green technology and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA for normalization. The primers used for real time PCR analysis were the following: NRF-2α forward 5′-AGACAGAAGCCAAACAGGAG-3′, NRF-2α reverse 5′-TGTTCCACAATGCTTTCTTC-3′ (GenBank™ accession number NM_001108841), β-F_1 forward 5′-AGAGATGAGTGTTGAACAGG-3′ and β-F_1 reverse 5′-TACAGAATAACCACCATGGG-3′ (GenBank™ accession number NM_134364); GAPDH forward 5′-GCATGGCCTTCCGTGTTCC-TACC-3′, GAPDH reverse 5′-GCCGCCTGCTTCACCACCTTCT-3′ (GenBank™ accession number NM_017008.3).

2.9. Isolation of total proteins from liver

Total proteins from liver were obtained by homogenizing a piece of rat liver (about 0.5 g) in a medium containing 210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 5 mM Tris/HCl pH 7.5 plus protease inhibitors.

2.10. Electrophoresis and immunoblotting procedures

Proteins were separated on 15% (for mitochondrial proteins) or 10% (for total proteins from liver) SDS-PAGE gels [23]. The separated proteins were transferred to nitrocellulose membranes and incubated with primary antibodies all diluted in 20 mM Tris/HCl, pH 7.6, 0.14M NaCl, 0.5% Tween 20 (TBS-T). After washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies (Pierce) which were visualized using the chemiluminescence kit (GE Healthcare). The blots were then exposed to Hyperfilm and signals were quantified by densitometry as for RPA.

2.11. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as in [29]. Chromatin complexes were immunoprecipitated for 12–18 h at 4 °C with 10 μg of anti-NRF-

2α antibody (Santa Cruz Biotechnology), or goat IgG or rabbit IgG overnight at 4 °C on a rotating wheel. After immunoprecipitation performed as in [30] with non-specific IgGs or with antibody against NRF-2α, initial PCRs were conducted with serial dilutions of input material from each immunoprecipitation to establish the appropriate cycling conditions and accurately compare template content across treatments (not shown). Primers were designed to amplify a 486 bp fragment (−503/−17 bp upstream the ATG translation initiation codon) of the proximal promoter region in the rat β-F_1 gene. Primer sequences used in PCR reaction were the following: β-F_1ChIP forward (5′-TAAAGACCCTCCAGGTTTCC-3′) and β-F_1ChIP Reverse (5′-GACTGAGCGCTCCAGCGGTC-3′). The PCR reaction was performed with 2 μL of immunoprecipitate, in a final volume of 25 μL, 100 nM of each primer, and 1× PCR mix (Sigma) in a Thermal Cycler (Applied Biosystem). Samples were incubated for an initial denaturation at 94 °C for 60 s, followed by 30 cycles at 94 °C for 20 s, 62 °C for 20 s, and 72 °C for 30 s. PCR products were separated on a 1.5% (w/v) agarose gel and analysed by ethidium bromide staining. The stained gel was visualized and the PCR products from three individual experiments were quantified using a Versadoc imaging system (Bio-Rad Laboratories).

2.12. Reporter vector construction and transient transfection assay

The DNA fragment (486 bp) of rat ATP synthase β-F_1 promoter was obtained by PCR using a rat genomic DNA as template and the primers forward 5′-GAATTCGGTACCTAAAGACCCTCCAGGTTTCC-3′ and reverse 5′-GAATTCCTCGAGACTGAGCGCTCCAGCGGTC-3′, located at 503 bp and 37 bp, respectively, upstream the ATG codon. The amplification product was digested with *Kpn*I and *Xho*I, then subcloned into the same sites of pGL3 basic vector (Promega), obtaining the pATP5B486 construct. Rat hepatoma cell line H4IIE was maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin G (100 units/mL) and streptomycin (100 μg/mL) at 37 °C under 5% CO_2 atmosphere. Transfection and luciferase assays were carried out as described in [30]. H4IIE cells were co-transfected with pATP5B486 (1.5 μg/well) and the *Renilla* luciferase reference plasmid, pGL4.73 (0.02 μg/well), control for transfection efficiency, by using FuGENE 6 transfection reagent (Roche Diagnostics); then, the cells were incubated for 24 h. After transfection, to evaluate the effect of T_2 on rat β-F_1 promoter activity, cells were incubated in DMEM medium supplemented with 10% (v/v) charcoal-treated FBS and 10^{-7} M T_2. For transcriptional activation by NRF-2α, H4IIE cells were transiently co-transfected with p503ATP5B and pGL4.73 reference plasmid as described above, together with either pCMV-NRF2α (Geneservice, United Kingdom) (0.1 μg/well) encoding the NRF-2α transcription factor, or pcDNA3.1 control empty vector.

2.13. Statistical analysis

Data are means ± standard error (SE) of the indicated number of experiments. The results were computed with Excel (Microsoft). Comparison was made using one way analysis of variance (ANOVA) followed by a *post-hoc* Tukey's B test. All statistical analyses were performed using SPSS/PC computer program. In all instances $P<0.05$ was taken as the lowest level of significance.

3. Results

3.1. Thyroid state of treated rats

In hypothyroid rats the serum level of FT_3 decreased by 62% (1.32 ± 0.35 vs. 3.42 ± 0.61 pmol/L of euthyroid, $n=5$) and FT_4 level by about 90% (0.21 ± 0.05 vs. 2.93 ± 0.33 μg/dL of euthyroid, $n=5$). The reduction in FT_3 and FT_4 levels in the hypothyroid rats confirmed the effectiveness of treatment with PTU plus IOP in inducing a severe hypothyroid status [14]. Notably, chronic T_2 administration to hypothyroid rats did not significantly affect either FT_3 [14] and FT_4 serum levels (data not shown).

3.2. Effect of T_2 on mitochondrial respiratory rates and ADP/O ratio

Consistent with previous data [21] state 4 and state 3 oxygen consumption was reduced (~50%) in mitochondria from hypothyroid as compared to euthyroid rats (Table 1). A significant decrease (~30%) in CCCP-induced uncoupled respiration was also observed in mitochondria from hypothyroid rats. Treatment of hypothyroid rats with T_2 significantly increased respiratory rates in state 4, state 3 and uncoupled state, restoring rates to those of euthyroid rats.

The level of coupling between the phosphorylation activity and the mitochondrial respiration is represented by ADP/O ratio. This parameter significantly increased in hypothyroid in comparison to control animals while it was almost unaffected in hypothyroid + T_2. However, no significant variation was observed in RCI values between the different groups of rats, thus indicating that the quality of mitochondrial preparations from control and treated rats was similar [31].

3.3. T_2 effect on the ATP synthase activity

The observed changes in respiratory rate in T_2-treated hypothyroid rats could reflect changes in the activity of oxidative phosphorylation. Therefore, the rate of ATP synthesis was investigated. ATP synthesis was significantly reduced (−34%) in succinate-supplemented mitochondria from liver of hypothyroid as compared to euthyroid rats (Fig. 1A). T_2 administration to hypothyroid rats restored ATP synthesis to the level observed in euthyroid rats.

To verify whether the increased ATP synthesis observed upon T_2 treatment could be ascribed to a direct effect of the hormone on F_oF_1-ATP synthase, we measured oligomycin-sensitive ATP hydrolase activity in ESMP, as this activity does not depend on the other respiratory chain components [21]. Moreover, ESMP should permit to study the effects of T_2 on ATP synthase, differentiating them from the effects on adenine nucleotide translocator or adenine nucleotide content [20]. Fig. 1B shows a decrease (−36%) of the oligomycin-sensitive ATP hydrolase activity in ESMP from hypothyroid compared to ESMP from euthyroid rats. ATP hydrolase activity in ESMP from hypothyroid + T_2 showed a recovery to the value seen in euthyroid rats (Fig. 1B).

3.4. T_2 effect on transcription and translation of ATP synthase

The catalytic β-F_1 subunit of the F_oF_1-ATP synthase is encoded, unlike other components of oxidative phosphorylation complexes [21], by a single copy gene [32], ubiquitously expressed in mammalian cells. The ATP synthase β-F_1 represents a nuclear gene encoding for a mitochondrial inner membrane protein known to respond to THs [22]. In order to study the molecular events possibly responsible for the observed changes in F_oF_1-ATP synthase activity, the levels of β-F_1

Table 1
Effect of 3,5-diiodothyronine on rat-liver mitochondrial respiratory rates.

Rats	State 4	State 3	CCCP-stimulated	RCI	ADP/O
Euthyroid	18.9 ± 1.5[a]	125.0 ± 6.7[a]	186 ± 7.5[a]	6.6 ± 0.8	1.3 ± 0.10[a]
Hypothyroid	9.4 ± 1.6[b]	68.5 ± 9.6[b]	136 ± 6.5[b]	7.3 ± 0.9	1.7 ± 0.05[b]
Hypothyroid+T_2	20.1 ± 2.3[a]	132.8 ± 10.1[a]	199 ± 11.5[a]	6.6 ± 1.5	1.3 ± 0.15[a]

Succinate-dependent oxygen consumption was measured by a Clark oxygen electrode. Respiratory rates are expressed as natoms oxygen min^{-1} mg of mitochondrial $protein^{-1}$. Data represent the means ± SE of 6 different experiments performed with duplicate samples. Values sharing a different letter differ significantly. RCI: respiratory control index.

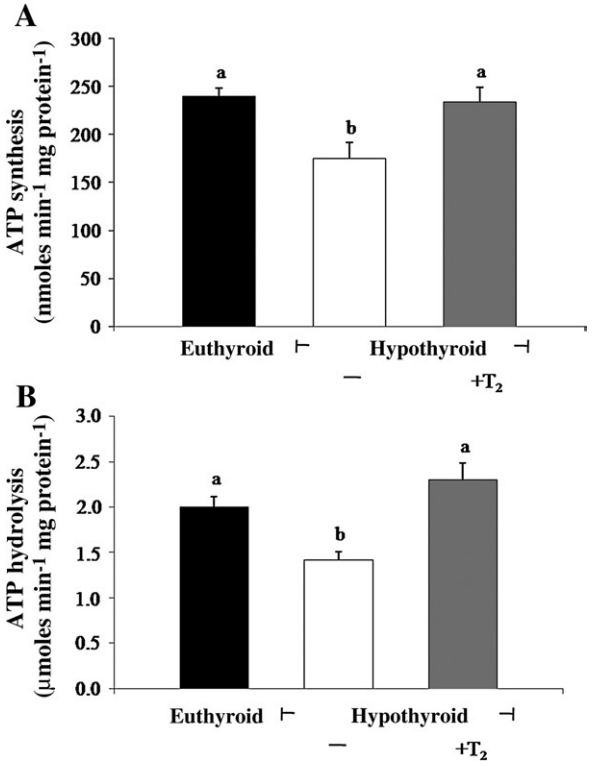

Fig. 1. F_oF_1-ATP synthase activity in rat-liver mitochondria and submitochondrial particles of T_2-treated hypothyroid rats. (A) ATP synthesis was measured on freshly isolated rat-liver mitochondria. Data represent the means ± SE of 6 different experiments performed with duplicate samples. (B) Sub-mitochondrial particles were prepared in the presence of EDTA (ESMP) and oligomycin-sensitive ATP hydrolysis was measured with ATP 1 mM using an ATP-regenerating system. Data represent the means ± SE of 5 different experiments performed with duplicate samples. Values sharing a different letter differ significantly.

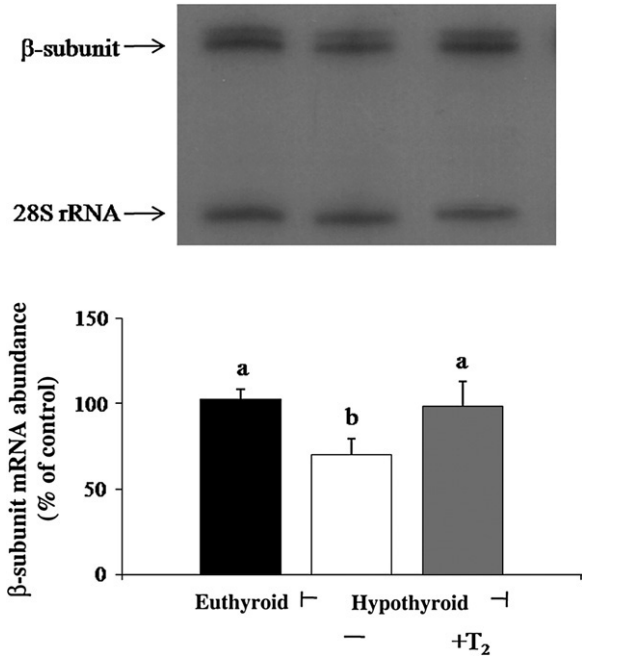

Fig. 2. β-subunit mRNA abundance in rat liver of T_2-treated hypothyroid rats. Total liver RNA (10 μg) was hybridized with ^{32}P-labeled β-subunit and 28S rRNA (used for the normalization) antisense probes. The bars represent the optical scan of the autoradiogram. The data are expressed as percentage of β-subunit abundance of euthyroid rats (100 %) and are means ± SE of 4 different experiments. Values sharing a different letter differ significantly.

mRNA in liver from control and treated rats were determined by RPA. Using a β-F_1 probe, it was found that the amount of protected β-F_1 mRNA decreased (−30%) in hypothyroid relative to euthyroid rats (Fig. 2). Interestingly, treatment of hypothyroid rats with T_2, which is generally believed to operate by a non-nuclear mechanism [reviewed in 5], restored the β-F_1 mRNA content to euthyroid levels. In accordance with a previous report [21], T_3, administered under the same experimental conditions of T_2, significantly increased the level of ATP synthase β-subunit mRNA (data not shown). The amount of 28S rRNA, used for normalization, was unmodified.

In order to verify whether the changes in β-F_1 mRNA level were associated with modification in the protein content, western blot analysis of α/β subunits of ATP synthase was performed. Fig. 3A shows that the immunodetected α/β proteins decreased (−40%) in hypothyroid mitochondria, whereas they were raised by T_2 to the level of the euthyroid control.

The levels of F_oI-PVP and OSCP, two stalk subunits necessary for the correct functioning of ATP synthase [19] were also analyzed. As obtained for α/β, the content of F_oI-PVP and OSCP subunits markedly decreased in hypothyroid rats and was restored to euthyroid levels by T_2 treatment (Fig. 3A and B). Porin was used for normalization (Fig. 3) as its amount is unmodified upon thyroid hormone treatment [23].

3.5. T_2 increases NRF-2α mRNA abundance and protein level in liver from hypothyroid rats

GA-binding protein/nuclear respiratory factor-2, belonging to ETS transcription factor family, is an ubiquitously expressed nuclear transcription factor involved in a broad range of cellular processes, such as the activation of some nuclear genes encoding for mitochondrial proteins [33–35].

To acquire further insight into the mechanism by which T_2 increases transcription of β-F_1 gene, NRF-2α mRNA and protein levels were quantified in rat liver. T_2 administration to hypothyroid rats increased (about 38%) NRF-2α mRNA abundance when compared to hypothyroid animals (Fig. 4A). Similarly to mRNA, the NRF-2α protein level also augmented (Fig. 4B) following T_2 treatment. Protein disulphide-isomerase (PDI) was used for normalization since its amount is not affected in rat liver by the thyroid state [22].

In order to evaluate whether T_2 effect on NRF-2α and β-F_1 gene expression was evident already after short time T_2 treatment, a time course analysis of NRF-2α and β-F_1 mRNA level was carried out. Hypothyroid rats were treated with a single T_2 dose and liver from individual rats was dissected at various times after hormone injection. Increase of NRF-2α mRNA abundance was already evident after 30 min, reaching the maximum 1 h after T_2 administration (Fig. 4C). Augmentation of β-F_1 mRNA level was also observed, being evident after 1 h and reached the maximum at 8 h (Fig. 4C). These data indicated that the NRF-2α induction preceded that of β-F_1 subunit, suggesting that NRF-2 might be involved in the control of this gene exerted by T_2.

3.6. Specific binding in vivo of NRF-2α to β-F1 promoter

To investigate whether NRF-2α binding to the proximal promoter of the endogenous β-F_1 gene is *in vivo* modulated by T_2, a ChIP assay was performed. Chromatin from liver of euthyroid, hypothyroid and T_2-treated hypothyroid rats was isolated. After immunoprecipitation with antibodies against NRF-2α, or with non-specific IgGs, PCRs were carried out. A 486 -bp fragment of the proximal β-F_1 promoter was amplified only when immunoprecipitation was performed with specific anti-NRF-2α antibody (Fig. 4D). By contrast, amplimers were not detected in immunoprecipitation experiments performed with non-specific IgGs (data not shown). In T_2-treated rats the

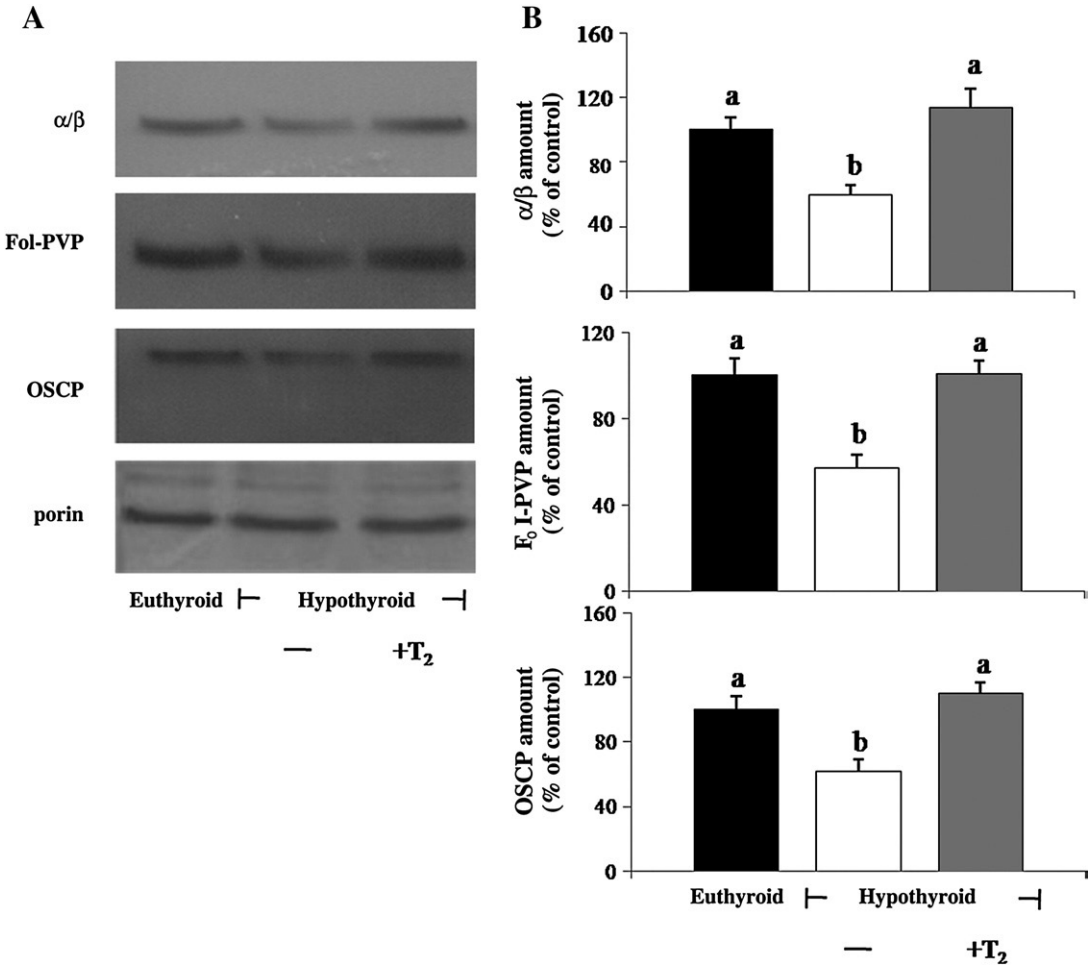

Fig. 3. Effect of T_2 treatment on the expression of α/β, F_oI-PVP and OSCP subunits of ATP synthase in rat-liver mitochondria. Liver-mitochondrial proteins (15 μg), isolated from different rat groups, were separated by electrophoresis and transferred onto nitrocellulose membranes. The latter were incubated with antibodies against α/β, F_oI-PVP and OSCP subunits of ATP synthase. Panel A shows a representative experiment. Panel B shows the histograms representing the means ± SE of the values obtained from densitometric analysis of 6 different experiments after normalization with porin. The data are expressed as percentage of the protein level of euthyroid rats (100 %). Values sharing a different letter differ significantly.

binding of NRF-2α to the promoter noticeably increased when compared with the control.

3.7. T_2 treatment and NRF-2α overexpression activate the β-F_1 promoter activity

To investigate the effect of T_2 treatment and NRF-2α overexpression on the transcriptional activity of the rat β-F_1 gene, reporter assays were carried out. To this aim, the construct pATP5B486, containing the 486 bp fragment (−503/−17 bp upstream the ATG translation initiation codon) of the proximal promoter region of the rat β-F_1 gene, fused to the luciferase reporter gene, was synthesized. H4IIE cells were transiently co-transfected with pATP5B486 together with either the pCMV-NRF-2α or the empty vector pcDNA3.1, then T_2 or vehicle was added to the medium. As a control, a set of transfection experiment with the empty pGL3-basic vector was carried out. As shown in Fig. 5, T_2 treatment of H4IIE cells augmented the β-F_1 promoter activity by approx 80%. NRF-2α overexpression increased promoter activity with a similar extent. A higher activation (~130%) of β-F_1 promoter was observed in H4IIE cells transfected with pCMV-NRF2α and cultured in the presence of T_2. Neither NRF-2α overexpression nor T_2 treatment caused a significant increment of luciferase activity in H4IIE cells transfected with the empty pGL3-basic control plasmid.

4. Discussion

It has been reported that THs directly influence the mitochondrial phosphorylation process by modulating the phosphorylation machinery rather than the respiratory chain [20,21].

While until a few years ago only T_4 and T_3 were considered the only active thyroid hormones, evidence has also been reported on the biological action of T_2 [2-5]. The effect of T_2 is not restricted to mammalian species since mitochondrial energy metabolism in liver and muscle of the goldfish is altered by T_2 treatment [36]. It has been proposed that T_2 is able to increase the mitochondrial respiration rate with a mechanism of action different from that of T_3 [11]. T_3 exerts its "long-term" effects mainly controlling the rate of transcription of genes encoding for different proteins of cellular energy metabolism [34,37]. T_2 effects, unlike those of T_3, are often reported as protein synthesis-independent and they are more rapid than those of T_3 [5].

The direct action of T_2 on mitochondria is supported by findings of specific binding sites for T_2 in subunit Va of COX. On the other hand, it has been indicated that the mitochondrial phosphorylation system is not primarily affected by acute T_2 administration [38].

The present findings, with T_2 chronically administered to hypothyroid rats, clearly indicate that T_2, besides the increase in oxygen consumption, significantly affects F_oF_1-ATP synthase activity in liver mitochondria.

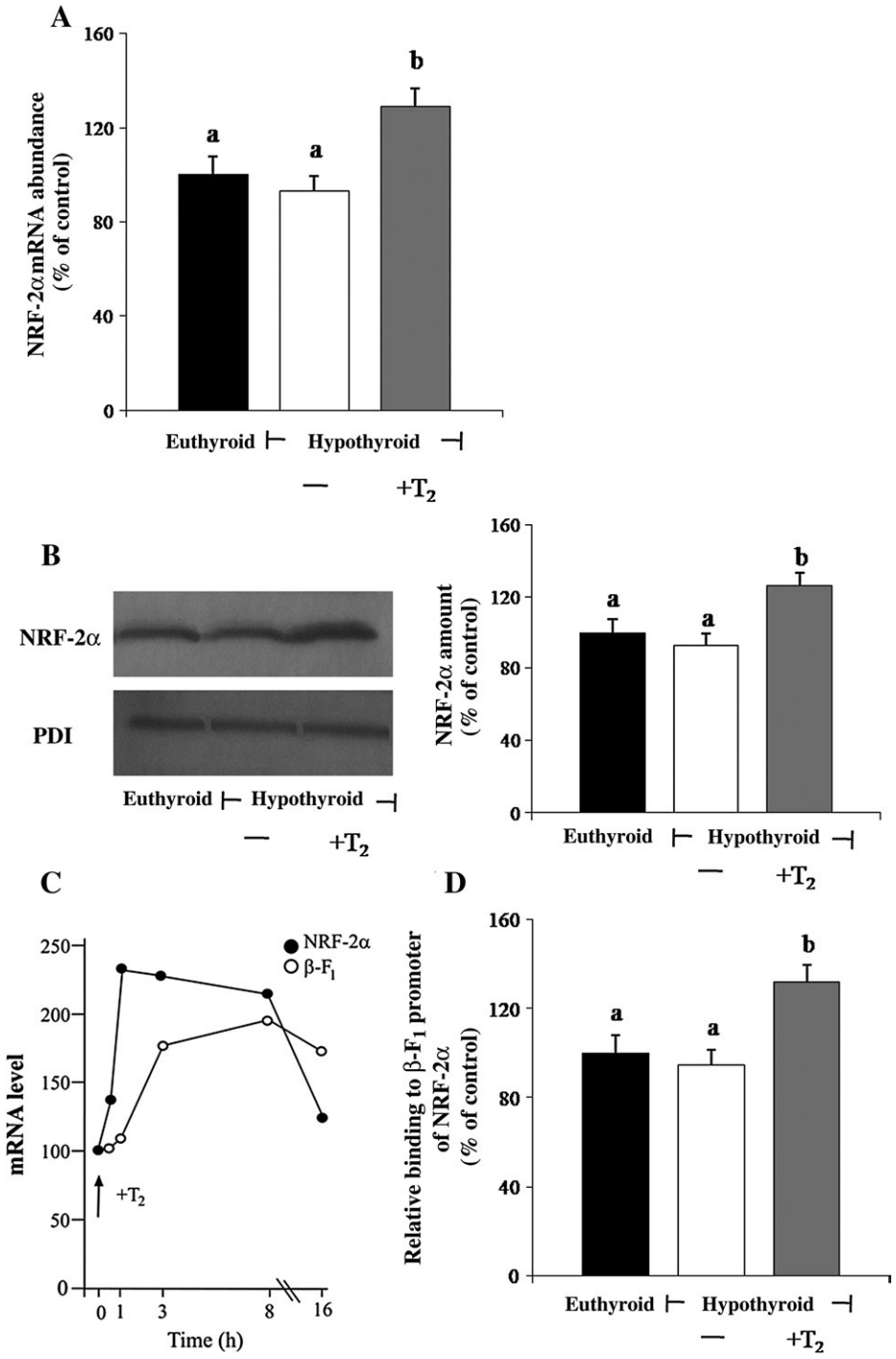

Fig. 4. Effects of T_2 on mRNA abundance, protein level and binding to β-F_1 promoter of NRF-2 α-subunit in liver from hypothyroid rats. (A) The histogram represents NRF-2 α-subunit mRNA abundance, determined by using qRT-PCR and expressed as relative amounts (glyceraldehydes-3-phosphate dehydrogenase as a reference) in liver from euthyroid, hypothyroid and hypothyroid+T_2 rats. Data are expressed as percentage of NRF-2 α-subunit mRNA abundance of euthyroid rats (100%) and are means ± SE of 4 different experiments. (B) Total proteins from liver (50 μg), isolated from different rat groups, were separated by electrophoresis, transferred onto nitrocellulose membranes and immunoblotted using anti-NRF-2 α-subunit antibody. The histogram shows the means ± SE of the values obtained from densitometric analysis of 3 different experiments. The data are expressed as percentage of the protein level of euthyroid rats (100%). (C) Time course of NRF-2α and β-F_1 mRNA induction in the liver of hypothyroid rats after T_2 treatment. Hypothyroid rats were injected with a single dose of T_2 and killed at the indicated times. Normalized value of β-F1 and NRF-2α transcript levels were reported as percentage of corresponding values determined in hypothyroid rats at time 0 (arrow). Data are from one of three independent experiments carried out with similar results. (D) *In vivo* chromatin immunoprecipitation assay was performed using liver from euthyroid, hypothyroid and T_2-treated hypothyroid rats. Results were quantified by densitometric analysis of the PCR products separated on agarose gels (data not shown). The values were derived from the average density of the PCR products from hypothyroid or T_2-treated hypothyroid rats, compared with the average density of those from euthyroid rats (100%). Values sharing a different letter differ significantly.

We can reasonably exclude the possibility that the observed effects exerted by the injected T_2 could be attributed to its metabolic products. The experimental design (PTU + IOP administration) we used, basically the same as that employed in Goglia's laboratory [3,14], is of advantage in allowing the observed effects to be directly attributed to the iodothyronine actually administered.

The present work shows that chronic T_2 administration to hypothyroid rats resulted in an increased amount of several

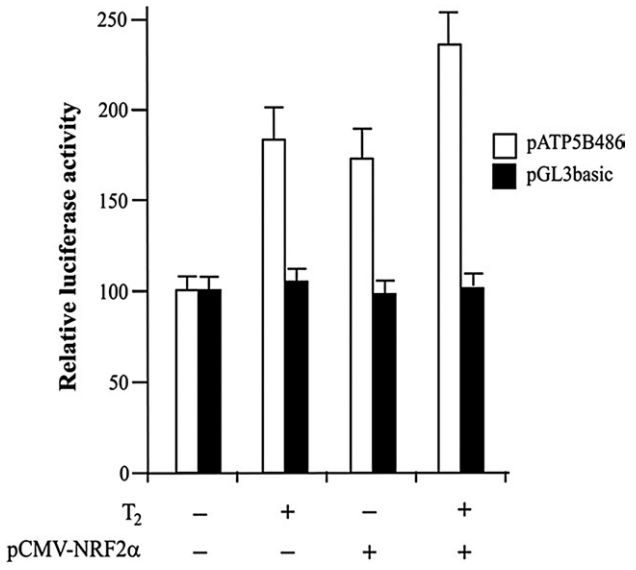

Fig. 5. Effects of T_2 and NRF-2α overexpression on β-F1 promoter activity in H4IIE cells. H4IIE cells were transiently co-transfected with pATP5B486, pGL3 basic empty vector, and pCMV-NRF2α. After transfection, cells were incubated in DMEM medium with or without T_2 for 24 h. Normalized luciferase activity was expressed as percentage of value obtained in the control cells, which were transfected with the empty control vector pcDNA3.1 and incubated without T_2 hormone. Values are means ± S.E., $n = 4$.

fundamental subunits (i.e. α, β, F_oI-PVP and OSCP) of the F_oF_1-ATP synthase (Fig. 3). In addition, our results show that the catalytic β-subunit mRNA abundance increased upon chronic T_2 treatment (Fig. 2). Therefore, the T_2-induced enhancement of the β-subunit expression suggests a T_2-mediated nuclear effect. This finding is in some way surprising as it has been shown that T_2 has substantially low affinity for all nuclear thyroid receptor (TRα1, TRα2, TRβ1, TRβ2) [2]. Thus, to clarify this incongruity and to investigate the molecular mechanism of T_2 effect on β-F_1 expression, we analyzed the induction of the NRF-2α, a nuclear transcription factor ubiquitously expressed and involved in a broad range of cellular processes, such as the activation of some nuclear-encoded mitochondrial genes. NRF-2 is unique among ETS factors family members because it is an obligate multimeric protein complex [39,40]. It consists of two distinct proteins (NRF-2α and NRF-2β) which form a tetrameric $\alpha_2\beta_2$ complex. Between the two subunits, only NRF-2α has been shown to be implicated in the T_3-induced up-regulation of β-F_1 gene [22]. Our data clearly indicate that T_2 significantly increased NRF-2α protein level and mRNA abundance (Fig. 4A and B). The latter finding was further supported by the time course experiments, indicating a rapid and strong increment of NRF-2α-subunit mRNA abundance in response to T_2 injection. β-F_1 mRNA content also increased and reached the maximum later than NRF-2 α-subunit mRNA (Fig. 4C). Therefore, the T_2-mediated induction of NRF-2α preceded that of β-F_1 subunit, suggesting that NRF-2 might be involved in the modulation of the control β-F_1 gene by T_2. To note that similar results have been reported after T_3 injection [22].

Since NRF-2 has the DNA binding domain located in the α-subunit and the transcriptional activation domain in the β-subunit, ChIP assay was conducted using antibody against NRF-2α. ChIP assay results (Fig. 4D) support the hypothesis that T_2 up-regulates β-F_1 gene expression not only augmenting mRNA and protein contents of the specific transcription factor NRF-2α, but also increasing its binding to β-F_1 promoter.

Reporter assay demonstrated that β-F_1 promoter activity was stimulated in transfected H4IIE cells by both T_2 treatment and NRF-2α overexpression (Fig. 5). To note that a higher stimulation of β-F_1 promoter has been observed in H4IIE cells transfected with NRF-2α construct (pCMV-NRF-2α) and cultured in T_2-supplemented medium (Fig. 5). Taken together, these data suggest that T_2 could activate NRF-2α not only increasing its mRNA abundance (Fig. 4C) but probably also through a post-translational modification of the protein. However, we cannot rule out that besides NRF-2α, other transcription factors might be implicated in T_2-mediated induction of β-F_1 expression. Further experiments would give more insights into the role of T_2 on β-F_1 expression.

Our results on T_2-induced stimulation of the transcription rate of F_oF_1-ATP synthase can be related to the finding that *in vivo* T_2 administration augmented mRNA abundance for malic enzyme in rat liver [2]. T_2 was also effective in stimulating growth hormone (GH) mRNA level in GH_3 cells, even if T_2 was 100 fold less potent than T_3 [2]. On the other hand, down-regulation of $TR\beta_2$ mRNA was similar for both iodothyronines [2]. The common opinion that T_2 action is often associated with its "short-term" effects [5] could be ascribed to the fact that only few studies so far have been reported on chronic T_2 treatment of animals. It has been hypothesized that, because of the poor permeability of plasma membrane for iodothyronines, only after chronic treatment of hypothyroid animals it is possible to show an effect of T_2 in some tissues such as the liver [41]. So far, the physio-pathological role of T_2 is not completely defined. The results here reported on F_oF_1-ATP synthase activity and expression strongly support the hypothesis that T_2 might be important in physiological situations requiring additional energy expenditure, such as exposure to cold [5]. It has been reported [42] that conditions in which a "low T_3 state" occurs, such as a nonthyroidal illness and brain tumors, are characterized by higher T_2 concentrations. This phenomenon may represent a compensatory/defense mechanism activated to maintain a metabolic-clinical euthyroidism in patients with reduced T_3 levels [42].

Overall, our results represent the first evidence that the F_oF_1-ATP synthase activity and expression is up-regulated in liver by chronic T_2 administration to hypothyroid rats. Our data support the notion that T_2-transcriptional activation of ATP synthase is indirect, being the nuclear-mediated T_2-effects exerted, at least in part, through the activation of NRF-2α. However, it is worth to underline that our findings might be specific for the liver and, at the moment, they cannot be simply extended to other tissue.

The present study [see also 5,7, 10–15] provides further evidence of thyromimetic activity of T_2, raising doubts on the uniqueness of T_3 as the only active thyroid hormone.

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