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Redox regulation of thyroid hormone-induced Kupffer cell-dependent IκB-α phosphorylation in relation to inducible nitric oxide synthase expression

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Abstract

Thyroid hormone-induced calorigenesis promotes oxidative stress in the liver with higher respiratory burst activity in Kupffer cells, which could increase the expression of redox-sensitive genes. Our aim was to test the hypothesis that L-3,3′,5-triiodothyronine (T_3) triggers inducible nitric oxide synthase (iNOS) expression in rat liver by upstream mechanisms involving the inhibitor of κB (IκB) kinase activation. T_3 administration (daily doses of 0.1 mg/kg for three consecutive days) induced a calorigenic response, with maximal increases in the content of hepatic thiobarbituric acid reactants or protein carbonyls and NOS activity at 48–72 h after treatment, compared to control values. In this time interval, the serum levels of tumor necrosis factor-α (TNF-α; ELISA) are enhanced, concomitantly with higher liver IκB-α phosphorylation (Western blot analysis), NF-κB DNA binding (electrophoretic mobility shift assay), and iNOS mRNA expression (reverse transcription-polymerase chain reaction). These changes and the increase in hepatic NOS activity are abolished by the administration of either α-tocopherol (100 mg/kg) or the Kupffer cell inactivator gadolinium chloride (10 mg/kg) prior to T_3. It is concluded that T_3-induced oxidative stress triggers the redox upregulation of liver iNOS expression through a cascade initiated by TNF-α produced by Kupffer cells and involving IκB-α phosphorylation and NF-κB activation, a response that may represent a defense mechanism by protecting the liver from cytokine-mediated lethality and ROS toxicity.

Keywords: *Thyroid hormone, oxidative stress, IκB-α, inducible nitric oxide synthase, liver, Kupffer cells*

Abbreviations: *ELISA, enzyme-linked immunoabsorbent assay, EMSA, electrophoretic mobility shift assay, GdCl3, gadolinium chloride, iNOS, inducible nitric oxide (NO) synthase, IKK, inhibitor of κB (IκB) kinase, NF-κB, nuclear factor-κB, ROS, reactive oxygen species, RT-PCR, reverse transcription-polymerase chain reaction, TBARs, thiobarbituric acid reactants, TNF-α, tumor necrosis factor-α.*

Introduction

Upregulation of the components of the respiratory apparatus is considered the molecular basis of thyroid calorigenesis, characterized by a higher capacity of oxidative phosphorylation, with the consequent increase in ATP generation being partially compensated by a decrease in the efficiency of ATP synthesis due to intrinsic uncoupling [1,2]. In the liver, acceleration of energy metabolism by T_3 involves an enhanced generation of reactive oxygen species (ROS), which determines a higher consumption of cellular antioxidants and inactivation of enzymes affording antioxidant protection, thus inducing oxidative stress [3]. In these conditions, Kupffer cell function is stimulated, as evidenced by the higher phagocytosis and increased respiratory burst activity

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observed in liver perfusion studies [4]. These alterations correlate with the hyperplasia and hypertrophy of Kupffer cells found histologically, that may contribute to the oxidative stress [4] and the TNF-α response developed [5].

Several physiological effects of T_3 within cells are exerted at the level of transcription, triggered by the interaction of liganded T_3 receptors with T_3-responsive elements, which regulate nuclear and mitochondrial expression of respiratory genes [6]. Upregulation of gene expression by nuclear T_3 receptors may involve stimulation of intermediate activators such as nuclear respiratory factor-1 (NRF-1), NRF-2, and stimulatory protein-1 [7], and/or coactivators such as peroxisome proliferator-activated receptor γ coactivator 1 [8]. Recently, data was reported to support the hypothesis that T_3 can achieve the redox regulation of gene transcription, either as an alternate or a secondary mechanism to the nuclear T_3 receptor pathway [9]. In fact, T_3 administration to rats activates hepatic NF-κB [9], a redox-sensitive factor for the transcriptional activation of several genes, including those for cytokines [10,11]. The T_3-induced increase in NF-κB DNA binding is characterized by being (i) coincidental with the onset of thyroid calorigenesis and enhancement in hepatic respiration, (ii) suppressed by the antioxidants α-tocopherol and N-acetylcysteine, (iii) abrogated by GdCl$_3$, a selective Kupffer-cell inactivator [12], and (iv) associated with induced mRNA expression of the NF-κB-responsive genes for tumor necrosis factor-α (TNF-α) and interleukin (IL)-10, which is correlated with higher serum levels of the cytokines [9].

In addition to the higher ROS production, that is an early response occurring within 24 h of the administration of a single dose of T_3 [3,4], liver nitric oxide (NO) generation exhibits a delayed time course profile, with significant increases being observed after the second (47%) and third (70%) daily consecutive doses of T_3 [13]. This effect of hyperthyroidism on liver NO synthase (NOS) activity is reversed toward control values after 3 days of hormone withdrawal, concomitantly with normalization of thyroid calorigenesis [13]. In view of these considerations, and that NF-κB has prominent transcriptional control over expression of the inducible NOS (iNOS) gene in Kupffer cells, endothelial cells, and hepatocytes [14], the aim of this work was to test the hypothesis that T_3-induced oxidative stress triggers liver iNOS expression by upstream mechanisms involving the activation of the inhibitor of κB (IκB) kinase complex, IKK. For this purpose, thyroid calorigenesis, parameters related to oxidative stress in the liver, and hepatic NOS activity were assessed after the administration of T_3 (daily dose of 0.1 mg/kg for three consecutive days), in relation to the serum levels of TNF-α and liver IκB-α phosphorylation, NF-κB DNA binding, and iNOS mRNA levels. The involvement of oxidative stress and Kupffer cells in iNOS expression was evaluated in separate groups of control rats and hyperthyroid animals subjected to either α-tocopherol [15] or GdCl$_3$ [12] pretreatment, respectively.

Materials and methods

Animals and treatments

Female Sprague-Dawley rats (Bioterio Central, ICBM, Facultad de Medicina, Universidad de Chile) weighing 200–300 g were housed on a 12 h light/dark cycle and were provided with rat chow and water *ad libitum*. Animals received daily ip injections of 0.1 mg of T_3/kg body weight for up to three consecutive days or equivalent volumes of hormone vehicle (0.1 N NaOH, controls) and studies were performed at the indicated times after treatment. Separate groups of rats were subjected to either (i) 100 mg of α-tocopherol/kg i.p. 17 h prior to T_3 or (ii) 10 mg of GdCl$_3$/kg i.v. 24 h before T_3, and studies were carried out at the indicated times after hormone treatment. Thyroid calorigenesis was assessed by the rectal temperatures of the animals measured with a thermocouple (Cole-Parmer Instrument, Chicago, IL, USA). All animals used were cared according to the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (NHI publication No. 86–23).

Assay for NOS activity and parameters related to oxidative stress

Animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the liver was perfused *in situ* with 150 ml of a cold solution containing 150 mM KCl and 5 mM Tris, pH 7.4, in order to remove blood. Liver samples were homogenized on ice and centrifuged at 100,000g for 1 h at 4°C, as previously described [13]. Liver supernatants were used immediately for NOS activity assay [16] in which the oxidation of oxyhemoglobin to methemoglobin by NO is monitored spectrophotometrically at 401 nm versus 411 nm. NOS activity was calculated by using the absorption coefficient of methemoglobin (38,600 M^{-1} cm^{-1}) for the wavelength pair 401 minus 411 [13,16] and results were expressed as nmol NO/mg protein/min.

Liver protein oxidation was assayed by the reaction of 2,4-dinitrophenylhydrazine with protein carbonyls according to Reznick and Packer [17], lipid peroxidation was measured by the thiobarbituric acid assay (TBARs) as described by Buege and Aust [18], and the protein content of liver homogenates was determined according to Lowry et al. [19].

Enzyme-linked immunoabsorbent assay (ELISA) for TNF-α

Serum from rat blood was obtained by cardiac puncture, separated and stored at $-80°C$, and TNF-α levels were measured by ELISA (UltraSensitive Cytoscreen KRC3013 kit, Biosource International, Camarillo, CA, USA) according to manufacturer's specifications. Serum samples containing high TNF-α levels were repeated after dilution to ensure assay results within the standard curve.

Western blot analysis of IκB-α and phospho-IκB-α (Ser32)

At selected times after T₃ treatment, liver samples (100–500 mg) frozen in liquid nitrogen were homogenized and resuspended in a buffer solution pH 7.9 containing 10 mM HEPES, 1 mM EDTA, 0.6% Nonidet P-40, 150 mM NaCl, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 2 mM orthovanadate). Soluble protein fractions (50 μg) were separated on 12% polyacrylamide gels using SDS-PAGE [20] and transferred to nitrocellulose membranes [21], which were blocked for 1 h at room temperature with TBS-containing 5% non-fat dry milk. The blots were washed with TBS-containing 0.1% Tween 20 and hybridized with rabbit polyclonal antibody for rat IκB-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by rabbit polyclonal antibody for rat phospho-IκB-α (Ser32) (Cell Signaling Technology, Beverly, MA, USA), and mouse monoclonal antibody for rat β-actin (internal control) (ICN Biomedicals, Inc., Aurora, OH, USA). After extensive washing, the antigen–antiboby complexes were detected using horseradish peroxidase labeled goat anti-rabbit IgG or goat anti-mouse IgG and a SuperSignal West Pico Chemiluminescence kit detection system (Pierce, Rockford, IL, USA).

Electromobility shift assay (EMSA)

Nuclear protein extracts from liver samples were prepared according to Deryckere and Gannon [22]. The samples were subjected to EMSA for assessment of NF-κB DNA binding using the iNOS NF-κB probe 5″-GCACACCCTACTGGGGACTCTCCCT-3″ (Invitrogen Corp., Carlsbad, CA, USA), labeled with α-³²P-dCTP using the Klenow DNA Polymerase Fragment I (Invitrogen Corp., Carlsbad, CA, USA), as previously described [23]. The specificity of the reaction was determined by a competition assay using 100-fold molar excess of unlabeled DNA probe. The subunit composition of DNA binding protein was confirmed by supershift assay using specific antibodies from goat and rabbit IgG raised against NF-κB p50 and p65 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Samples were loaded on nondenaturating 6% polyacrylamide gels and run until the free probe reached the end of the gel; NF-κB bands were detected by autoradiography and quantified by densitometry using Scion Image (Scion Corp., Frederick, MD, USA).

Isolation of hepatic rna and reverse transcription-polymerase chain reaction (RT-PCR) assay for iNOS mRNA

Total liver RNA was extracted with TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) [24] and quantified by measurement of ultraviolet absorption at 260 nm. For RTPCR assay of mRNA, first-strand cDNA was synthesized from total RNA (3 μg) using SuperScript RNAse H⁻ Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA, USA) and random hexamer primers [pd(N)₆] (Promega, Madison, WI, USA). cDNA was amplified in a PCR reaction using Taq DNA polymerase (Invitrogen Corp., Carlsbad, CA, USA) in the presence of primers specific for rat iNOS. Nucleotide sequences for sense and antisense primers used were 5″-CAACAACACAGGATGAC-CCTAA-3″ and 5″-GGTAGGTTCCTGTTGTTT-CTAT-3″ (Invitrogen Corp., Carlsbad, CA, USA), respectively. In these conditions, a 417 bp sequence between +133 and +550 bp of rat iNOS cDNA was amplified. To control the relative amount of total mRNA transcribed in each reverse transcriptase reaction, an RNA 18S invariant standard [Classic II 18S Internal Standards (324 bp); Ambion, The RNA Co., Austin, TX, USA] was used. PCR conditions included denaturation, annealing, and extension at 94, 56, and 72°C, for 30, 30, and 60 s, respectively, for 38 cycles. PCR products were electrophoresed on 2% agarose gels containing ethidium bromide, visualized by UV-induced fluorescence, and analyzed by densitometry using Scion Image (Scion Corp., Frederick, MD, USA) [9].

Statistical analysis

Values shown are means ± SEM for the number of separate animals indicated. One-way ANOVA and the Newman-Keuls, test assessed the statistical significance of differences between mean values ($p < 0.05$).

Results

Effects of T₃ administration on the rectal temperature of the animals and liver oxidative stress status and NOS activity

Administration of T₃ to fed rats induced a calorigenic response shown by the significant increase in the rectal temperature of the animals in the 10–74 h time period studied, as compared with euthyroid rats (Figure 1A). In these conditions, T₃ resulted in a progressive
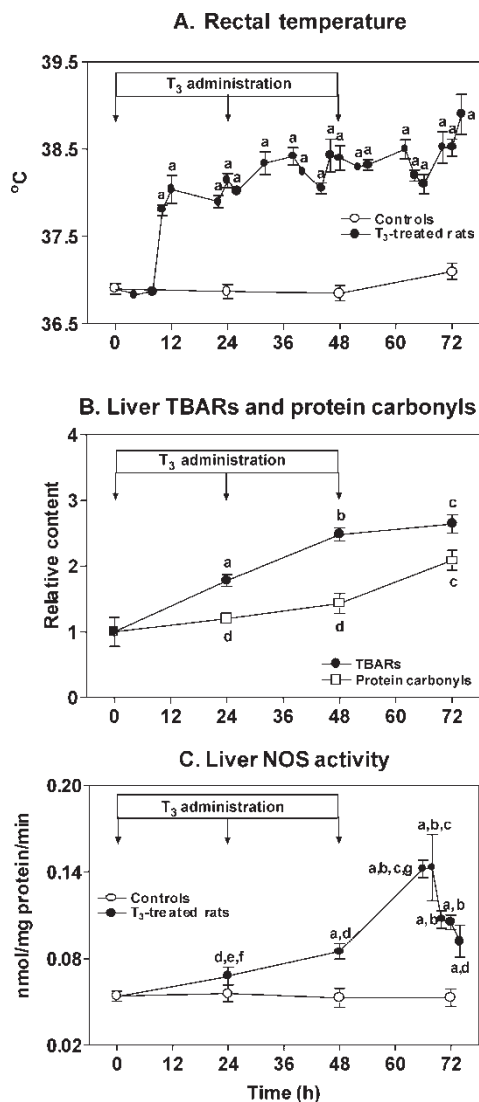
Figure 1.　*Time course study of the effects of T_3 administration on the rectal temperature of the animals (A), liver TBARs and protein carbonyl contents (B), and hepatic NOS activity (C)*. Data are means ± SEM for three to fifteen different animals. Significance studies: (A) [a]$p < 0.05$ compared with control values (at 0, 24, 48, and/or 72 h) and T_3-treated rats at 4 and 8 h; (B) [a]$p < 0.05$ versus controls (at time zero) and T_3-treated rats at 48 and 72 h; [b]$p < 0.05$ compared with controls (at time zero) and T_3-treated rats at 24 h; [c]$p < 0.05$ versus controls (at time zero) and T_3-treated rats at 24 and 48 h; [d]$p < 0.05$ compared with controls (at tome zero) and T_3-treated rats at 72 h; (C) [a]$p < 0.05$ versus control values (at time 0, 24, 48, and/or 72 h); $p < 0.05$ compared with T_3-treated animals at [b]24 h, [c]48 h, [d]66–68 h, [e]70 h, [f]72 h, and [g]74 h. Mean values for TBARs [0.35 ± 0.08 ($n = 4$) nmol/mg of protein] and for protein carbonys [1.05 ± 0.05 ($n = 9$) nmol/mg of protein] in control rats at time zero were arbitrarily set to unity, and values at other time points were normalized to this (B).

enhancement in the content of TBARs and protein carbonyls of the liver (Figure 1B), parameters that exhibited maximal increases at 72 h following hormone administration and are significantly correlated ($r = 0.81$, $p < 0.05$). At 24 h after the first dose of T_3, liver NOS activity was not modified over control values, but it was significantly elevated in the 48–74 h

time period studied involving additional T_3 doses, with a maximal effect being observed at 66–68 h (Figure 1C).

T_3 *Administration promotes liver iNOS expression. Effect of α-tocopherol or GdCl$_3$ pretreatment*

In the 48–74 h time interval studied, showing significant enhancement in liver NOS activity by T_3 treatment (Figure 1C), the levels of TNF-α in serum are also elevated, with significant increases being found at 58–66 h after hormone administration, compared with control values (Figure 2A). Following 58–70 h after T_3 treatment, the hepatic levels of phosphorylated IκB-α were significantly enhanced over control values, with maximal effects being observed at 62 h (Figure 2B). This effect of T_3 was elicited concomitantly with a diminution in the content of non-phosphorylated IκB-α (Figure 2B), without changes in the total content of hepatic IκB-α, as shown by the 96–101% recovery of IκB-α in the liver of T_3-treated animals compared with control rats. Under these conditions, T_3 led to a significant enhancement in the DNA binding activity of NF-κB (Figure 2C) and in the levels of iNOS mRNA (Figure 2D) of the liver, over control values. Thyroid hormone-induced increases in either serum TNF-α levels (at 64 h; Figure 3A), liver IκB-α phosphorylation and NF-κB DNA binding (at 62 h; Figure 3B), or hepatic iNOS mRNA levels and NOS activity (at 70 and 68 h, respectively; Figure 3C), were abrogated by pretreatment with both α-tocopherol and GdCl$_3$. Similar results were obtained after the administration of daily injections of α-tocopherol (100 mg/kg) 17 h prior to each dose of T_3 for three consecutive days (data not shown). Experiments with GdCl$_3$ were carried out using a single dose 24 h prior to T_3, considering that repopulation of Kupffer cells starts 4 days after its administration and is completed after 7 days [12].

Discussion

Data presented in this work support the contention that thyroid hormone induces upregulation of gene expression in the liver through a redox signaling mechanism triggered at the Kupffer cell level [5,9], resulting in the enhancement of iNOS expression. In the time course studies shown, we have established that significant increases in liver NOS activity occurs in the 48–72 h time period after T_3 administration, with maximal effects being observed at 66–68 h. This effect of thyroid hormone is elicited in thermogenic conditions with maximal accumulation of products of hepatic lipid peroxidation and protein oxidation, thus evidencing the attainment of significant oxidative stress in the liver. In these prooxidant conditions, the serum levels of TNF-α are significantly elevated at 58–66 h after T_3 treatment, a response that is triggered by oxidative stress in Kupffer cells due to
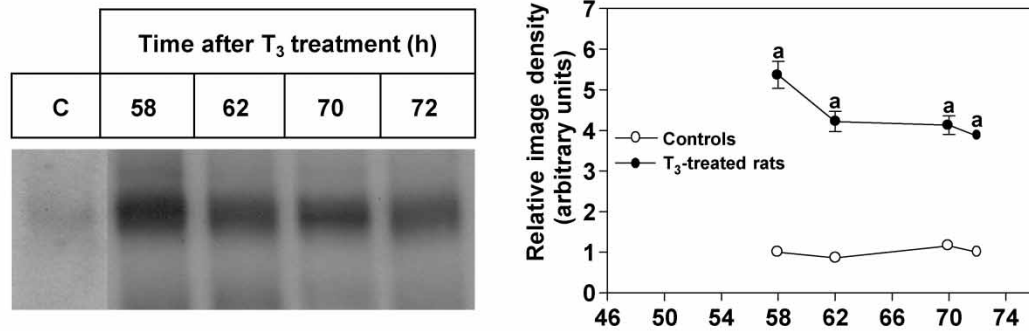
A. Serum TNF- levels
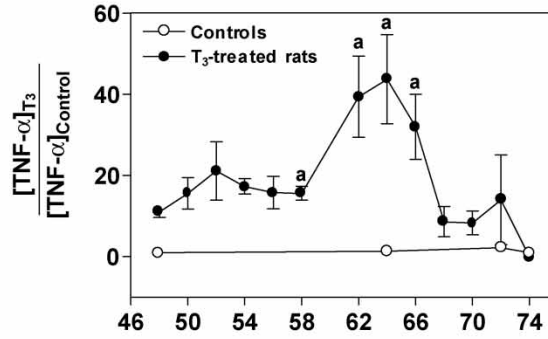
B. Liver IκB-α phosphorylation
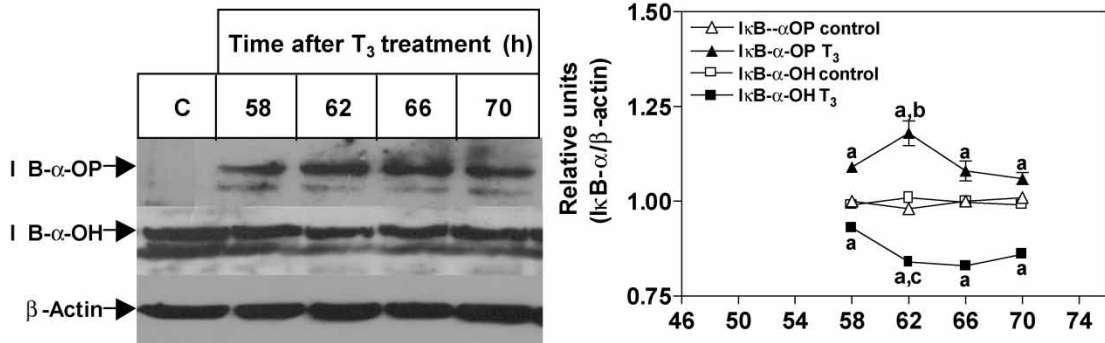
C. Liver NF-κB DNA binding
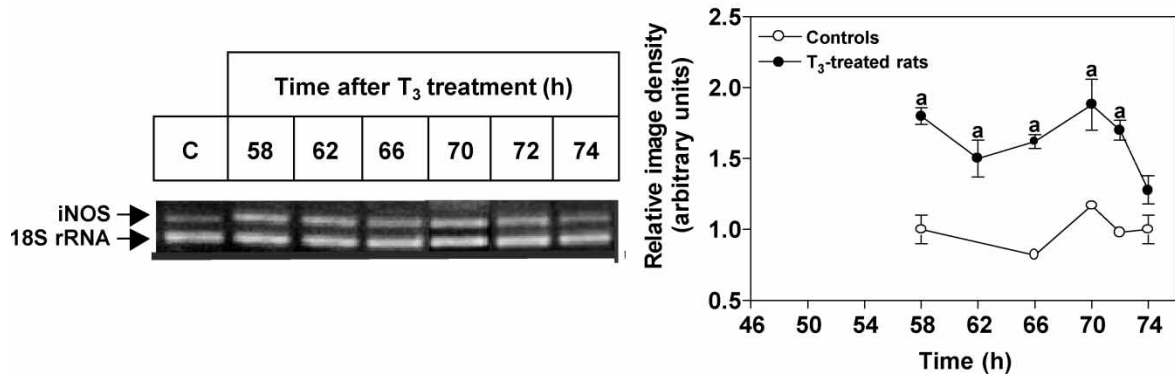
D. Liver iNOS mRNA levels

Figure 2.   *Time course study of the effects of T_3 administration on the serum levels of TNF-α (A) and liver IκB-α phosphorylation (B), NF-κB DNA binding (C), and iNOS mRNA levels (D).* Data are means ± SEM for three to seven different animals. (B) Representative SDS-PAGE of liver soluble protein fractions from control rats [C] and T_3-treated animals at different times after treatment for detection of nonphosphorylated IκB-α (IκB-α-OH), phosphorylated IκB-α (IκB-α-OP), and β-actin [left panel] and the respective densitometric quantification expressed as the IκB-α-OH(OP)/β-actin ratios to compare lane-to-lane equivalency in total protein content [right panel]. (C) Representative autoradiograph of NF-κB DNA binding evaluated by EMSA using nuclear extracts from livers of control rats [C] and T_3-treated animals at
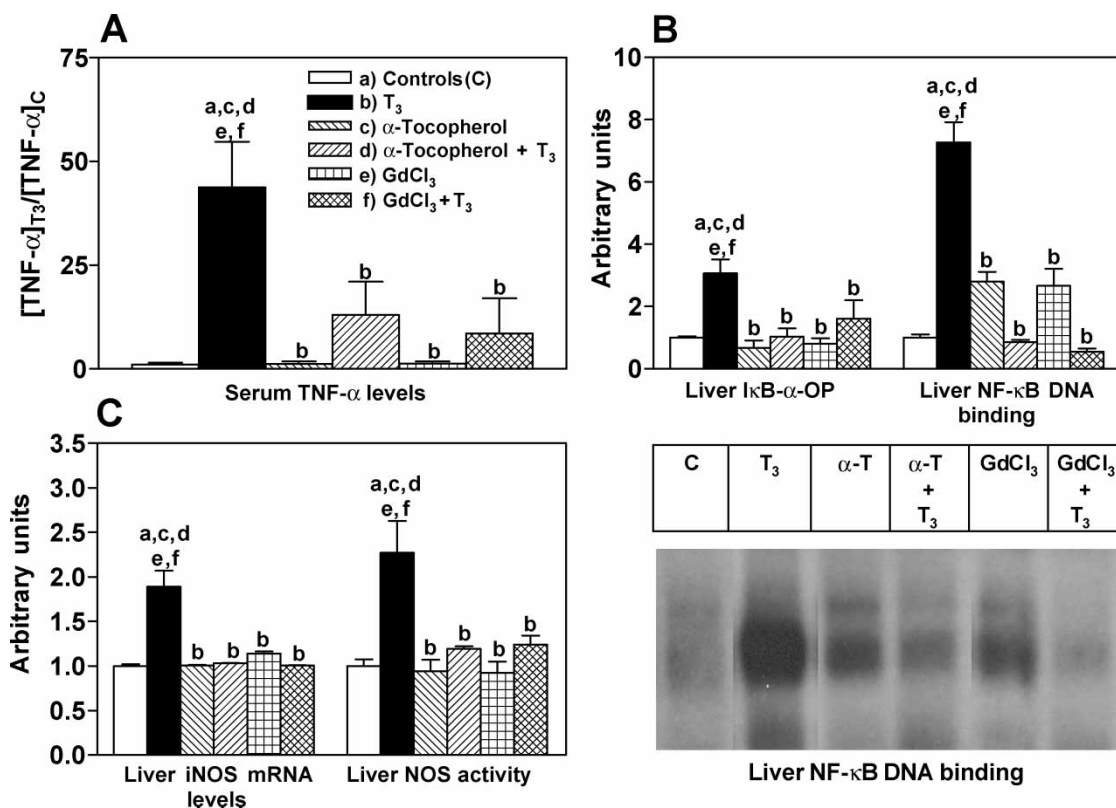
Figure 3.   *Effects of α-tocopherol and GdCl₃ pretreatment on T₃-induced serum TNF-α levels at 64 h (A), liver IκB-α phosphorylation and NF-κB DNA binding at 62 h (B), and hepatic iNOS mRNA levels (at 70 h) and NOS activity (at 68 h) (C)*. Data are means ± SEM for three to fifteen different animals. Liver IκB-α phosphorylation and iNOS mRNA content are expressed as indicated in the legend for Figure 2. In (B), densitometric quantification of relative NF-κB binding (upper panel) and representative autoradiographs (lower panel) evaluated by EMSA are shown. Average data from control rats were set to unity, and values from the experimental conditions studied were normalized to this. Significance studies ($p < 0.05$) are shown by the letters identifying each experimental group.

its abolishment by α-tocopherol and GdCl₃ pretreatment. T₃ elicits an early TNF-α response that is maximal at 22 h [5] and that levels off at 24 h (unpublished data), probably due to the concomitant IL-10 expression [9] which downregulates pro-inflammatory cytokine production [25,26]. In rat liver, Kupffer cells are the most important source of TNF-α [27] that is produced and released in response to several stimuli including ROS, primarily generated by activated NADPH oxidase [28].

TNF-α released from Kupffer cells exhibits autocrine and paracrine actions in the liver through interactions with two surface receptors in target cells, namely, TNF-α receptor 1 (TNFR-1) and TNFR-2 in order to mediate TNF-α-dependent signals from

cell membrane to nucleus [29]. Signal cascades operating after TNF-α-TNFR-1 coupling play an important role in the homeostatic response of the liver to oxidative stress, triggering defense and reparative processes against injury under conditions of moderate prooxidant status and low levels of transient TNF-α expression [11,30,31]. Data reported indicate that T₃-induced TNF-α production occurred concomitantly with significant increases in liver IκB-α serine 32 phosphorylation, suggesting the activation of the IKK complex [10]. The process requires the recruitment of several adaptor molecules including TNFR-associated death domain, TNFR-associated factor-2, and receptor-interacting protein causing the activation of NF-κB-inducing kinase,

different times after treatment [left panel] and the respective densitometric quantification of relative NF-κB binding [right panel]. (D) Representative agarose gel electrophoresis of the RT-PCR products for iNOS mRNA (417 bp) and 18S rRNA (324 bp) after ethidium bromide staining in total hepatic RNA samples from control rats [C] and T₃-treated animals at different times after treatment [left panel] and the respective densitometric quantification of RT-PCR products of the mRNA of iNOS expressed as the iNOS mRNA/18S rRNA ratios to compare lane-to-lane equivalency in total RNA content [right panel]. Average data from all control rats shown at the first experimental time were set to unity, and values for control animals and T₃-treated rats at other time points were normalized to this. Significance studies: [a]$p < 0.05$ compared with controls values in (A), (C), and (D); (B): [a]$p < 0.05$ versus the respective control values for IκB-α-OH or IκB-α-OP; [b]$p < 0.05$ compared with T₃-treated rats at 58, 66, and 70 h; [c]$p < 0.05$ versus T₃-treated animals at 58 h.

which in turn associates and activates the IKK complex [10,29,32]. This pathway, and the proposed TNF-α-dependent p65 phosphorylation at serine 529 [33], may contribute to the enhancement in the activating potential of NF-κB observed in the 58–72 h time interval after T$_3$ administration.

T$_3$-induced NF-κB DNA binding is associated with upregulation of iNOS expression and the increase in liver NOS activity, parameters that are suppressed by α-tocopherol and GdCl$_3$ pretreatment. High levels of NO are expected in conditions of iNOS expression, which may constitute a defense mechanism avoiding cytotoxicity from oxidative stress. This can be achieved through different actions including: (i) scavenging of ROS to reduce the oxidation potential that deteriorates biomolecules and activates NF-κB, (ii) nitrosylation of NF-κB p50 to diminish the DNA binding of NF-κB, and/or (iii) induction of the synthesis and/or stabilization of IκB [10,34]. In agreement with this proposal, T$_3$-induced oxidative stress is limited to enhanced efflux of lactate dehydrogenase from the liver to the sinusoidal space, probably due to higher ROS mediated membrane lipid peroxidation [35], without morphological evidence of liver injury, except for Kupffer cell hyperplasia and hyperthrophy [4,36]. Alternatively, antioxidant defense mechanisms may involve the upregulation of the T$_3$-responsive genes for uncoupling proteins (UCPs) [37,38], including hepatic UCP-2 induction [39]. Firstly, mild uncoupling by UCPs decreases the mitochondrial membrane potential below a critical level, thus increasing O$_2$ consumption and blunting O$_2^{-\cdot}$/H$_2$O$_2$ production [38]. This contention is based on the finding that mitochondrial H$_2$O$_2$ generation is greatest in state 4, a condition involving a high degree of reduction of the carriers, compared with that in the state 3 [40]. Secondly, UCPs may allow the transport of peroxidized insaturated fatty acid anions, in addition to native fatty acid anions, from the inner to outer side of the inner mitochondrial membrane [37], with the consequent decrease in the oxidative damage to mitochondrial DNA and proteins [37,38].

In conclusion, redox upregulation of liver iNOS expression by T$_3$ is attained through a cascade initiated by TNF-α produced by Kupffer cells and involving IκB-α phosphorylation and NF-κB activation, a response that may represent a defense mechanism against T$_3$-induced oxidative stress by protecting the liver from cytokine mediated lethality and ROS toxicity. This proposed pathway seems to be a secondary mechanism of ROS induced by T$_3$ administration, and its involvement in the expression of other redox-regulated genes or its direct relation with nuclear and mitochondrial transcription systems triggered by T$_3$ remain to be established.

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