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Redox up-regulated expression of rat liver manganese superoxide dismutase and Bcl-2 by thyroid hormone is associated with inhibitor of κB-α phosphorylation and nuclear factor-κB activation

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Abstract

Recently, we demonstrated that 3,3',5-triiodothyronine (T3) induces oxidative stress in rat liver, with enhancement in the DNA binding of nuclear factor-κB (NF-κB) and the NF-κB-dependent expression of tumor necrosis factor-α (TNF-α). In this study, we show that T3 administration (daily doses of 0·1 mg/kg i.p. for three consecutive days) elicited a calorigenic response and higher liver O_2 consumption rates, with increased serum levels of TNF-α (ELISA), liver inhibitor of κB (IκB-α) phosphorylation (Western blot analysis), and hepatic NF-κB DNA binding (EMSA) at 56–72 h after treatment. Within this time interval, liver manganese superoxide dismutase (MnSOD) activity and the protein expression of MnSOD and Bcl-2 are enhanced. These changes are abrogated by the administration of α-tocopherol (100 mg/kg i.p.) prior to T3. It is concluded that T3 treatment leads to the redox upregulation of MnSOD and Bcl-2 in rat liver, in association with TNF-α release and activation of the IκB-α kinase/NF-κB cascade, which may constitute a protective mechanism against free radical toxicity involving cell death signaling.

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Introduction

Superoxide radical ($O_2{}^{\bullet-}$) and hydrogen peroxide (H_2O_2) are primary reactive oxygen species (ROS) generated as by-products in electron transfer processes, particularly mitochondrial respiration and microsomal cytochrome P450 monoxygenation (Videla 2000). Specialized cells in direct relation to their functions also produce ROS, as is the case of the NADPH oxidase (NOX2) involved in the bactericidal mechanism of phagocytes or the dual oxidase p138^{Tox} (DUOX2) that supports thyroid hormone biosynthesis in thyroid cells (Lambeth *et al.* 2000). When generated at high levels, ROS are cytotoxic and mutagenic due to their high chemical reactivity that produces substantial oxidative modifications in unsaturated lipids, proteins, and DNA, with loss of their functions and cell viability (Dröge 2002, Martindale & Holbrook 2002). In addition, accumulating evidence suggests that production of low levels of ROS (Thannickal & Fanburg 2000, Dröge 2002, Martindale & Holbrook 2002), and lipid oxidation products of ROS-dependent reactivity (Poli *et al.* 2004, Leonarduzzi *et al.* 2004), may play a role in signal transduction processes. This can be accomplished through reversible oxidative modifications of critical amino acid residues within the functional domain of proteins, particularly cysteine residues, which may alter the activity of enzymes such as kinases and phosphatases or the DNA binding of transcription factors involved in cell signaling (Thannickal & Fanburg 2000, Martindale & Holbrook 2002, Poli *et al.* 2004).

Thyroid hormone is essential for growth, development, and regulation of energy metabolism (Oppenheimer *et al.* 1996). Hyperthyroidism in vertebrates increases their basal metabolic rate due to higher O_2 consumption by target tissues such as liver (Fernández *et al.* 1985), leading to enhanced ROS generation (Videla 2000). 3,3',5-Triiodothyronine (T3)-induced pro-oxidant activity (i) occurs at mitochondrial (Fernández & Videla 1993a), microsomal (Fernández *et al.* 1985), and cytosolic (Huh *et al.* 1998) levels, (ii) involves generation of reactive nitrogen species (RNS) in addition to ROS (Fernández *et al.* 1997), and (iii) accounts for 16–25% of the net increase in total O_2 consumption of the liver (Fernández & Videla 1993b). The latter respiratory component may include O_2 equivalents employed both in ROS/RNS generation and in the free radical–dependent oxidation of unsaturated fatty acids (Fernández *et al.* 1985, Landriscina

et al. 1988, Venditti *et al.* 1997, Huh *et al.* 1998), proteins (Tapia *et al.* 1999), and DNA (Andican *et al.* 2004). Enhancement in liver mitochondrial H_2O_2 production is also observed in the transition from hypothyroid to hyperthyroid state as a function of the content of autoxidisable electron carriers (Venditti *et al.* 2003), an effect that is mimicked by cold-induced hyperthyroidism (Venditti *et al.* 2004). In addition to these changes that are presumed to occur primarily at the parenchymal cell level, T3 administration to rats led to hyperplasia and hypertrophy of Kupffer cells, with the consequent enhancement in respiratory burst activity (Tapia *et al.* 1997). The latter process is mainly due to the activity of the ROS generator NOX2, with a smaller contribution of nitric oxide ($^\bullet$NO) synthase (NOS) (Wang *et al.* 1993), being the increase in hepatic NOS activity induced by T3 partially inhibited by the Kupffer cell inactivator gadolinium chloride (GdCl3) (Fernández *et al.* 1997). This liver free radical activity induced by hyperthyroid state is coupled to a diminution in the antioxidant potential of the tissue in experimental animals and man, leading to oxidative stress (Videla 2000) and activation of the redox-sensitive transcription factor NF-κB (Tapia *et al.* 2003).

In the liver, NF-κB has prominent transcriptional control over expression of a number of genes, such as those for cytokines, chemokines, and adhesion molecules (Flohé *et al.* 1997, Tsukamoto & Lin 1997), type I acute-phase proteins (Ramadori & Christ 1999), anti-apoptotic proteins (Ashkenazi & Dixit 1998), and enzymes. Among the latter group, manganese superoxide dismutase (MnSOD) constitutes a major protective mechanism against ROS by removing $O_2^{\bullet-}$ from both mitochondrial and the cytosol, otherwise inducing oxidant injury or acting as pro-apoptotic factors (Oberley 2004). In view of these considerations, and the substantial enhancement in liver mitochondrial ROS generation by hyperthyroidism (Fernández & Videla 1993*a*, Venditti *et al.* 2003), the aim of the present study was to test the hypothesis that T3-induced pro-oxidant activity stimulates the expression of liver MnSOD and Bcl-2 through upstream processes involving the activation of the inhibitor of κB (IκB) kinase complex (IKK) and the transcription factor NF-κB. For this purpose, hepatic MnSOD activity and the protein expression of MnSOD and Bcl-2 were studied in relation to the serum levels of tumor necrosis factor-α (TNF-α) and liver IκB-α phosphorylation and NF-κB DNA binding, both in control rats and animals given T3, and in separate groups subjected to α-tocopherol treatment prior to hormone administration.

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 i.p. injections of 0·1 mg of T3/kg body weight for three consecutive days or equivalent volumes of hormone vehicle (0·1 mol/l NaOH, controls) and studies were performed at the indicated times after treatment. Thyroid hormone-induced calorigenesis was assessed by the rectal temperatures of the animals measured with a thermocouple (Cole-Parmer Instrument, Chicago, IL, USA), the basal rate of O_2 consumption by the liver was determined polarographically in perfusion studies (Fernández & Videla 1993*b*, Tapia *et al.* 1997), and blood samples were taken from the tail vein for the measurement of serum T3 levels by GammaCoat (I^{125})T3 Radioimmunoassay (assay sensitivity limit of 9 ng T3/dl, between-assay variation of 4%, and intra-assay variation of 3·5% at 33 ng/dl and 2·7% at 290 ng/dl; Baxter Healthcare, Cambridge, MA, USA). Separate groups of rats were subjected to 100 mg of α-tocopherol/kg i.p. (Camandola *et al.* 1999) 17 h prior to the first dose of T3, and studies were carried out at the indicated times after hormone treatment. In these groups, the levels of α-tocopherol in plasma were measured by reverse phase high performance liquid chromatography (HPLC) after extraction with hexane, according to Shearer (1986). For this purpose, a Simmetry C-18 column (Waters Corporation, Milford, MA, USA), 3·5 μm, with dimensions of 4·6 × 100 mm was employed. Separation was done using a flow rate of 1 ml/min of the mobile phase (7% v/v dichloromethane in methanol) and detection was performed at 292 nm. All animals used were cared for according to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals by the National Academy of Sciences (National Institutes of Health publication No. 86–23).

Assay for MnSOD activity

The animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and the livers were perfused with 150 ml of a cold solution containing 150 mM KCl and 5 mM Tris pH 7·4 in order to remove blood. Mitochondrial pellets obtained by differential centrifugation were suspended in a buffer containing 210 mM mannitol, 70 mM sacarose, 2 mM HEPES, and 0·5 g/l serum bovine albumin, pH 7·4, and MnSOD activity was measured by the MnSOD-sensitive autoxidation of hematoxylin at 560 nm, in a reaction medium containing 0·05 M sodium phosphate buffer pH 7·5, 0·1 mM EDTA, and 5 mM hematoxylin (Martin *et al.* 1987). Results are expressed as units/mg mitochondrial protein (1 unit corresponds to the amount of enzyme producing 50% inhibition of the rate of hematoxylin autoxidation at 30 °C), standardized with purified MnSOD (Sigma Chemical Co., St Louis, MO, USA). Although mitochondrial fractions may contain traces of CuZnSOD, this enzyme activity is significantly diminished after the administration of T3 for 3 consecutive

days (Fernández *et al.* 1988), thus minimizing possible contamination artifacts. The protein content was determined according to Lowry *et al.* (1951).

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, phospho-Iκ-α (Ser32), Bcl-2, and MnSOD

At selected times after T3 treatment, liver samples (100–500 mg) frozen in liquid nitrogen were homogenized and suspended 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 phenyl-methylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mM orthovanadate). Soluble protein fractions (50 μg) were separated on 12% polyacrylamide gels using SDS–PAGE (Laemmli 1970) and transferred to nitrocellulose membranes (Towbin *et al.* 1979), 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). For Bcl-2, samples were hybridized with mouse monoclonal antibody for rat Bcl-2 (BD Transduction Laboratories, San Jose, CA, USA). In the case of MnSOD, mitochondrial suspensions (25 μg) were separated on 13% SDS–PAGE and resolved with mouse monoclonal antibody for rat MnSOD (BD Transduction Laboratories, San Jose, CA, USA). In all determinations, mouse monoclonal antibody for rat β-actin (ICN Biomedicals, Inc., Aurora, OH, USA) was used as internal control. 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 (1994). The samples were subjected to EMSA for assessment of NF-κB DNA binding using the NF-κB probe 5′-GATCTCAGAGGG GACTTTCCGAG-3′ (Genset Corp., La Jolla, CA,

USA), labeled with α-^{32}P-dCTP using the Klenow DNA Polymerase Fragment I (Invitrogen Corp., Carlsbad, CA, USA), as previously described (Videla *et al.* 2004). 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 nondenaturing 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).

Statistical analysis

Values shown are means ± S.E.M. 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

The administration of T3 to rats as depicted in Fig. 1A resulted in significant increases in serum T3 levels over control values (Controls, 51 ± 3 ($n = 10$) ng/dl; T3-treated rats at 24 h, 354 ± 61 ($n = 6$) ($P < 0·05$ vs controls); T3-treated rats at 48 h, 307 ± 26 ($n = 5$) ($P < 0·05$ vs controls); T3-treated rats at 72 h, 288 ± 15 ($n = 8$) ($P < 0·05$ vs controls). Under these conditions, T3 treatment induced a calorigenic response with significantly higher rectal temperatures either at 24 and 48 h after treatment or in the 5674 h time period studied, over control values (Fig. 1A). In the latter experimental time interval, T3 treatment led to (i) an increase (44%) in the average rate of O_2 consumption of the liver compared with control values (controls, 1·84 ± 0·07 ($n = 8$) μmol/g liver/min; T3-treated rats, 2·65 ± 0·12 ($n = 9$); $P < 0·05$) and (ii) significantly higher TNF-α levels in serum, which returned to control values at 74 h (Fig. 1B).

To examine the effect of T3 treatment on the expression of MnSOD and Bcl-2 in rat liver, assessment of hepatic IκB-α serine 32 phosphorylation, NF-κB activation, and MnSOD and Bcl-2 protein expression was performed. Fig. 2A shows significant 1740% increases in IκB-α phosphorylation at 58–72 h after T3 administration, expressed as the quotient of phosphorylated IκB-α/non phosphorylated IκB-α ratios in T3-treated and control rats, with the concomitant 2·7 to 6·6-fold enhancement in the DNA binding capacity of NF-κB (Fig. 2B). Under these conditions, T3 led to the upregulation of liver MnSOD, as evidenced by the 12–34% increase in protein expression (Fig. 3A, upper panel), with the concomitant 125–160% enhancement in the specific activity of the
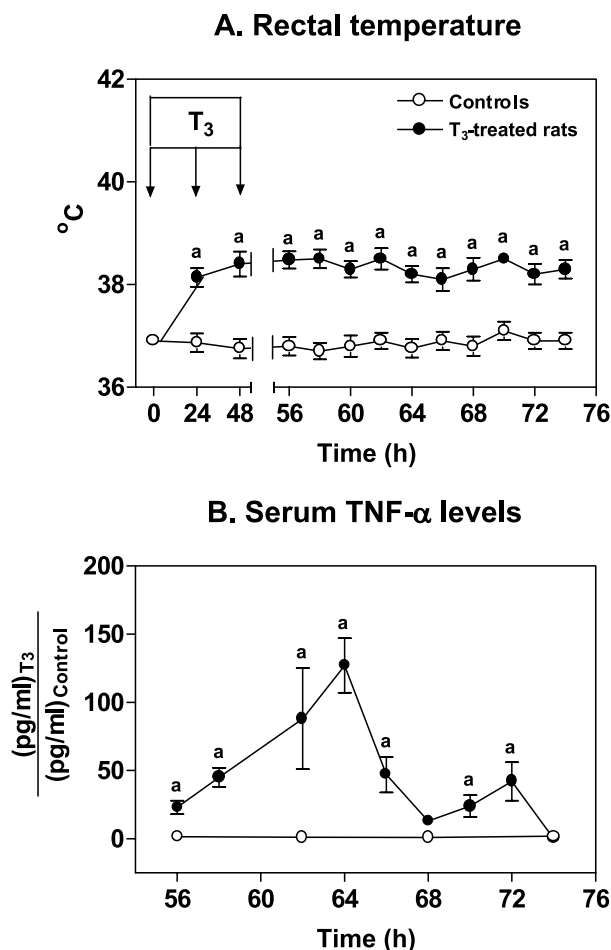
A. Rectal temperature

B. Serum TNF-α levels

Figure 1 Time course study of the effects of T3 administration on the rectal temperature of the animals (A) and the serum levels of tumor necrosis factor-α (TNF-α) (B). Fed rats received daily i.p. injections of 0·1 mg T3/kg body weight for three consecutive days or hormone vehicle (arrows) and studies were carried out at the indicated times. Data are means ± S.E.M. for three to fifteen different animals per group. Significance studies (one-way ANOVA and the Newman–Keuls' test): [a]$P<0.05$ compared with control values. In (B), values in control rats at time zero were arbitrarily set to unity, and values in control animals and T3-treated rats at other time points were normalized to this.

enzyme (Fig. 3B, upper panel), both at 68–72 h after hormone administration. Also, hepatic Bcl-2 protein expression is enhanced after T3 treatment, a response that is 2·7-fold higher than control values at 68–70 h (Fig. 4, upper panel). T3-induced increases in both MnSOD protein expression (Fig. 3A, lower panel) and activity (Fig. 3B, lower panel) or in Bcl-2 protein expression (Fig. 4, lower panel) at 70 h after hormone administration are abolished by pretreatment with α-tocopherol. At this experimental time, rats subjected to α-tocopherol pretreatment exhibit higher levels of α-tocopherol in serum over control values, irrespective of T3 administration ((a)

controls, $12\cdot1 \pm 0\cdot6$ ($n=5$) μM; (b) T3-treated rats, $12\cdot9 \pm 1\cdot9$ ($n=5$); (c) α-tocopherol, $19\cdot9 \pm 0\cdot6$ ($n=7$) ($P<0\cdot05$ vs a and b); (d) α-tocopherol and T3, $19\cdot3 \pm 1\cdot2$ ($n=4$) ($P<0\cdot05$ vs a and b)). Abrogation of T3-induced serum TNF-α levels, liver IκB-α phosphorylation, and hepatic NF-κB DNA binding by α-tocopherol administration prior to hormone treatment, have recently been reported by our group (Fernández *et al.* 2005).

Discussion

Mitochondria are primarily concerned with intracellular energy homeostasis, but also play crucial roles in the regulation of apoptotic and necrotic cell death, Ca^{2+} homeostasis, or ROS generation. Acceleration of energy metabolism, as a consequence of thyroid calorigenesis, leads to a higher production of ROS at the mitochondrial level (Fernández & Videla 1993*a*, Venditti *et al.* 2003), which may represent potential proapoptotic and/or necrotic factors (Lemasters *et al.* 1999). Under these conditions, aerobic cells have different mechanisms for minimizing or re-establishing the original redox state, including the upregulation of the expression of genes encoding for proteins with antioxidant activity (Dröge 2002). Data presented in this work indicate that thyroid hormone upregulates gene expression in the liver through a redox signaling mechanism resulting in the induction of two nuclear encoded proteins, namely, the mitochondrial matrix enzyme MnSOD (Oberley 2004) and the anti-apoptotic protein Bcl-2, predominantly located in mitochondrial membranes (Tsujimoto 2003). These effects of T3 elicited at 68–72 h after hormone administration (i) occur under thermogenic conditions with increased liver O_2 consumption rates underlying a higher oxidative stress status (Videla 2000) and (ii) involve a TNF-α response triggered by oxidative stress in Kupffer cells, due to its abrogation by α-tocopherol or N-acetylcysteine (Fernández *et al.* 2002, 2005) and GdCl3 pretreatment (Fernández *et al.* 2005). Although T3-induced calorigenic effects are significant at 24 h, in the experimental design employed, the redox upregulation of liver MnSOD and Bcl-2 occurs at 68–72 h after treatment, coinciding with the maximum oxidative stress status reached (Fernández *et al.* 2005). This is evidenced by the higher lipid peroxidation and protein carbonylation responses induced by T3 at 72 h over those found at 24 h (Fernández *et al.* 2005), thus supporting the contention that the redox regulation of gene expression depends on the pro-oxidant level attained (Martindale & Holbrook 2002) within a given time interval (Dröge 2002). T3-induced TNF-α response was paralleled by the activation of the IKK complex, as shown by the significant enhancement in liver IκB-α phosphorylation. This process, that is accomplished after TNF-α/TNF-α receptor 1 coupling requiring the recruitment of several adaptor molecules and the activation

A. Liver IκB-α phosphorylation
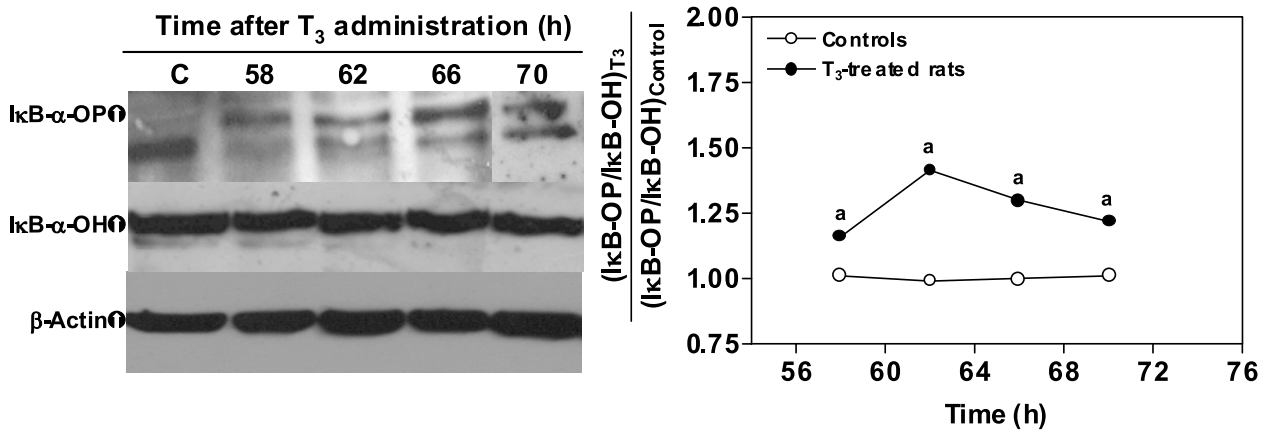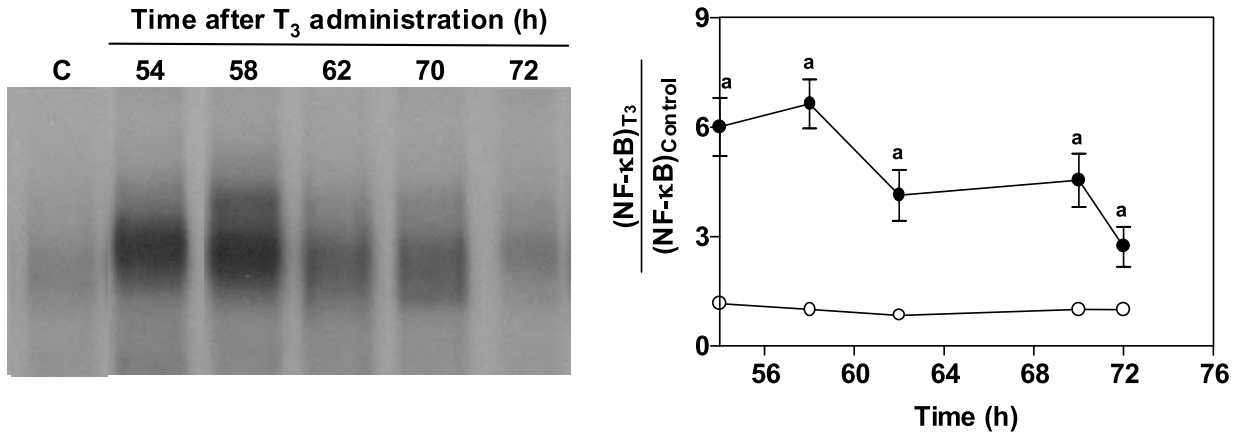

B. Liver NF-κB DNA binding

Figure 2 Time course study of the effects of T3 administration on rat liver inhibitor of κB-α (IκB-α) phosphorylation (A) and nuclear factor-κB (NF-κB) DNA binding (B). Data are means ± S.E.M. for three different animals per experimental group. (A) Representative SDS-PAGE of liver soluble protein fractions from control rats (C) and T3-treated animals at different times after treatment for detection of phosphorylated IκB-α (IκB-α-OP), nonphosphorylated IκB-α (IκB-α-OH), and β-actin [left panel] and the respective densitometric quantification expressed as the ratios of (IκB-α-OP)/(IκB-α-OH) in T3-treated rats over controls, previously normalized for β-actin to compare lane-to-lane equivalency in total protein content [right panel]. (B) Representative autoradiograph of NF-κB DNA binding evaluated by EMSA using nuclear extracts from livers of control rats (C) and T3-treated animals at different times after treatment [left panel] and the respective quantification of relative NF-κB DNA binding [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 experimental times were normalized to this. Significance studies (one-way ANOVA and the Newman–Keuls' test): [a]$P<0.05$ compared with control values.

of NF-κB-inducing kinase (Garg & Aggarwal 2002), may play a role in the enhancement of NF-κB DNA binding found after T3 administration, although the participation of other signaling kinases cannot be discarded.

Enhancement of NF-κB DNA binding upregulates the expression of numerous inducible genes, including those for MnSOD (Jiang *et al.* 2004) and Bcl-2 (Tamatani *et al.* 1999). T3-induced liver NF-κB DNA binding led to increased expression of MnSOD, an effect that involves a higher activity of the enzyme and that is abolished by α-tocopherol pretreatment. Upregulation of MnSOD

expression by T3 represents an effective protective mechanism against T3-induced liver $O_2^{\bullet -}$ generation, which occurs not only in mitochondria (Fernández & Videla 1993*a*, Venditti *et al.* 2003), but also in microsomes (Fernández *et al.* 1985) and the cytosol (Huh *et al.* 1998), as enhancement in MnSOD in mitochondria will increase $O_2^{\bullet -}$ removal from mitochondria and the cytosol. The latter process is due to $O_2^{\bullet -}$ diffusion into the positively charged mitochondrial intermembrane space, followed by $O_2^{\bullet -}$ protonation to form hydroperoxyl radical, which reaches the mitochondrial matrix for dismutation
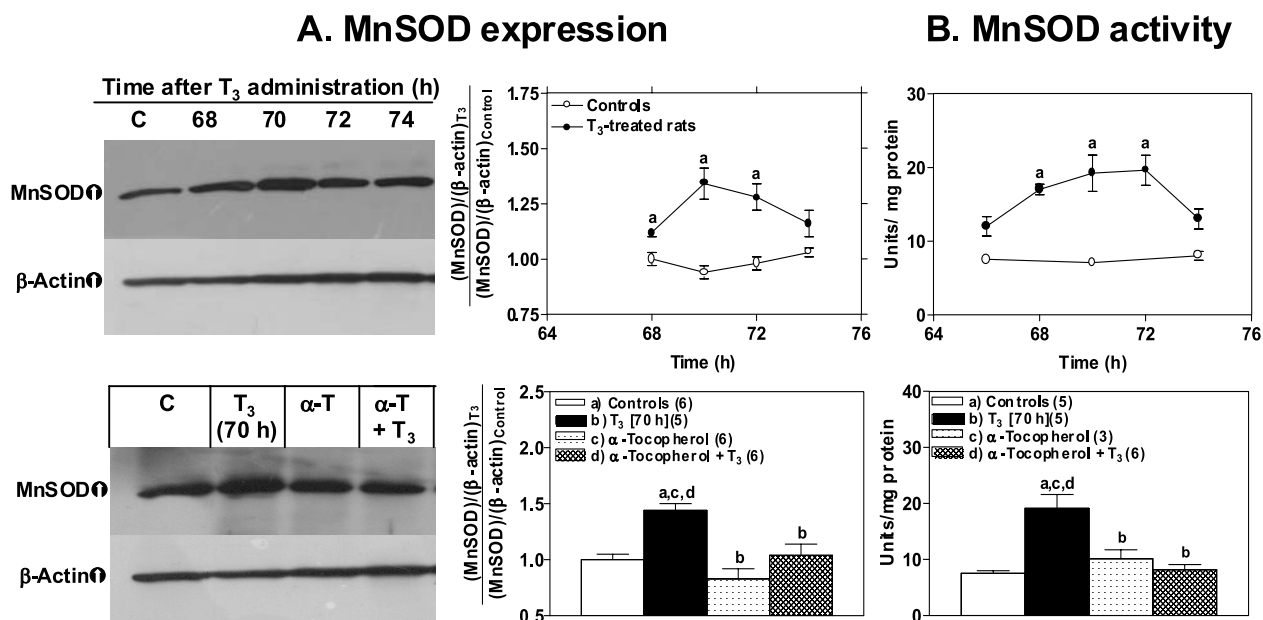
A. MnSOD expression

B. MnSOD activity

Figure 3 Time course study of the effects of T3 administration on liver manganese superoxide dismutase (MnSOD) protein expression (A) and MnSOD activity (B), and the effect of α-tocopherol (α-T) pretreatment at 70 h after hormone treatment. Data are means ± S.E.M. for three to seven different animals per experimental group. (A) Representative SDS-PAGE of liver soluble protein fractions from control rats (C) and T3-treated animals at different times after treatment for detection of MnSOD and β-actin [upper-left panel] and the respective densitometric quantification expressed as the ratios (MnSOD/ β-actin) in T3-treated rats over control animals [upper-right panel]. The lower-left panel shows representative SDS-PAGE for the effect of α-tocopherol treatment prior to T3 on MnSOD protein expression, at 70 h after hormone administration, with the respective densitometric quantification shown in the lower-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 experimental times were normalized to this. Significance studies (one-way ANOVA and the Newman–Keuls′ test): ${}^{a}P<0.05$ compared with control values. (B) Spectrophotometric determination of liver MnSOD activity at different times after T3 administration [upper panel] and the effect of α-tocopherol pretreatment at 70 h after hormone treatment [lower panel]. Significance studies ($P<0.05$) are indicated by the letters identifying each experimental group.

(Guidot *et al.* 1995). It is interesting to note that T3-induced liver MnSOD expression is coordinated with the upregulation of inducible NOS (iNOS) (Fernández *et al.* 2005). Under these conditions, the effective scavenging of $O_2^{•-}$ by MnSOD will minimize its reaction with NO, thus favoring the maintenance of high NO levels that are important to avoid prolonged NF-κB activation by oxidative stress and the consequent excessive pro-inflammatory cytokine expression. This negative feedback signaling mechanism for NF-κB may involve the diminution of its DNA binding capacity by nitrosylation of NF-κB p50 and/or the enhancement in IκB availability by synthesis or stabilization (Laroux *et al.* 2000).

In addition to liver MnSOD expression, T3 administration achieved the redox upregulation of hepatic Bcl-2, in association with the activation of the IKK/NF-κB pathway. Although Bcl-2 function is primarily concerned with the control over apoptosis commitment in disease development, overexpression of Bcl-2 is also related to the regulation of the intracellular redox state (Voehringer & Meyn 2000). This is evidenced by the increase in the total antioxidant capacity, diminishing ROS generation (Tyurina *et al.* 1997, Wiedau-Pazos *et al.* 1998) and the

oxidation of proteins and unsaturated fatty acids (Hockenbery *et al.* 1993, Virgili *et al.* 1998) in Bcl-2 overexpressing cells, which were ascribed to a higher intracellular availability of reduced glutathione (GSH) (Voehringer & Meyn 2000). In agreement with this proposal, previous work by our group showed that elevation of basolateral γ-glutamyl transferase (γ-GT) ecto-activity is related to the recovery of hepatic GSH content after depletion by T3 administration, by supplying the precursors for intracellular GSH synthesis (Carrión *et al.* 1993). Bcl-2 overexpression is correlated with the expression of CD53, a member of the membrane-spanning tetraspanin 4 (TM4) family of surface proteins that facilitate the interaction and activity of membrane-bound proteins, including γ-GT (Voehringer & Meyn 2000). Enhancement in sinusoidal γ-GT by T3 occurs under conditions of enhanced hepatic GSH biosynthetic capacity (Fernández & Videla 1996), with higher rates of the NADPH-generating enzyme glucose-6-phosphate dehydrogenase to support oxidized glutathione (GSSG) reduction into GSH (Giavarotti *et al.* 1998).

Collectively, data presented indicate that T3 administration elicits the redox upregulation of MnSOD and
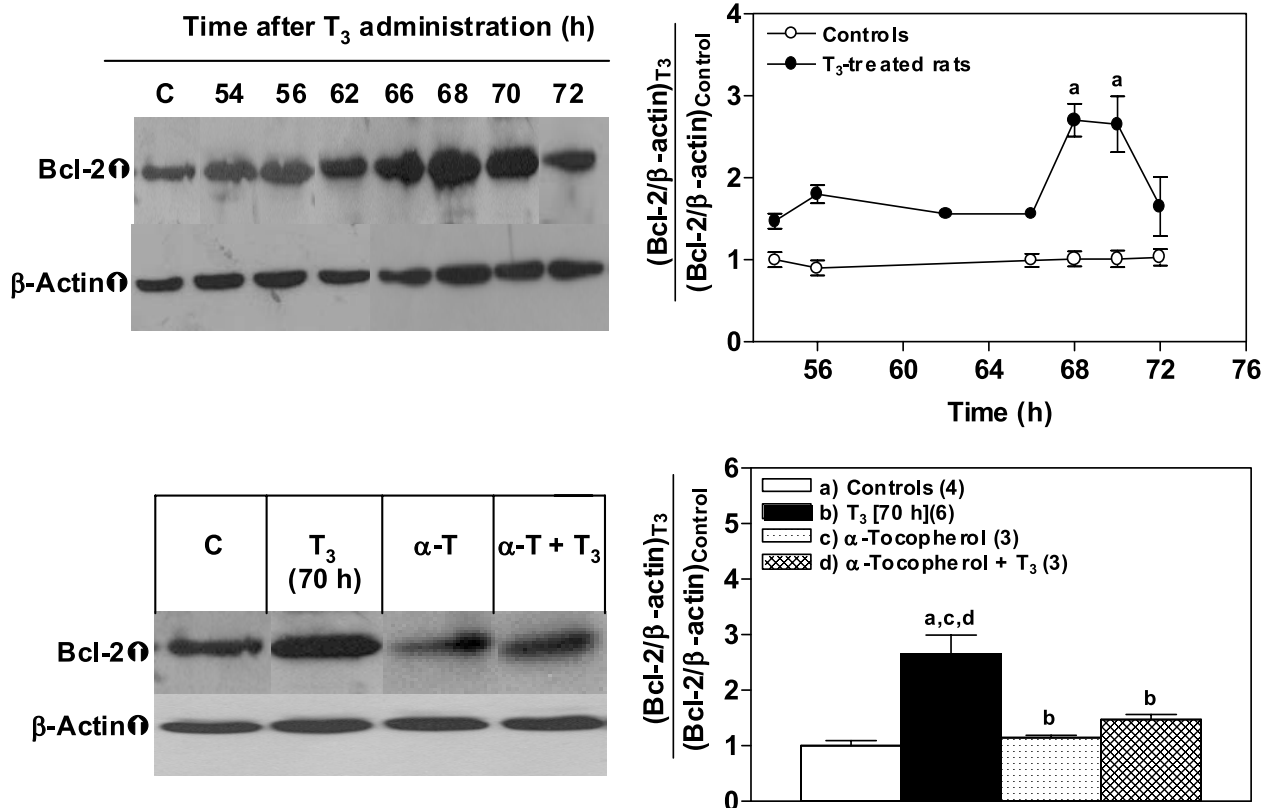
Liver Bcl-2 expression

Figure 4 Time course study of the effects of T3 administration on liver Bcl-2 protein expression and the effect of α-tocopherol (α-T) pretreatment at 70 h after hormone treatment. Data are means ± S.E.M. for three to six different animals per experimental group. Representative SDS-PAGE of liver soluble protein fractions from control rats (C) and T3-treated animals at different times after treatment for detection of Bcl-2 and β-actin [upper-left panel] and the respective densitometric quantification expressed as the ratios (Bcl-2/β-actin) in T3-treated rats over control animals [upper-right panel]. The lower-left panel shows representative SDS-PAGE for the effect of α-tocopherol treatment prior to T3 on Bcl-2 after hormone administration, with the respective densitometric quantification shown in the lower-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 experimental times were normalized to this. Significance studies (one-way ANOVA and the Newman–Keuls' test): [a]$P < 0.05$ compared with control values [upper-right panel] or $P < 0.05$ as shown by the letters identifying each experimental group [lower-right panel].

Bcl–2 in rat liver, in association with the Kupffer cell–dependent release of TNF-α and activation of the IKK/ NF-κB cascade. These adaptive responses, and that of hepatic iNOS previously reported (Fernández *et al.* 2005), represent protective mechanisms against ROS toxicity that underlies the oxidative stress status induced in the liver by thyroid calorigenesis, and that might involve cell death signaling.

Acknowledgements

References

Andican G, Gelisgen R, Civelek S, Seven A, Seymen O, Altug T, Yigit G & Burcak G 2004 Oxidative damage to nuclear DNA in hyperthyroid rat liver: inability of vitamin C to prevent the damage. *Journal of Toxicology and Environmental Health* **67** 413–420.

Ashkenazi A & Dixit VM 1998 Death receptors: signaling and modulation. *Science* **281** 1305–1308.

Camandola S, Aragno M, Cutrín JC, Tamagno E, Danni O, Chiarpotto E, Parola M, Loenarduzzi G, Biasi F & Poli G 1999 Liver AP-1 activation due to carbon tetrachloride is potentited by 1,2-dibromoethane but is inhibited by α-tocopherol or gadolinium chloride. *Free Radical Biology and Medicine* **26** 1108–1116.

Carrión Y, Fernández V & Videla LA 1993 Influence of thyroid hormone administration on hepatic glutathione content and basolateral γ-glutamyltransferase ectoactivity in the isolated perfused rat liver. *Biochemical Pharmacology* **45** 2527–2535.

Deryckere F & Gannon F 1994 A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. *Biotechniques* **16** 405.

Dröge W 2002 Free radicals in the physiological control of cell function. *Physiological Reviews* **82** 47–95.

Fernández V, Barrientos X, Kipreos K, Valenzuela A & Videla LA 1985 Superoxide radical generation, NADPH oxidase activity, and cytochrome P-450 content of rat liver microsomal fractions in an experimental hyperthyroid state: relation to lipid peroxidation. *Endocrinology* **117** 496–501.

Fernández V & Videla LA 1993a Influence of hyperthyroidism on superoxide radical and hydrogen peroxide production by rat liver submitochondrial particles. *Free Radical Research Communications* **18** 329–335.

Fernández V & Videla LA 1993b 3,3',5-Triiodothyronine-induced hepatic respiration: effects of desferrioxamine and allopurinol in the isolated perfused rat liver. *Toxicology Letters* **69** 205–210.

Fernández V & Videla LA 1996 Hepatic glutathione biosynthetic capacity in hyperthyroid rats. *Toxicology Letters* **89** 85–89.

Fernández V, Llesuy S, Solari L, Kipreos K, Videla LA & Boveris A 1988 Chemiluminescent and respiratory responses related to thyroid hormone-induced liver oxidative stress. *Free Radical Research Communications* **5** 77–84.

Fernández V, Cornejo P, Tapia G & Videla LA 1997 Influence of hyperthyroidism on the activity of nitric oxide synthase in the rat. *Nitric Oxide: Biology and Chemistry* **1** 463–468.

Fernández V, Videla LA, Tapia G & Israel Y 2002 Increases in tumor necrosis factor-α in response to thyroid hormone-induced liver oxidative stress in the rat. *Free Radical Research* **36** 719–725.

Fernández V, Tapia G, Varela P & Videla LA 2005 Redox regulation of thyroid hormone-induced Kupffer cell-dependent IκB-α phosphorylation in relation to inducible nitric oxide synthase expression. *Free Radical Research* **39** 411–418.

Flohé L, Brigelius-Flohé R, Saliou C, Traber MG & Packer L 1997 Redox regulation of NF-kappa B activation. *Free Radical Biology and Medicine* **22** 1115–1126.

Garg AK & Aggarwal BB 2002 Reactive oxygen intermediates in TNF signaling. *Molecular Immunology* **39** 509–517.

Giavarotti KAS, Rodrigues L, Rodrigues T, Junqueira VBC & Videla LA 1998 Liver microsomal parameters related to oxidative stress and antioxidants systems in hyperthyroid rats subjected to acute lindane treatment. *Free Radical Research* **29** 35–42.

Guidot DM, Repine JE, Kitlowski AD, Flores SC, Nelson SK, Wright RM & McCord JM 1995 Mitochondrial respiration scavenges extramitochondrial superoxide anion via a nonenzymatic mechanism. *Journal of Clinical Investigation* **96** 1131–1136.

Hockenbery DM, Oltavi ZN, Yin XM, Milliman CL & Korsmeyer SJ 1993 Bcl-2 functions in an antioxidant pathway to prevent programmed cell death. *Cell* **75** 241–251.

Huh K, Kwon TH, Kim JS & Park JM 1998 Role of the hepatic xanthine oxidase in thyroid dysfunction: effect of thyroid hormones in oxidative stress in rat liver. *Archives of Pharmacological Research* **21** 236–240.

Jiang B, Xu S, Hou X, Pimentel DR, Brecher P & Cohen RA 2004 Temporal control of NF-κB activation by ERK differentially regulates interleukin-1β-induced gene expression. *Journal of Biological Chemistry* **279** 1323–1329.

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–685.

Lambeth JD, Cheng G, Arnold RS & Edens WA 2000 Novel homologs of gp91phox. *Trends in Biochemical Sciences* **25** 459–461.

Landriscina C, Petragallo V, Morini P & Marcotrigiano GO 1988 Lipid peroxidation in rat liver microsomes. I. Stimulation of the NADPH-cytochrome P450 reductase-dependent process in hyperthyroid state. *Biochemistry International* **17** 385–393.

Laroux FS, Lefer DJ, Kawachi S, Scalia R, Cockrell AS, Gray L, van der Heyde H, Hoffman JM & Grisham MB 2000 Role of nitric oxide in the regulation of acute and chronic inflammation. *Antioxidants and Redox Signaling* **2** 391–396.

Lemasters JJ, Qian T, Bradham CA, Brenner DA, Cascio WE, Trost LC, Nishimura Y, Nieminen AL & Herman B 1999 Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. *Journal of Bioenergetics and Biomembranes* **31** 305–319.

Leonarduzzi G, Robbesyn F & Poli G 2004 Signaling kinases modulated by 4 hydroxynonenal. *Free Radical Biology and Medicine* **37** 1694–1702.

Lowry OH, Rosebrough N, Farr A & Randall R 1951 Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275.

Martin JP Jr, Dailey M & Sugarman E 1987 Negative and positive assays of superoxide dismutase based on hematoxylin autoxidation. *Archives of Biochemistry and Biophysics* **255** 329–336.

Martindale JL & Holbrook NJ 2002 Cellular response to oxidative stress: signaling for suicide and survival. *Journal of Cellular Physiology* **192** 1–15.

Oberley TD 2004 Mitochondria, manganese superoxide dismutase, and cancer. *Antioxidants and Redox Signaling* **6** 483–487.

Oppenheimer JH, Schwartz HL & Strait KA 1996 The molecular basis of thyroid hormone action. In *Werner and Ingbar's The Thyroid: a Fundamental and Clinical Text*, edn 7, pp 162–184. Eds LE Braverman & RD Utiger. New York: Lippincott-Raven.

Poli G, Leonarduzzi G, Biasi F & Chiarpotto E 2004 Oxidative stress and cell signalling. *Current Medicinal Chemistry* **11** 1163–1182.

Ramadori G & Christ B 1999 Cytokines and the hepatic acute-phase response. *Seminars in Liver Disease* **19** 141–155.

Shearer MJ 1986 Vitamins. In *HPLC of Small Molecules. A Practical Approach*, pp 157–219. Ed CK Lim. Oxford: IRL Press.

Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizumo T & Tohyama M 1999 Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFκB activation in primary hippocampal neurons. *Journal of Biological Chemistry* **274** 8531–8538.

Tapia G, Pepper I, Smok G & Videla LA 1997 Kupffer cell function in thyroid hormone-induced liver oxidative stress in the rat. *Free Radical Research* **26** 267–279.

Tapia G, Cornejo P, Fernández V & Videla LA 1999 Protein oxidation in thyroid hormone-induced liver oxidative stress: relation to lipid peroxidation. *Toxicology Letters* **106** 209–214.

Tapia G, Fernández V, Varela P, Cornejo P, Guerrero J & Videla LA 2003 Thyroid hormone-induced oxidative stress triggers nuclear factor-κB and cytokine gene expression in rat liver. *Free Radical Biology and Medicine* **35** 257–265.

Thannickal VJ & Fanburg BL 2000 Reactive oxygen species in cell signaling. *American Journal of Physiology − Lung Cellular and Molecular Physiology* **279** L1005–L1028.

Towbin H, Staehelin T & Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *PNAS* **76** 4350–4354.

Tsujimoto Y 2003 Cell death regulation by the Bcl-2 protein family in the mitochondria. *Journal of Cell Physiology* **195** 158–167.

Tsukamoto H & Lin M 1997 The role of Kupffer cells in liver injury. In *Cells of the Hepatic Sinusoid*, vol 6, pp 244–250. Eds E Wisse, DL Knook & C Balabaud. Leiden, The Netherlands: The Kupffer Cell Foundation.

Tyurina YY, Tyurin VA, Carta G, Quinn PJ, Schor NF & Kagan VE 1997 Direct evidence for antioxidant effect of Bcl-2 in PC12 rat pheochromocytoma cells. *Archives of Biochemistry and Biophysics* **344** 413–423.

Venditti P, Balestrieri M, Di Meo S & De Leo T 1997 Effect of thyroid state on lipid peroxidation, antioxidant defenses, and susceptibility to oxidative stress in rat tissues. *Journal of Endocrinology* **155** 151–157.

Venditti P, De Rosa R & Di Meo S 2003 Effect of thyroid state on H_2O_2 production by rat liver mitochondria. *Molecular and Cellular Endocrinology* **205** 185–192.

Venditti P, De Rosa R & Di Meo S 2004 Effect of cold–induced hyperthyroidism on H_2O_2 production and susceptibility to stress conditions of rat liver mitochondria. *Free Radical Biology and Medicine* **36** 348–358.

Videla LA 2000 Energy metabolism, thyroid calorigenesis, and oxidative stress: functional and cytotoxic consequences. *Redox Report* **5** 265–275.

Videla LA, Tapia G, Varela P, Cornejo P, Guerrero J, Israel Y & Fernández V 2004 Effects of acute γ–hexachlorocyclohexane intoxication in relation to the redox regulation of nuclear factor–κB, cytokine gene expression, and liver injury in the rat. *Antioxidants and Redox Signaling* **6** 471–480.

Virgili F, Santini MP, Canali R, Polakowska RR, Heads A & Perozzi G 1998 Bcl-2 overexpression in the HaCat cell line is associated with a different fatty acid composition and sensitivity to oxidative stress. *Free Radical Biology and Medicine* **24** 93–101.

Voehringer DW & Meyn RE 2000 Redox aspects of Bcl-2 function. *Antioxidants and Redox Signaling* **2** 537–550.

Wang J-F, Komarov P & de Groot H 1993 Luminol chemiluminescence in rat macrophages and granulocytes: the role of NO, $O_2{}^-$/H_2O_2, and HOCl. *Archives of Biochemistry and Biophysics* **304** 189–196.

Wiedau–Pazos M, Trudell JR, Altenbach C, Kane A, Hubbell WT & Bredesen DE 1998 Expression of bcl-2 inhibits cellular radical generation. *Free Radical Research* **24** 205–212.