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Increases in Tumor Necrosis Factor-α in Response to Thyroid Hormone-induced Liver Oxidative Stress in the Rat

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Thyroid hormone-induced calorigenesis contributes to liver oxidative stress and promotes an increased respiratory burst activity in Kupffer cells, which could conceivably increase the expression of redox-sensitive genes, including those coding for cytokines. Our aim was to test the hypothesis that L-3,3′,5-triiodothyronine (T_3)-induced liver oxidative stress would markedly increase the production of TNF-α by Kupffer cells and its release into the circulation. Sprague–Dawley rats received a single dose of 0.1 mg T_3/kg or vehicle (controls) and determinations of liver O_2 consumption, serum TNF-α, rectal temperature, and serum T_3 levels, were carried out at different times after treatment. Hepatic content of total reduced glutathione (GSH) and biliary glutathione disulfide (GSSG) efflux were measured as indices of oxidative stress. In some studies, prior to T_3 injection animals were administered either (i) the Kupffer cell inactivator gadolinium chloride ($GdCl_3$), (ii) the antioxidants α-tocopherol and *N*-acetyl-L-cysteine (NAC), or (iii) an antisense oligonucleotide against TNF-α (ASO TJU-2755). T_3 elicited an 80-fold increase in the serum levels of TNF-α at 22 h after treatment, which coincided with the onset of thyroid calorigenesis. Pretreatment with $GdCl_3$, α-tocopherol, NAC, and ASO TJU-2755 virtually abolished this effect and markedly reduced T_3-induced liver GSH depletion and the increases in biliary GSSG efflux. It is concluded that the hyperthyroid state in the rat increases the circulating levels of TNF-α by actions exerted at the Kupffer cell level and these are related to the oxidative stress status established in the liver by thyroid calorigenesis.

Keywords: Thyroid hormone; Oxidative stress; Kupffer cell; TNF-α; Rat liver

INTRODUCTION

The aerobic metabolism is characterized by a steady-state production of prooxidant oxygen (ROS) and nitrogen (RNS) reactive species balanced by a similar rate of their removal by antioxidants. The continuous regeneration of antioxidant capacity is required to maintain homeostasis, otherwise an imbalance in the prooxidant/antioxidant equilibrium is established in favor of prooxidants, a condition referred to as oxidative stress that may lead to a number of pathophysiological events.[1,2] The relationship between thyroid calorigenesis and oxidative stress[3] has been studied in view of the direct correlation between the basal metabolic rate and the lipid peroxidative potential of tissues in various mammalian species.[4] In the liver, oxidative stress coupled to thyroid calorigenesis results from an accelerated energy metabolism involving an increased generation of ROS and RNS,[3,5] with the consequent depression of antioxidant stores[3,6] and increases in lipid peroxidation and protein oxidation.[3] Kupffer cell function is significantly augmented in experimental hyperthyroidism, as evidenced by the enhancement in phagocytosis and an increased respiratory burst activity as assessed in liver perfusion studies.[7] These alterations correlate with the hyperplasia and hypertrophy of Kupffer cells observed histologically and may contribute to

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the enhanced oxidative stress developed.[7] Hyperthyroidism in man is also associated with a prooxidant condition as shown by an increase in the levels of circulating and urinary thiobarbituric acid reactants and spontaneous chemiluminescence in urine, over values in euthyroid subjects.[3] These changes, and the significant reduction in plasma concentration of the antioxidants α-tocopherol, ascorbic acid, coenzyme-Q_{10}, and thiols, are either normalized or reduced following thyrostatic therapies in hyperthyroid patients.[3]

In addition to the direct actions leading to the oxidative damage of essential biomolecules, oxidative stress may alter the expression of redox-sensitive genes involved in adaptive physiological responses or may contribute to the genesis of some diseases[8] through ROS-mediated activation of redox-sensitive transcription factors such as NF-κB[9] and activator protein-1 (AP-1).[10] These signal transduction pathways seem to be operative in macrophages, as evidenced by the strong activation of NF-κB elicited by hydrogen peroxide generated in the respiratory burst of rat alveolar macrophages and the mouse macrophages J774A.1 cell line, which is largely due to NADPH oxidase activity.[11] In Kupffer cells, NF-κB and AP-1 are known to have a major transcriptional control over the expression of a number of key effector molecules,[12] including cytokines such as tumor necrosis factor-α (TNF-α), a pleiotropic regulatory peptide able to trigger death signals and loss of hepatocyte viability.[2,13]

In the present study, our aim was to test the hypothesis that thyroid hormone-induced oxidative stress in the liver augments TNF-α production by Kupffer cells. For this purpose, serum TNF-α levels were determined in control rats and in animals administered L-3,3′,5,-triiodothyronine (T_3) under several conditions, including pretreatment with the Kupffer cell inactivator gadolinium chloride ($GdCl_3$),[14] the antioxidants α-tocopherol and *N*-acetyl-L-cysteine (NAC), and the administration of an antisense oligonucleotide targeting the primary RNA transcript of TNF-α.[15] The levels of TNF-α were correlated with changes in parameters related to thyroid calorigenesis and liver oxidative stress.

MATERIALS AND METHODS

Materials

The antisense oligonucleotide TJU-2755[15] was synthesized by Hybridon (Cambridge, MA) and consists of a 21-mer phosphorothioate-modified oligonucleotide with the sequence (5′TGATCCA CTCCCCCCTCCACT-3′) complementary to the 3′-untranslated region of rat TNF-α mRNA. All other reagents and chemicals used were of reagent grade, purchased either from Sigma Chemical (St. Louis, MO) or from Merck (Chile).

Animals and Treatments

Female Sprague–Dawley rats (Instituto de Salud Pública, Santiago, Chile) weighing 200–300 g were housed on a 12 h light/dark cycle and were provided rat chow (Champion S.A., Santiago, Chile) and water *ad libitum*. Animals received a single ip injection of 0.1 mg of T_3/kg body weight or equivalent volumes of vehicle (0.1N NaOH)(controls) and studies were carried out at the indicated times after treatment. Separate groups of rats were subjected to the following pretreatments prior to hormone administration: (i) 10 mg of $GdCl_3$/kg iv 24 h before a single dose of T_3, (ii) 100 mg of α-tocopherol/kg ip 17 h prior to T_3, (iii) 1 g of NAC/kg ip 30 min before T_3, or (iv) daily doses of 10 mg/kg of TJU-2755 iv for two consecutive days, 24 h prior T_3, and studies were carried out 22 h after hormone administration. At the indicated experimental times, the rectal temperature of the animals was measured with a thermocouple (Cole-Parmer Instrument, Chicago, IL), and blood samples were taken from the tail vein for the determination of serum T_3 levels by the GammaCoat™ (I^{125}) T_3 Radioimmunoassay (Baxter Healthcare, Cambridge, MA). All animals used were cared 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).

Enzyme-linked Immunoabsorbent Assay (ELISA) for TNF-α

Animals were anesthetized with sodium pentobarbital (50 mg/kg, ip) and serum from blood obtained by cardiac puncture was separated and stored at −80°C. ELISA was conducted by using UltraSensitive Cytoscreen™ KRC3013 kit (Biosource International, Camarillo, CA) according to manufacturer's specifications. For comparison purposes, a separate group of animals was given 50 μg of lipopolysaccharide (LPS)/kg iv (from *Escherichia coli* serotype 055:B5) and blood samples were taken 90 min after treatment. Serum samples containing high TNF-α levels were repeated after dilution to assure assay results within the standard curve.

Liver O_2 Consumption and Parameters Related to Oxidative Stress

The rate of O_2 consumption by the liver was determined polarographically in perfusion studies, by means of a Clarke-type oxygen electrode placed in the perfusion line carrying the effluent perfusate from the vena cava, as previously reported.[6,7] At the

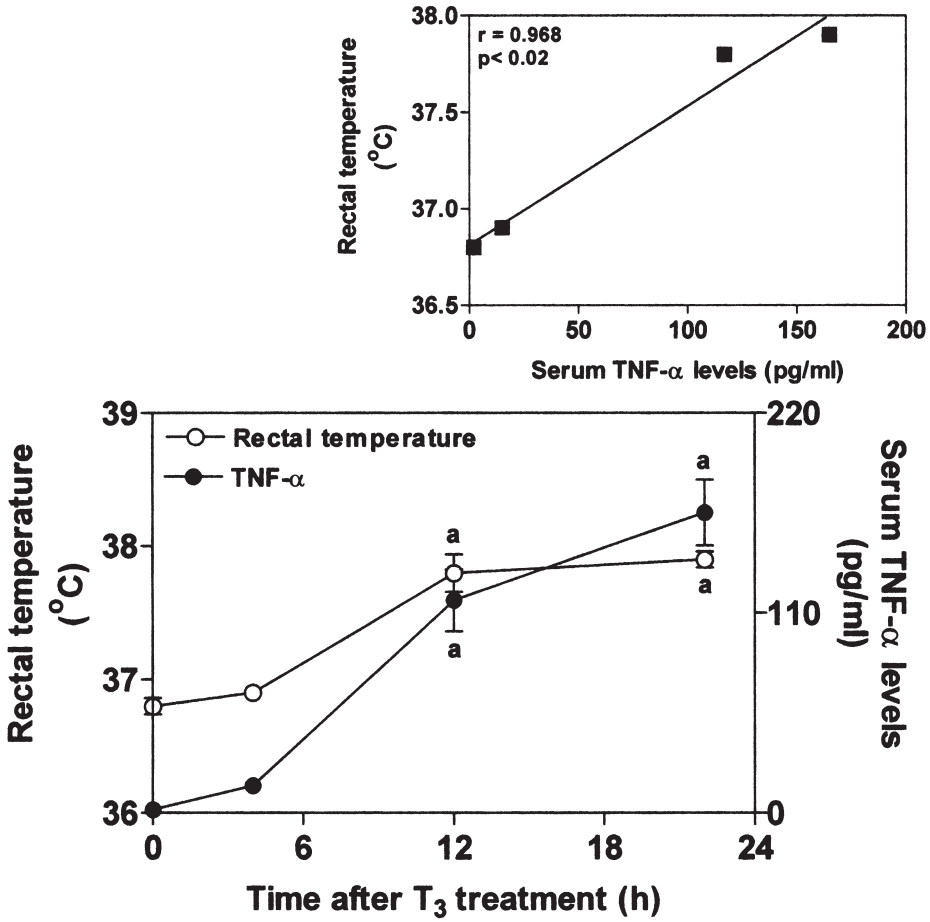

FIGURE 1 Time course study of the effect of L-3,3′,5-triiodothyronine (T_3) administration on the rectal temperature of the animals and the serum of TNF-α in the rat. Animals were given a single dose of 0.1 mg T_3/kg ip or equivalent volumes of vehicle (0.1N NaOH) (controls at time zero), and studies were carried out at the indicated times. Values shown correspond to means ± SEM for 3–12 animals per experimental time. [a]Significantly different ($p < 0.05$) compared to both control values shown at time zero and T_3-treated rats at 4 h after treatment, assessed by one-way ANOVA and the Newman–Keuls' test. Inset: correlation between rectal temperature of the animals and the respective serum levels of TNF-α in controls rats and animals given T_3 at different times after treatment.

end of perfusion, liver samples were taken to determine the hepatic content of total reduced glutathione (GSH) equivalents by the enzymatic assay of Tietze.[16] In separate groups of animals, the biliary release of glutathione disulfide (GSSG) was evaluated as an additional indicator of oxidative stress.[17] For this purpose, the peritoneal cavity of anesthetized rats (50 mg of sodium pentobarbital/kg ip) was opened, the bile duct was cannulated, and bile was collected for the estimation of the bile flow and the spectrophotometric determination of GSSG.[17]

Statistics

Values shown correspond to the means ± SEM for the number of separate experiments indicated. One-way ANOVA and the Newman–Keuls' test assessed the statistical significance of differences between mean values.

RESULTS

Time Course of the Effect of T_3 Administration on Serum T_3 Levels, Rectal Temperature of the Animals, Liver O_2 Consumption, and Serum TNF-α Levels

Administration of T_3 to fed rats elicited a calorigenic response evidenced by the significant enhancement ($p < 0.05$) in the rectal temperature of the animals, which reached maximal values at 12 and 22 h after hormone treatment (Fig. 1), comparable to those observed at 24 h.[5,7] At 22 h after T_3 treatment, thyroid calorigenesis coincides with significant increases both in the basal rate of O_2 consumption of the liver [controls, 2.00 ± 0.05 ($n = 6$) μmol/g liver/min; T_3-treated rats, 2.39 ± 0.04 ($n = 7$); $p < 0.05$] and in serum T_3 levels [controls, 62 ± 4 ($n = 5$) ng/dl; T_3-treated rats, *340 ± 51* ($n = 10$); $p < 0.05$]. Under these conditions, serum TNF-α levels are greatly enhanced by T_3 treatment, with an 80-fold increase being observed at 22 h after hormone

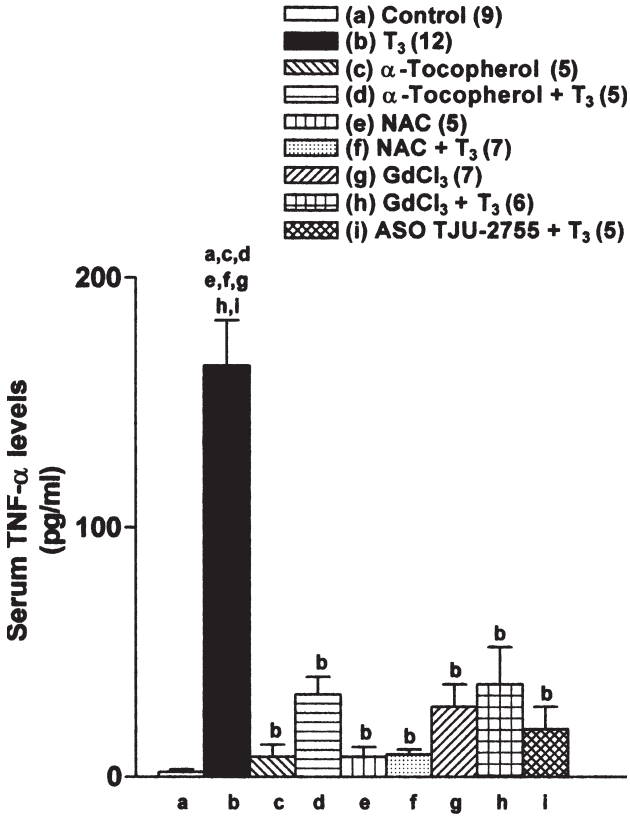

FIGURE 2 Effect of pretreatment with α-tocopherol, *N*-acetylcysteine (NAC), gadolinium chloride ($GdCl_3$), and antisense oligonucleotide TJU-2755 on T_3-induced increase in serum TNF-α levels at 22 h after hormone administration in the rat. Values shown correspond to means ± SEM for the number of rats indicated in parentheses. The significance of the differences between mean values ($p < 0.05$), assessed by one-way ANOVA and the Newman–Keuls' test, is shown by the letters identifying each experimental group.

administration (Fig. 1), in comparison with a 155-fold enhancement achieved by LPS [controls, 2 ± 1 ($n = 9$) pg/ml; LPS, 311 ± 58 ($n = 8$); $p < 0.05$], a well known inducer of TNF-α production.[18] Values of serum TNF-α levels obtained under the various conditions used show a significant direct correlation with the respective rectal temperature of the animals (Fig. 1, inset).

Effect of Pretreatment with α-tocopherol, *N*-acetylcysteine (NAC), Gadolinium Chloride ($GdCl_3$), or Antisense Oligonucleotide (ASO) TJU-2755 on T_3-induced Increase in Serum TNF-α Levels. Changes in Parameters Related to Thyroid Calorigenesis and Liver Oxidative Stress 22 h after Hormone Administration

Euthyroid animals subjected to α-tocopherol, NAC, or $GdCl_3$ had serum TNF-α levels comparable to control values (Fig. 2). T_3-induced increases in serum TNF-α levels at 22 h after hormone treatment [T_3 − average control = 163 ± 19 pg/ml ($n = 12$)] are significantly reduced by 85% ($p < 0.05$) by

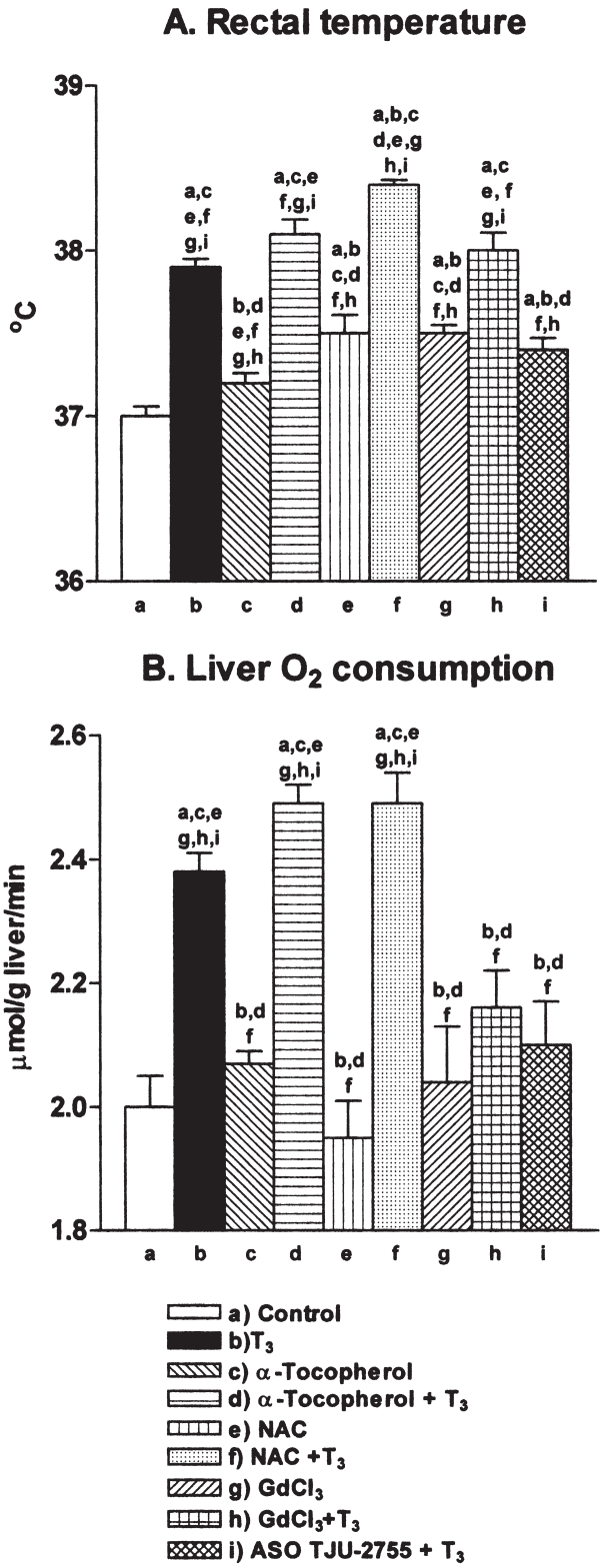

FIGURE 3 Effect of pretreatment with α-tocopherol, *N*-acetylcysteine (NAC), gadolinium chloride ($GdCl_3$), and antisense oligonucleotide TJU-2755 on T_3-induced changes in (A) the rectal temperature of the animals and (B) the basal rate of O_2 consumption of the liver at 22 h after hormone administration in the rat. Values shown correspond to means ± SEM for 4–19 animals per experimental group. The significance of the differences between mean values ($p < 0.05$), assessed by one-way ANOVA and the Newman–Keuls' test, is shown by the letters identifying each experimental group.

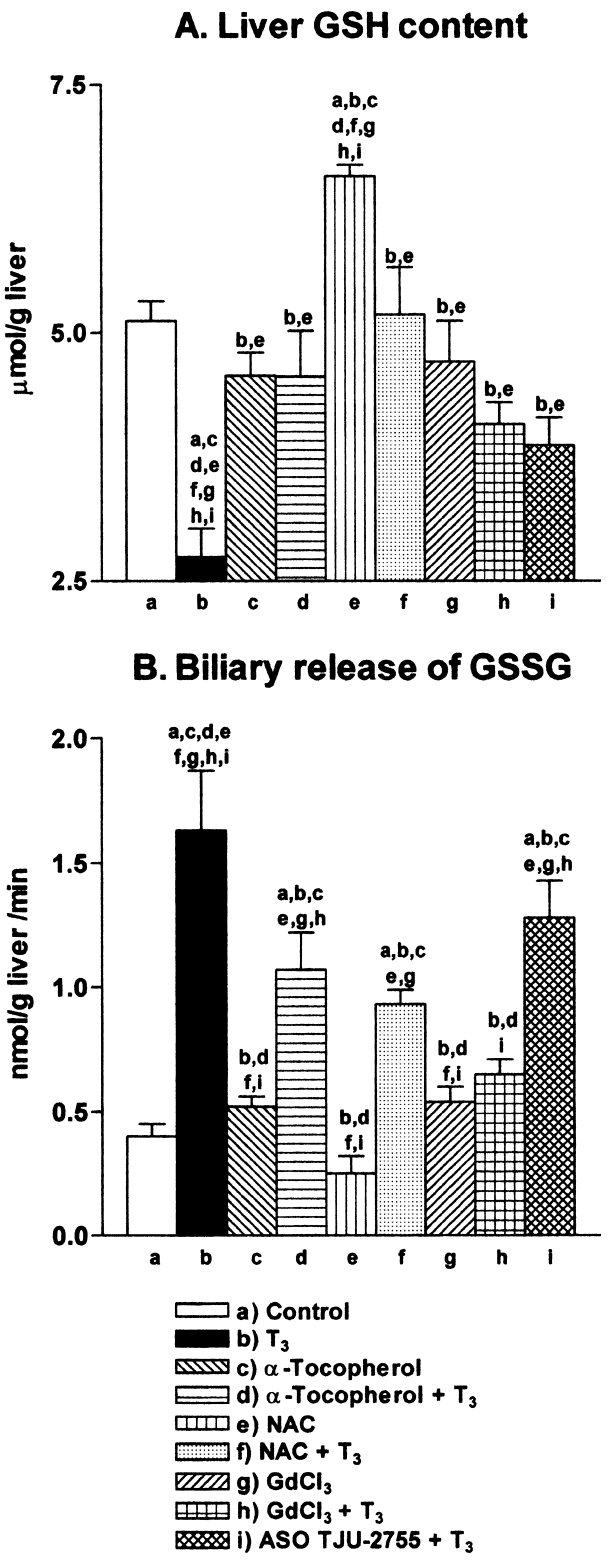

FIGURE 4 Effect of pretreatment with α-tocopherol, *N*-acetylcysteine (NAC), gadolinium chloride ($GdCl_3$), and antisense oligonucleotide TJU-2755 on T_3-induced changes in (A) the content of reduced glutathione (GSH) of the liver and (B) the biliary release of oxidized glutathione (GSSG) at 22 h after hormone administration in the rat. Values shown correspond to means ± SEM for 3–7 animals per experimental group. The significance of the differences between mean values ($p < 0.05$), assessed by one-way ANOVA and the Newman–Keuls' test, is shown by the letters identifying each experimental group.

pretreatment with either α-tocopherol [(α-tocopherol/T_3) − (average α-tocopherol) = 25.2 ± 7.0 ($n = 5$)], by 99% ($p < 0.05$) by NAC [(NAC/T_3) − (average NAC) = 1.3 ± 2.3 ($n = 7$)], and by 94% ($p < 0.05$) by $GdCl_3$ [($GdCl_3/T_3$) − (average $GdCl_3$) = 9.5 ± 14.6 ($n = 6$)] (Fig. 2). Similarly, pretreatment with the antisense oligonucleotide TJU-2755 decreased by 90% ($p < 0.05$) the elevation of serum TNF-α levels by T_3 [(ASO TJU-2755/T_3) − (average control) = 16.6 ± 8.9 pg/ml ($n = 5$)] (Fig. 2).

Twenty two hours after thyroid hormone administration, T_3-induced increase in the rectal temperature of the animals [T_3 − average control = 0.92 ± 0.05°C ($n = 19$)] which was not modified by pretreatment with either α-tocopherol [(α-tocopherol/T_3) − (average α-tocopherol) = 0.89 ± 0.09 ($n = 10$)] or NAC [(NAC/T_3) − (average NAC) = 0.86 ± 0.03 ($n = 14$)], was significantly diminished by 46% ($p < 0.05$) by pretreatment with $GdCl_3$ [($GdCl_3/T_3$) − (average $GdCl_3$) = 0.50 ± 0.11 ($n = 11$)] and reduced by 50% ($p < 0.05$) by the antisense oligonucleotide TJU-2755 [(ASO TJU-2755/T_3) − (average control) = 0.46 ± 0.07 ($n = 8$)] (Fig. 3A). Showing a similar pattern, the T_3-induced enhancement in the rate of O_2 consumption by the liver [(T_3 − average control) = 0.38 ± 0.05 μmol/liver/min ($n = 7$)] was not altered in animals pretreated with α-tocopherol [(α-tocopherol/T_3) − (average α-tocopherol) = 0.42 ± 0.03 ($n = 5$)] or NAC [(NAC/T_3) − (average NAC) = 0.53 ± 0.05 ($n = 4$)], whereas it was significantly reduced by 68% ($p < 0.05$) and 76% ($p < 0.05$) in rats pretreated with either $GdCl_3$ [($GdCl_3/T_3$) − (average $GdCl_3$) = 0.12 ± 0.06 ($n = 5$)] or antisense oligonucleotide TJU-2755 [(ASO TJU-2755/T_3) − (average control) = 0.09 ± 0.07 ($n = 4$)], respectively (Fig. 3B).

Administration of T_3 elicited a significant 46% diminution in the content of hepatic GSH [T_3 − average control = −2.38 ± 0.29 μmol/g liver ($n = 4$)] (Fig. 4A) and a 307% enhancement in the biliary release of GSSG [T_3 − average control = 1.23 ± 0.24 nmol/g liver/min ($n = 5$)] (Fig. 4B) at 22 h after hormone treatment, indicating thyroid hormone-induced oxidative stress in the liver.[3,6] Liver GSH depletion by T_3 was significantly diminished by 99% ($p < 0.05$) and 41% ($p < 0.05$) by pretreatment with α-tocopherol [(α-tocopherol/T_3) − (average α-tocopherol) = 0.002 ± 0.46 μmol/g liver ($n = 5$)] or NAC [(NAC/T_3) − (average NAC) = −1.49 ± 0.20 ($n = 5$)] (Fig. 4A). Pretreatment with $GdCl_3$ inhibited the T_3-induced depletion of GSH by 74% ($p < 0.05$) [($GdCl_3/T_3$) − (average $GdCl_3$) = −0.63 ± 0.22 ($n = 7$)], while pretreatment with antisense oligonucleotide TJU-2755 inhibited the GSH depletion by 48% ($p < 0.05$) [(ASO TJU-2755/T_3) − (average control) = −1.24 ± 0.17 ($n = 4$)] (Fig. 4A). In addition, T_3-induced enhancement in the biliary efflux of GSSG was also significantly

reduced in rats pretreated with α-tocopherol [(α-tocopherol/T_3) − (average α-tocopherol) = 0.55 ± 0.14 nmol/g liver/min ($n = 3$); 55% decrease; $p < 0.05$], NAC [(NAC/T_3) − (average NAC) = 0.67 ± 0.06 ($n = 6$); 46% decrease; $p < 0.05$], $GdCl_3$ [($GdCl_3/T_3$) − (average $GdCl_3$) = 0.10 ± 0.06 ($n = 5$); 91% decrease; $p < 0.05$], or antisense oligonucleotide TJU-2755 [(ASO TJU-2755/T_3) − (average control) = 0.85 ± 0.05 ($n = 3$); 31% decrease; $p < 0.05$)] (Fig. 4B).

DISCUSSION

Kupffer cells, the most abundant population of macrophages in the body,[19] are the main source of TNF-α,[20] a cytokine considered to be a most important inflammatory mediator.[18] Data reported in this study demonstrate that the development of a hyperthyroid state in the rat by T_3 administration increases the circulating levels of TNF-α by actions exerted at the Kupffer cell level, which are related to the oxidative stress status established in the liver by thyroid hormone. The T_3-induced enhancement in serum TNF-α levels is primarily due to Kupffer cell activity since this response is virtually abolished by pretreatment with $GdCl_3$. This rare earth metal is known to selectively eliminate large Kupffer cells in the periportal region of the acinus,[14] thus significantly blocking both colloidal carbon phagocytosis[7,21] and the carbon-induced respiratory burst activity in the liver.[7] With regard to the mechanism(s) involved in T_3-induced TNF-α production, the enhancement in the oxidative stress status of the liver by thyroid calorigenesis, comprising a concomitant increase in Kupffer cell activity,[7] seems to play a role. This is evidenced by the substantial reduction in T_3-induced serum levels of TNF-α by pretreatment with the antioxidants α-tocopherol and NAC, associated with a virtual abolition of the T_3-induced liver GSH depletion and elevation in the biliary release of GSSG as indices related to oxidative stress.[6,17] Whether all hepatic cells are subjected to oxidative stress following T_3 administration cannot be determined from the present study. However, it is likely that this effect is shared by all hepatic cell populations as both hepatocytes and cells lining the hepatic sinusoids exhibit nuclear binding proteins for thyroid hormones that mediate actions such as thyroid calorigenesis.[22] Thus, data presented, along with early studies, strongly suggest that thyroid hormone increases TNF-α levels in serum through an oxygen radical-mediated upregulation of gene expression in Kupffer cells leading to enhanced synthesis of the cytokine. It is of interest that an interplay between the oxidative stress status in different hepatic cells appears to exist, since TNF-α generated by Kupffer cells significantly affects the oxidative stress in hepatocytes. This is seen by the reduction in biliary GSSG efflux induced by T_3 in rats pretreated with the anti TNF-α antisense and $GdCl_3$, Kupffer cell inactivator that diminishes, T_3-induced liver GSH depletion and the increases in lipid peroxidation.[7] These two agents also reduced the rate of hepatic O_2 consumption induced by T_3 that most likely represents the oxidative capacity of hepatocytes, which accounts for 90% of liver weight. While depletion of Kupffer cells by $GdCl_3$, may lead to the reduced production in a number of cytokines and prostanoids, the 21-mer antisense anti TNF-α is expected to be specific.[15] Data presented are in line with earlier observations that increased in liver O_2 consumption induced by ethanol is blocked both by Kupffer cell depletion[23] as well as by antithyroid medication,[24,25] and that ethanol-induced liver injury is not seen in knockout animals for TNF-α receptor-1, which is responsible for the activation of death signals and apoptosis after combining with TNF-α.[26]

As a pleiotropic cytokine mediating several physiological and pathological processes,[2,13] TNF-α is defined as an endogenous pyrogen due to its ability to act directly on the hypothalamus, leading to activation of responses that decrease heat loss and increase heat production.[27] Thyroid hormone-induced TNF-α levels in serum at 12 h after treatment coincided with a significant increase in the rectal temperature of the animals. The maximal TNF-α response observed at 22 h after T_3 treatment is abolished by pretreatment with either $GdCl_3$ and ASO TJU-2755 or with antioxidants, an effect that is accompanied by reduction in thyroid calorigenesis and liver O_2 uptake by the former pretreatments but not by α-tocopherol or NAC. Thus, TNF-α seems to play a role in the onset of thyroid calorigenesis, whereas dissociation between this effect of TNF-α and those related to T_3-induced energy wasting might occur at latter times after the initial T_3 treatment. In these latter conditions, thyroid calorigenesis involves the stimulation of both nuclear and mitochondrial gene expression with induction of the components of the respiratory apparatus and uncoupling proteins, which determines higher rates of ATP production partially compensated by intrinsic uncoupling.[3] This view is in agreement with the contention that mechanisms underlying the initiation and those that sustain the febrile response to circulating pyrogens are probably different.[28]

Collectively, data presented indicate that T_3 elicits a major increase in TNF-α production, comparable to that seen after the administration of endotoxin (LPS), an effect which is mediated by oxidative stress at the Kupffer cell level. The studies may also explain how the hepatotoxic effect of some drugs such as acetaminophen, carbon tetrachloride, or ethanol is increased by hyperthyroidism and blunted by antithyroid medication.[24,29,30] These toxins promote

oxidative stress by increasing radical production or by reducing ROS protective mechanisms[31–35] and display hepatotoxic effects that are mediated by Kupffer cell function and TNF-α release.[36–39] The mechanisms underlying the early TNF-α response induced by T_3 are currently under study in our laboratory.

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