Research Article

Thyroid Hormone-Induced Cytosol-to-Nuclear Translocation of Rat Liver Nrf2 Is Dependent on Kupffer Cell Functioning

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Cytotoxicity of Kupffer cells has been implicated in ischemia-reperfusion (IR) injury, an inflammatory model underlying drastic ROS generation [5], whereas preconditioning strategies against IR injury have been associated with moderate increases in ROS production [6, 7].

Thyroid hormone- (L-3,3′,5-triiodothyronine, T3) induced calorogenic effects involving ROS generation in the liver has been proposed as a preconditioning mechanism for IR injury [8]. T3-induced ROS generation occurs at different subcellular sites of hepatocytes and in the respiratory burst of Kupffer cells, triggering the activation of the transcription factors nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), and activating protein 1 (AP-1). Under these conditions, the redox upregulation

1. Introduction

Kupffer cells reside in liver sinusoids representing approximately 35% of hepatic nonparenchymal cells. These liver macrophages have scavenger receptors that are essential for eliminating blood borne bacteria [1]. In addition, activated Kupffer cells produce and release several mediators including cytokines, lipid substances, and reactive oxygen species (ROS), which can function locally or systemically to mediate immune responses [1]. These responses play a key role in the homeostatic adaptation to liver injury; however, if dysregulated, they can induce acute or chronic liver damage [2–4]. Cytotoxicity of Kupffer cells has been implicated in
of Kupffer cell-dependent expression of cytokines (tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6) is achieved, which upon interaction with specific receptors in hepatocytes trigger the expression of antioxidant enzymes (manganese superoxide dismutase, inducible nitric oxide synthase) [9, 10], antiapoptotic proteins (Bcl-2) [9], and acute-phase proteins (haptoglobin, β-fibrinogen) [11]. These responses, and the promotion of hepatocyte and Kupffer cell proliferation observed [12, 13], represent cytoprotective effects reestablishing redox homeostasis, promoting cell survival, and protecting the liver against IR injury [7].

Activation of nuclear factor-κB-related factor 2 (Nrf2) also affects cytoprotection, in addition to NF-κB, STAT3-, and AP-1-dependent signaling pathways, a transcription factor whose cytosol-to-nuclear translocation has been recently found to be triggered by T3 administration through a redox-dependent mechanism [14]. Nrf2 signaling is characterized by its sensitivity to low levels to ROS [15], controls the expression of antioxidant components, detoxification enzymes, membrane transporters, or 26 S proteasome components, and interplays with NF-κB affording anti-inflammatory responses [16–19]. Thus, the cytoprotective effects of T3-induced liver Nrf2 activation may represent an alternate mechanism for liver preconditioning, a condition associated with Kupffer cell functioning that may constitute a new therapeutic option for liver surgery and liver transplantation in man using reduced-size grafts from living donors [7, 8, 20]. According to these considerations, the purpose of this study was to investigate whether T3-induced liver Nrf2 activation depends on the respiratory burst activity of Kupffer cells, a process related to ROS generation and liver homeostasis. For this purpose, Nrf2 activation, as assessed by cytosol-to-nuclear translocation, was determined in rat liver either without or with pretreatment with the Kupffer cell inactivator gadolinium chloride (GdCl3) [21] or with the selective NADPH oxidase inhibitor apocynin (1.5 mmol/L) added to the drinking water for 7 days prior to T3, a protocol shown to effectively inhibit NADPH oxidase activity in rats [22]. Studies were carried out 2 h after T3 administration in animals anesthetized with intraperitoneal (1 mL/kg) zolazepam chloride (25 mg/mL) and tiletamine chloride (25 mg/mL) (Zoletil-50; Virbac S/A, Carros, France). In the group of rats subjected to GdCl3-T3-combined treatment, levels of serum TNF-α were measured by ELISA (UltraSensitive Cytoscreen kit, Biosource International, Camarillo, CA, USA) according to manufacturer’s specifications. Experimental animal protocols and animal procedures complied with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 86-23, revised 1985) and were approved by Ethics Committee of the Faculty of Medicine, University of Chile (CBA 0269 FMUCH).

2.2. Kupffer-Cell Inactivation. Liver slices were obtained in anesthetized (Zoletil-50) rats at 24 to 72 h after-GdCl3, and kinetic changes of ED2-immunolabelled Kupffer cells were determined by immunohistochemistry using a commercial kit (AbD Serotec, Oxford, UK). Briefly, liver samples were fixed in phosphate-buffered formalin (pH 7.4) and incubated with a primary mouse antibody to ED2, followed by incubation with biotin-conjugated secondary goat antibody. Positive reactions were visualized with 3,3’-diaminobenzidine, and results are expressed as the number of cells determined in 10 different 0.7 mm² areas per liver from 3 rats per timepoint [23].

2.3. Liver Perfusion, Colloidal Carbon Uptake, and Carbon-Induced Respiratory Activity. Livers from animals anesthetized with Zoletil-50 were perfused with a solution containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 25 mM NaHCO3, and 10 mM glucose, equilibrated with and O2/CO2 mixture (19:1, vol/vol) to give pH 7.4, through a cannula placed in the portal vein. Perfusion was carried out at constant flow rates (3.5 to 4 mL/g liver/min) and temperature (36 to 37°C), without recirculation of the perfusate [13, 24]. After 15 min equilibration of perfused livers, O2 consumption (QO2) was determined in the effluent perfusate collected via a cannula placed in the vena cava and allowed to flow through a Clark-type oxygen electrode. For determination of colloidal carbon uptake by perfused livers, suspensions of India ink (Rotring, Hamburg, Germany) were prepared, dialysed, and infused between 30 to 45 min of perfusion at the concentration of 0.5 mg/mL. Per fusate samples were taken every 10 min in the presence and absence of the liver to measure the absorbance of colloidal carbon at 623 nm [24] (specific extinction coefficient of 0.97 [mg/mL]−1) [13]. Rates of carbon uptake were calculated from influent minus effluent concentration differences, referred to the perfusion flow. The respiratory burst activity induced by colloidal carbon infusion was assessed by the integration of the area under the QO2 curves between 30 and 45 min, and expressed as μmol O2/g liver [13]. These parameters were determined in control rats and in animals after 2 h of T3 administration.

2. Methods

2.1. Animal Treatments. Male Sprague-Dawley rats (Animal Facility of the Institute of Biomedical Sciences, Faculty of Medicine, University of Chile) weighing 180–200 g, housed on a 12-h light/dark cycle, and fed with rat chow and water ad libitum, received a single intraperitoneal dose of 0.1 mg of T3/kg body weight or equivalent volumes of the hormone vehicle 0.1 N NaOH (controls). Kupffer cells were selectively eliminated by a single intravenous injection of 10 mg of GdCl3/kg body weight [21] given 72 h before T3 administration, and control animals received equivalent volumes of saline. A separate group of rats was given the
and pretreatment for 72 h with GdCl$_3$ or for 7 days with apocynin prior to T$_3$.

2.4. Western Blot Analysis of Nrf2, Keap1, HO-1, GCLC, and Trx. Liver samples (100–500 mg) 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 phenylmethylsulfonyl fluoride, 1 mM EDTA, 0.6% Nonidet P-40, and 1 mM orthovanadate). Nuclear protein extracts (100 μg) and soluble protein fractions (60 μg) were mixed with sample loading buffer pH 6.8 (2% SDS, 0.0625 M Tris, 10% glycerol, and 2.5% β-mercaptoethanol) and heated at 95°C for 5 min, separated on 12% polyacrylamide gels using SDS-PAGE [25], and transferred to nitrocellulose membranes [26], which were blocked for 1 hour at room temperature with TBS-containing 5% bovine serum albumin. The blots were washed with TBS containing 0.1% Tween 20 and hybridized with either rabbit polyclonal antibodies for Nrf2, Keap1, HO-1, GCLC, and Trx (Abcam, Cambridge, MA), or mouse monoclonal antibodies for β-actin (ICN Biomedicals, Inc., Aurora, OH) and lamin A/C (BD Transduction Laboratories, San José, CA, USA). In all determinations, anti-β-actin was used as internal control for cytosolic fractions, whereas antilamin A/C was employed as internal control for nuclear fractions. In addition, the membranes were stained with anti-α-tubulin or anti-lamin A/C to confirm contamination of the cytosolic and nuclear fractions. After extensive washing, the antigen-antibody complexes were detected using horseradish peroxidase goat anti-rabbit IgG or goat anti-mouse IgG and antigen-antibody complexes were detected using horseradish peroxidase goat anti-rabbit IgG or goat anti-mouse IgG and a SuperSignal West Pico Chemiluminescence kit detection system (Pierce, Rockford, IL, USA). Bands were quantified by densitometry using Scion Image (Scion Corp., Frederick, MD).

2.5. Statistics. Data showing Gaussian distribution according to the Kolmogorov-Smirnov test are expressed as means ± SEM for the number of separate experiments indicated. One-way or two-way ANOVA and the Newman-Keuls test or Student’s t-test for unpaired data assessed the statistical significance ($P < 0.05$) of differences between mean values as indicated. All statistical analyses were computed employing GraphPad Prism™ version 2.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

Administration of the Kupffer cell inactivator GdCl$_3$ to euthyroid rats elicited a decrease in the number of ED2(+) cells, with 95% ($P < 0.05$) depletion observed at 72 h (Figure 1(a)), as assessed by immunohistochemical technique with ED2 antibody. Studies using the isolated perfused liver revealed that, at 72 h after treatment, GdCl$_3$ reduced by 86% and 83% ($P < 0.05$) the rate of colloidal carbon uptake (Figure 1(b)) and the associated carbon-induced respiratory activity (Figure 1(c)), respectively, compared to liver perfusions in the absence of carbon infusion. According to these results, the influence of Kupffer cells on T$_3$-induced liver Nrf2 activation was studied by giving T$_3$ at the time of maximal ED2(+) Kupffer-cell inactivation (72 h after GdCl$_3$), and studies on T$_3$ action were carried out 2 h after T$_3$ administration, time at which Nrf2 activation is attained [14].

Liver Nrf2 activation induced at 2 h after T$_3$ administration was evidenced by the significant 24% decrease in the content of cytosolic Nrf2 (Figure 2(a)) and 434% enhancement in that of nuclear Nrf2 (Figure 2(b)), with a 463% increase in nuclear/cytosolic Nrf2 ratio (Figure 2(c)). Treatment with GdCl$_3$ did not significantly modify the liver nuclear/cytosolic Nrf2 ratio when given alone (Figure 2(c)). However, cytosolic and nuclear Nrf2 levels after combined GdCl$_3$-T$_3$ protocol were comparable to control values (Figures 2(a) and 2(b)), leading to a net 91% decrease ($P < 0.05$) in the nuclear/cytosolic Nrf2 ratio compared to the net effect of T$_3$ alone (Figure 2(c), inset). Under these conditions, upregulation of liver HO-1 (Figure 2(d)), GCLC (Figure 2(e)), and Trx (Figure 2(f)) by T$_3$ was suppressed by the combined GdCl$_3$-T$_3$ treatment, without significant effects of GdCl$_3$ when given alone (Figures 2(d), 2(e) and 2(f)). These findings were observed concomitantly with 7.5-fold increase in the serum TNF-α levels by T$_3$, with a net 92% diminution being elicited by the combined GdCl$_3$-T$_3$ treatment (a control, 2 ± 1 (n = 9) pg TNF-α/mL; (b) T$_3$, 15 ± 1 (n = 3); (c) GdCl$_3$, 3 ± 2 (n = 3); (d) GdCl$_3$-T$_3$, 4 ± 2 (n = 3); (b) versus (a), (c), and (d), $P < 0.05$). Furthermore, liver Nrf2 inhibitor Keap1 levels in the cytosol exhibited 75% reduction in T$_3$-treated rats over controls (Figure 3(a)), whereas those of nuclear Keap1 were enhanced by 173% (Figure 3(b)), without significant changes in nuclear Keap1/Nrf2 ratios (Figure 3(c)). Net differences in the latter parameter indicate a substantial enhancement ($P < 0.05$) in animals subjected to combined GdCl$_3$-T$_3$ treatment [(GdCl$_3$ + T$_3$) − GdCl$_3$] compared to rats given T$_3$ alone [T$_3$− control] (Figure 3(c), inset).

Administration of apocynin to euthyroid rats resulted in 90% decrease ($P < 0.05$) in carbon-induced respiratory burst activity assessed in liver perfusion studies (Figure 4(a)), without significant changes in the rate of colloidal carbon uptake (Figure 4(b)). Liver Nrf2 activation by T$_3$ administration involved significant 48% decrease in the content of cytosolic Nrf2 (Figure 5(a)), 675% enhancement in nuclear Nrf2 levels (Figure 5(b)), and 14.9-fold increase in the serum TNF-α levels was suppressed in apocynin-T$_3$-treated animals (Figure 5(d)).

4. Discussion

Kupffer cell functioning assessed in the isolated perfused rat liver by means of colloidal carbon infusion allows the continuous estimation of the associated rate of carbon-particle
Gadolinium chloride (GdCl₃) administration is associated with suppression of Kupffer cell functioning in rat liver. Kinetics of Kupffer cell inactivation after GdCl₃ treatment (time zero) in livers from euthyroid rats by immunohistochemistry using ED2 antibody (a), rate of colloidal carbon uptake (b), and carbon-induced liver respiratory activity ($\Delta Q_{O_2}$) (c) assessed in isolated perfused livers at 72 h after GdCl₃ treatment. $\Delta Q_{O_2}$ was calculated by integration of the area under the O₂ consumption curves between 30 and 45 min perfusion (c). Values shown represent means ± SEM for 4 rats per experimental group. *P < 0.05 versus controls assessed by Student’s t-test for unpaired data.

The use of this model system provided evidence for the role of Kupffer cells in the hepatotoxicity of lindane [27], acetaminophen [28], and copper [29], as well as Kupffer cell function adaptation leading to hepatoprotection after T₃ administration [13, 20]. Data reported in this study indicate that T₃ administration up-regulates liver Nrf2 signaling depending on Kupffer cell functioning. Early (2 h) liver Nrf2 activation triggered by T₃ treatment evidenced by 4.63-fold enhancement in nuclear/cytosolic Nrf2 ratios denoting cytosol-to-nuclear Nrf2 translocation, occurred without significant changes in nuclear Keap1/Nrf2 ratios. These data indicate that T₃ achieves liver Nrf2 upregulation in a time interval at which Nrf2-dependent induction of its inhibitor Keap1 [30] does not occur, resulting in significant increases in the expression of the target genes controlled by Nrf2, namely, HO-1, GCLC, and Trx [18]. T₃-induced liver Nrf2 activation involves a redox-dependent mechanism, considering that cytosol to nuclear Nrf2 translocation is blocked by N-acetylcysteine pretreatment [14]. The redox activation of Nrf2 is associated with ROS produced due to acceleration of liver O₂ consumption by the calorigenic action of T₃ exerted on hepatocytes and Kupffer cells [7], but it also may involve ROS generated in the respiratory burst activity of hepatic macrophages [13]. The latter proposal underlies redox activation of NF-κB in Kupffer-cell of T₃-treated animals [31], with consequent expression and release of TNF-α, as reported in this study. Interaction of
Figure 2: Gadolinium chloride (GdCl₃) administration is associated with suppression of T₃-induced activation of liver Nrf2 signaling. Determinations were performed at 2 h after T₃ administration in rats pretreated with GdCl₃ for 72 h. (a) Levels of cytosolic Nrf2 protein (68 kDa), β-actin (43 kDa), α-tubulin (52 kDa), and lamin A/C (65 kDa); (b) levels of nuclear Nrf2 protein, lamin A/C, and α-tubulin; (c) nuclear/cytosolic Nrf2 content ratios and net effects of T₃ and GdCl₃ treatments (inset); (d) levels of heme oxygenase 1 (HO-1) protein (33 kDa); (e) levels of catalytic subunit of glutamate cysteine ligase (GCLC) protein (73 kDa); (f) levels of thioredoxin (Trx) protein (12 kDa). Values shown represent means ± SEM for 3 to 6 rats per experimental group. Significance (P < 0.05; two-way ANOVA and the Newman-Keuls' test) is indicated by the letters identifying each experimental group. Significance in the inset of (c) was calculated by Student’s t-test for unpaired data.
Figure 3: Gadolinium chloride (GdCl3) administration is associated with enhancement of liver Keap1/Nrf2 ratios over values in T3-treated rats. Determinations were performed at 2 h after T3 administration in rats pretreated with GdCl3 for 72 h. (a) Levels of cytosolic Keap1 protein (70 kDa) and β-actin (43 kDa); (b) levels of nuclear Keap1 protein and lamin A/C (65 kDa); (c) nuclear Keap1/Nrf2 content ratios and net effects of T3 and GdCl3 treatments (inset). Values shown represent means ± SEM for 4 to 6 rats per experimental group. Significance (P < 0.05; two-way ANOVA and the Newman-Keuls’ test) is indicated by the letters identifying each experimental group. Significance in the inset of (c) was calculated by Student’s t-test for unpaired data.

Figure 4: Apocynin administration is associated with suppression of colloidal carbon-induced liver respiratory activity (ΔQO2) assessed in isolated perfused livers from euthyroid rats (a), without altering carbon phagocytosis (b). Determinations were carried out 7 days after apocynin treatment. ΔQO2 was calculated by integration of the area under the O2 consumption curves between 30 and 45 min perfusion. Values shown represent means ± SEM for 4 rats per experimental group. Significance (P < 0.05) was calculated by Student’s t-test for unpaired data.
Figure 5: Apocynin administration is associated with diminution of T₃-induced activation of liver Nrf2 signaling. Determinations were performed at 2 h after T₃ administration in rats pretreated with apocynin for 7 days. (a) Levels of cytosolic Nrf2 protein (68 kDa) and β-actin (43 kDa); (b) levels of nuclear Nrf2 protein and lamin A/C (65 kDa); (c) nuclear/cytosolic Nrf2 content ratios and net effects of T₃ and apocynin treatments (inset); (d) levels of heme oxygenase 1 (HO-1) protein (33 kDa). Values shown represent means ± SEM for 3 to 4 rats per experimental group. Significance (P < 0.05; two-way ANOVA and the Newman-Keuls’ test) is indicated by the letters identifying each experimental group. Significance in the inset of (c) was calculated by Student’s t-test for unpaired data.
TNF-α with TNF-α receptor-1 in hepatocytes may trigger mitochondrial ROS production [32, 33], reinforcing that achieved by actions of T3 on hepatocyte energy metabolism. Under these conditions, Nrf2 activation may be achieved by direct action of ROS [17–19] or through ROS-dependent formation of cyclopentenone-containing J isoprostanes from polyunsaturated fatty acids, which release Nrf2 upon binding to Keap1 [34, 35]. In addition, increased formation of Nrf2/c-Jun complexes may occur due to the ability of TNF-α to induce c-Jun nuclear-binding activity [36], heterodimerization that is required for ARE-mediated transcriptional activation [37].

Dependency of T3-induced liver Nrf2 upregulation on Kupffer cells was demonstrated by inactivation of liver macrophages by GdCl3 [21] or inhibition of Kupffer-cell NADPH oxidase activity by apocynin [22, 38]. Administration of the GdCl3 72 h prior to T3 achieved almost complete elimination of ED2(+) cells, a Kupffer cell subpopulation characterized with an ED2 antibody recognizing a membrane antigen of resident macrophages such as Kupffer cells [39, 40]. Liver ED2(+) cells are described as mature macrophages [41], which are mainly located in periportal areas of the liver lobule [42]. These mature liver macrophages have higher lysosomal enzyme activities, phagocytic capacity, and production of TNF-α, interleukin-1 and prostaglandin E2 than smaller ED1(+) cells located in midzonal and central areas [1], a subpopulation of liver macrophages that is not modified by GdCl3 administration [20]. Under conditions of GdCl3-induced Kupffer-cell depletion, activation of Nrf2 and expression of HO-1, GCLC, and Trx by T3 were abolished, concomitantly with significant enhancement in nuclear Keap1/Nrf2 ratios. The latter finding suggests that the nuclear abundance of Keap1 is increased by combined GdCl3-T3 treatment, which may allow an efficient nuclear export mechanism to terminate T3-induced Nrf2 signaling [43]. In agreement with these views, T3-induced TNF-α response is abolished in rats subjected to combined GdCl3-T3 treatment, which may suppress Kupffer cell-dependent TNF-α-induced mitochondrial ROS component, otherwise altering Keap1 to a form which does not have anti-Nrf2 effects [19]. The role of Kupffer cells in T3-induced liver Nrf2 activation suggested by hepatic macrophage depletion after GdCl3 administration is further supported by experiments using apocynin prior to T3. Apocynin inhibits the assembly of the ROS generator NADPH oxidase in neutrophils and macrophages after metabolic conversion, without altering phagocytosis, in addition to its free-radical scavenging properties [38]. In addition, Kupffer cell activation by hepatic IR upregulates kidney Nrf2 signaling to avoid remote organ dysfunction, as treatment with GdCl3 prior to liver IR attenuates the TNF-α response induced, reducing the enhancement in renal levels of the Nrf2 activator 15-deoxy-

5. Conclusion

Data presented suggest that Kupffer cell functioning is essential for upregulation of liver Nrf2 stress response-signalin...


[42] L. Bouwens, M. Baekeland, R. de Zanger, and E. Wisse, "Quantitation, tissue distribution and proliferation kinetics of..."


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