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Kupffer-cell activity is essential for thyroid hormone rat liver preconditioning

G. Tapia, C. Santibáñez, J. Farías, G. Fuenzalida, P. Varela, L.A. Videla, V. Fernández*

Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Independencia 1027, Casilla 70000, Santiago 7, Chile

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ABSTRACT

We studied the role of Kupffer cell functioning in T_3 liver preconditioning against ischemia–reperfusion (IR) injury using the macrophage inactivator gadolinium chloride ($GdCl_3$) previous to T_3 treatment. Male Sprague–Dawley rats given a single i.p. dose of 0.1 mg T_3/kg were subjected to 1 h ischemia followed by 20 h reperfusion, in groups of animals pretreated with 10 mg $GdCl_3$/kg i.v. 72 h before T_3 or with the respective vehicles. IR resulted in significant enhancement of serum aspartate aminotransferase (3.3-fold increase) and tumor necrosis factor-α (93% increase) levels, development of liver damage, and diminished nuclear factor-κB DNA binding over control values. These changes, which were suppressed by the T_3 administration prior to IR, persisted in animals given $GdCl_3$ before T_3 treatment, under conditions of complete elimination of ED2(+) Kupffer cells achieved in a time window of 72 h. It is concluded that Kupffer cell functioning is essential for T_3 liver preconditioning, assessed in a warm IR injury model by hepatic macrophage inactivation.

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1. Introduction

Thyroid hormone (L-3,3′,5-triiodothyronine, T_3) exerts important actions on cellular energy metabolism, being mitochondria the major target for its calorigenic effects (Schwartz and Oppenheimer, 1978). Acceleration of O_2 consumption by T_3 is due to transcriptional activation of respiratory genes in target tissues such as liver, leading to increased production of reactive oxygen species (ROS) (Fernández and Videla, 1993; Venditti et al., 2003) and the consequent antioxidant depletion (Videla et al., 2007). The increase in ROS generation primarily occurs at mitochondrial (Fernández and Videla, 1993; Venditti et al., 2003) and other subcellular sites of hepatocytes (Fernández et al., 1985; Huh et al., 1998), and in Kupffer cells. In the latter case, higher ROS generation may be ascribed to up-regulation of respiratory genes such as that for mitochondrial glycerol-3-phosphate dehydrogenase (Videla et al., 2007) due to the existence of T_3 receptors in Kupffer cells (Sellitti et al., 1985), and enhancement in the respiratory burst activity due to NADPH oxidase activation by T_3 administration (Tapia et al., 1997). ROS may act as redox signals regulating important cellular functions such as protein activity and gene expression (Dröge, 2002; Poli et al., 2004). Indeed, ROS produced at the Kupffer-cell level activate redox-sensitive transcription factors such as nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), and activating protein 1 (AP-1), as evidenced by the abolishment of the T_3-induced DNA binding capacity of these proteins by antioxidants or Kupffer-cell inactivation (Videla et al., 2007; Tapia et al., 2006; Fernández et al., 2007a,b). Up-regulation of gene expression by T_3 is also accomplished in Kupffer cells, leading to an enhancement in the synthesis and release of the cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6, which in turn, trigger the expression of antioxidant enzymes, the anti-apoptotic protein Bcl-2, the acute-phase response, and hepatocyte proliferation (Videla et al., 2007; Tapia et al., 2006; Fernández et al., 2007a). These data indicate that T_3-induced calorigenesis triggers non-genomic effects leading to an expression pattern representing adaptive mechanisms to re-establish redox homeostasis and promote cell survival under conditions of ROS toxicity.

In agreement with these views, T_3-induced transient oxidative stress was reported to exert significant protection against ischemia–reperfusion (IR) liver injury (Fernández et al., 2007b), representing a novel preconditioning maneuver able to enhance the resistance of the liver to limit the detrimental effects of injurious stimuli (Casillas-Ramírez et al., 2006). Recently, the development of transient and reversible oxidative stress in the liver of rats acutely treated with T_3 was shown to have a causal role in protection against IR injury, as evidenced by the re-establishment of liver damage after the administration of N-acetylcysteine before T_3 (Fernández et al., 2008), in agreement with other strategies inducing redox imbalance (Casillas-Ramírez et al., 2006). T_3 liver preconditioning is associated with (i) the recovery of NF-κB and STAT3 DNA binding and the acute-phase response, which are lost during IR (Fernández et al., 2007b); and (ii) the enhanced cell proliferation induced, which may compensate for

* Corresponding author. Tel.: +56 2 9786256; fax: +56 2 7372783.
E-mail address: vfernand@med.uchile.cl (V. Fernández).

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liver cell lost due to IR-induced hepatocellular necrosis (Fernández et al., 2007a), features that may be related to Kupffer-cell activity (Tsukamoto, 2002). The objective of this study was to test the hypothesis that T_3-induced liver preconditioning depends on Kupffer cell functioning. For this purpose, T_3 liver preconditioning was determined in a model of partial hepatic IR injury in the rat, either without or with pretreatment with the Kupffer cell inactivator gadolinium chloride ($GdCl_3$) (Hardonk et al., 1992), the results of which were correlated with parameters related to cell death signaling, namely, TNF-α response and NF-κB activation.

2. Materials and methods

2.1. Animal treatments and model of partial hepatic IR injury

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*, were separated into eight experimental groups: (1) control-sham, (2) control-IR, (3) T_3-sham, (4) T_3-IR, (5) $GdCl_3$-control-sham, (6) $GdCl_3$-control-IR, (7) $GdCl_3$–T_3-sham, and (8) $GdCl_3$–T_3-IR. Animals received a single intraperitoneal dose of T_3 (0.1 mg/kg of body weight) or equivalent volumes of the hormone vehicle 0.1N NaOH (controls). Kupffer cells were selectively eliminated by a single intravenous injection of 10 mg of gadolinium chloride ($GdCl_3$)/kg body weight (Hardonk et al., 1992; Tapia et al., 1997), given 72 h before T_3 administration, and control animals received equivalent volumes of saline. At 48 h after T_3 treatment, rats were anesthetized with intraperitoneal (1 ml/kg) zolazepam chlorhydrate (25 mg/ml) and tiletamine chlorhydrate (25 mg/ml) (Zoletil 50; Virbac S/A, Carros, France). IR was induced by temporarily occluding the blood supply to the left and median lobes of the liver by means of a Schwartz clip (Fine Science Tools, Vancouver, BC, Canada) for 1 h followed by 20 h of reperfusion, and IR-controls were subjected to anesthesia and sham laparotomy (Fernández et al., 2007b). At the end of the reperfusion period, blood samples were obtained by cardiac puncture for serum AST and TNF-α (ELISA; Biosource International, Camarillo, CA, USA) assessment, and liver samples taken from the medial lobes were frozen in liquid nitrogen (assessment of NF-κB DNA binding), or fixed in phosphate-buffered formalin, embedded in paraffin, and stained with hematoxylin–eosin (morphology assessment). 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).

2.2. Kupffer-cell inactivation and repopulation

Liver slices were obtained from anesthetized (Zoletil 50) rats at 24–120 h post-$GdCl_3$ and kinetic changes of ED1- and ED2-immunolabeled 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 primary mouse antibodies to ED1 and ED2, followed by incubation with biotin-conjugated secondary goat antibodies. Positive reactions were visualized with 3,3′-diaminobenzidine (Ide et al., 2002; Gomes et al., 2004).

2.3. NF-κB electromobility shift assay

Nuclear protein extracts from liver samples were prepared and subjected to electromobility shift assay for assessment of NF-κB DNA binding, using the NF-κB probe 5′-GATCTCAGAGGGGACTTTCCGAG-3′ (Invitrogen Life Technologies, Carlsbad, CA, USA), labeled with [α-32P]dCTP using the Klenow DNA Polymerase Fragment I (Invitrogen Life Technologies), as described previously (Deryckere and Gannon, 1994; Tran-Thi et al., 1995; Fernández et al., 2005). The specificity of the reaction was determined by a competition assay using 100-fold molar excess of unlabeled DNA probes. 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).

2.4. Statistical analyses

Values shown represent the means ± SE for the number of separate experiments indicated. Net changes induced by IR under conditions of no treatment (group a, control) and after administration of T_3 (group b), $GdCl_3$ (group c), or $GdCl_3$ plus T_3 (group d) were calculated by subtracting individual values of control-IR minus the average value of control-sham, T_3-IR minus T_3-sham, $GdCl_3$-IR minus $GdCl_3$-sham, or $GdCl_3$–T_3-IR minus $GdCl_3$–T_3-sham, respectively. One-way ANOVA and the Newman–Keuls test assessed the statistical significance of differences between mean values. A P value of less than 0.05 was considered significant.

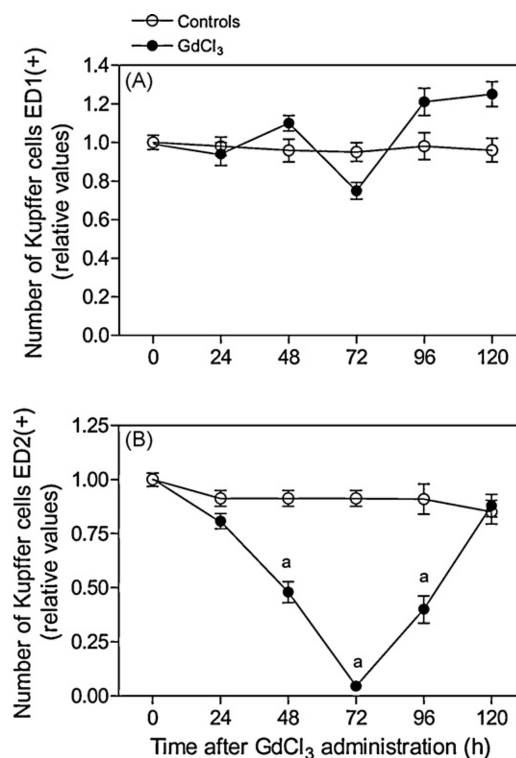

Fig. 1. Kinetics of Kupffer-cell inactivation and repopulation after gadolinium chloride ($GdCl_3$) administration (time zero) in livers from untreated rats by immunohistochemistry using ED1 (A) or ED2 (B) antibodies. Values shown represent number of cells expressed as means ± SEM determined in 10 different 0.7 mm^2 areas per liver from 3 rats per time-point. [a]$P < 0.05$ versus controls.

3. Results

3.1. Inactivation and repopulation of subpopulations of Kupffer cells

The use of immunohistochemical techniques with ED1 and ED2 antibodies allows the characterization of Kupffer cell subpopulations (Ide et al., 2002; Gomes et al., 2004). Administration of the Kupffer cell inactivator $GdCl_3$ did not significantly modify the number of ED1(+) cells (Fig. 1A), whereas that of ED2(+) cells was drastically reduced, with maximal depletion observed at 72 h after treatment ($P < 0.05$) (Fig. 1B). Repopulation of ED2(+) Kupffer cells was significant at 96 h after $GdCl_3$ administration, with complete recovery being found after 120 h (Fig. 1B). According to these results, the influence of Kupffer cells on T_3-induced liver preconditioning was assessed by giving T_3 at the time of maximal ED2(+) Kupffer-cell inactivation (72 h after $GdCl_3$), and the IR protocol was applied after 48 h of T_3 administration. At this experimental time, ED2(+) Kupffer-cell repopulation is completed (Fig. 1B) and T_3-induced liver oxidative stress is not observed (Fernández et al., 2007b), thus allowing the development of IR-induced oxidative stress and Kupffer cell functioning underlying IR liver injury (Casillas-Ramírez et al., 2006).

3.2. Serum AST and TNF-α levels and liver histology

One hour of partial hepatic ischemia induced by vascular clamping followed by reperfusion for 20 h achieved minimal mortality but extensive liver injury, as shown by significant 3.3-fold increase in serum AST levels, compared with sham-operated animals (Fig. 2A). In T_3-preconditioned rats, IR led to 1.7-fold enhancement in serum AST levels (Fig. 2A), leading to a net diminution of 97% in relation

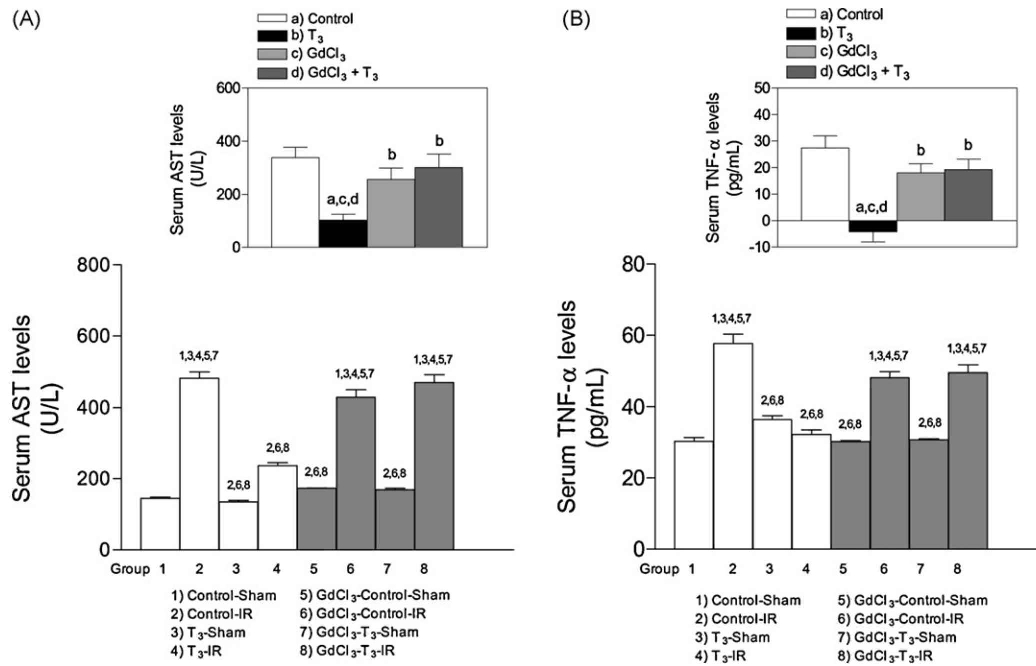

Fig. 2. Effect of gadolinium chloride ($GdCl_3$) administration on the serum levels of aspartate aminotransferase (AST) (A) and tumor necrosis factor-α (TNF-α) (B) after hepatic ischemia–reperfusion (IR) injury in unpreconditioned and T_3-preconditioned rats. Insets A and B: net changes induced by IR under conditions of no treatment (group a, control) and after administration of T_3 (group b), $GdCl_3$ (group c), and $GdCl_3$ plus T_3 (group d), calculated as described in Section 2. Values shown correspond to means ± SEM for 8–14 rats per group. Significance ($P < 0.05$) is shown by the numbers (or letters in the insets) identifying each experimental group.

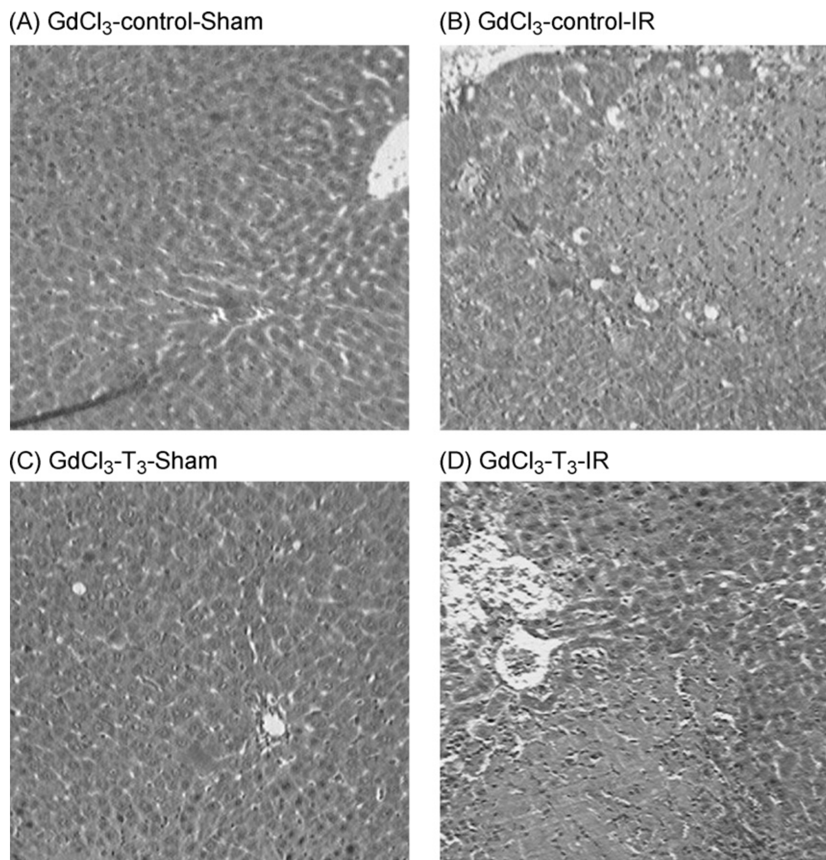

Fig. 3. Effect of gadolinium chloride ($GdCl_3$) administration on liver histology after hepatic ischemia–reperfusion (IR) injury in unpreconditioned and T_3-preconditioned rats. Representative liver sections from (A) a $GdCl_3$-control-sham-operated rat, (B) a $GdCl_3$-control animal subjected to IR, (C) a $GdCl_3$-pretreated T_3-treated sham-operated rat, and (D) a $GdCl_3$-pretreated T_3-treated animal subjected to IR (hematoxylin–eosin stained liver sections from a total of 3–4 rats per group; original magnification 40×).

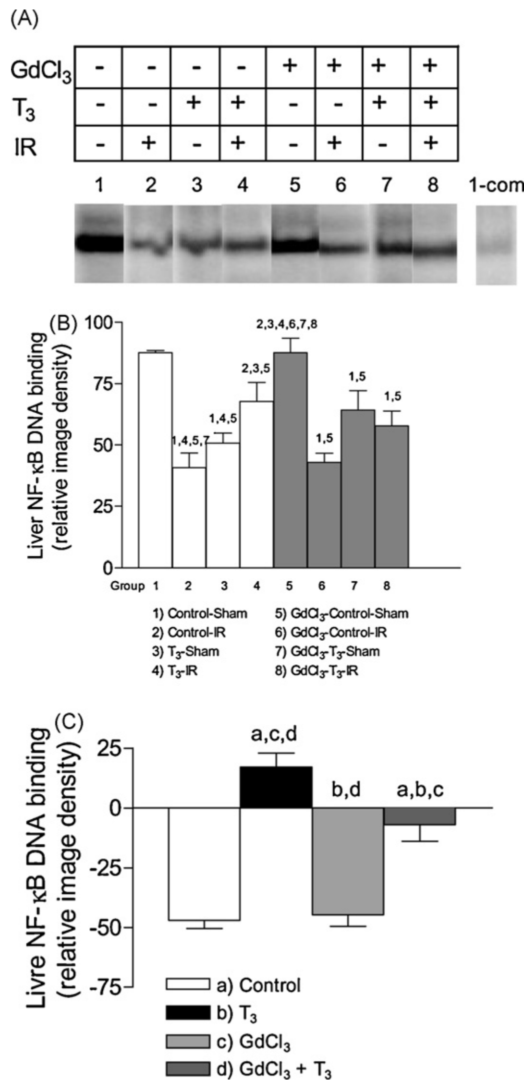

Fig. 4. Effect of gadolinium chloride ($GdCl_3$) administration on liver nuclear factor-κB (NF-κB) DNA binding on electromobility shift assay after hepatic ischemia–reperfusion (IR) injury in unpreconditioned and T_3-preconditioned rats. (A) Autoradiographs representing lanes loaded with 8 μg nuclear protein from an animal of each experimental group, as described in Fig. 1, and from rat number 1 in competition experiment with 100-fold molar excess of the unlabeled DNA probe (1-com). (B) Bar graphs corresponding to densitometric quantification of relative NF-κB DNA binding. (C) Net changes in NF-κB DNA binding induced by IR under conditions of no treatment (group a, control) and after administration of T_3 (group b), $GdCl_3$ (group c), and $GdCl_3$ plus T_3 (group d), calculated as described in Section 2. Values shown correspond to means ± SEM for 3–6 rats per group. Significance ($P < 0.05$) is shown by the numbers (B) or letters (C) identifying each experimental group.

to the unpreconditioned group (Fig. 2A, inset). After the administration of $GdCl_3$, IR led to 2.5-fold increase in serum AST levels over the $GdCl_3$-sham group and 2.8-fold enhancement over the $GdCl_3$–T_3-sham group (Fig. 2A), thus showing suppression of the preconditioning effect of T_3 (Fig. 2A, inset). Similar results are observed when serum ALT levels were assessed (data not shown). IR led to 93% ($P < 0.05$) increase in the serum levels of TNF-α over control values in unpreconditioned rats (Fig. 2B), an effect that was abolished by T_3 preconditioning (Fig. 2B, inset). Following $GdCl_3$ pretreatment, IR induced a 60% increase in serum TNF-α levels over the $GdCl_3$-sham group and 63% enhancement over the $GdCl_3$–T_3-sham group (Fig. 2B), thus eliminating the reducing effect of T_3 on IR-induced serum TNF-α response (Fig. 2B, inset). In agreement with serum transaminase data, control animals subjected to $GdCl_3$-sham (Fig. 3A) or to $GdCl_3$–T_3-sham (Fig. 3C) conditions exhibited normal liver histology, whereas IR in animals subjected to $GdCl_3$ pretreatment alone (Fig. 3B) and $GdCl_3$ pretreatment followed by T_3 administration (Fig. 3D) resulted in substantial distortion of liver architecture, with extensive centrolobulillar cell necrosis, congestive pericentral and periportal areas, and neutrophil infiltration. Liver fibrosis was not observed in any of the experimental groups studied.

3.3. Liver NF-κB DNA binding

IR diminished liver NF-κB DNA binding compared with control-sham-operated rats (Fig. 4A), with a net 54% decrease ($P < 0.05$, Fig. 4B), representing a reduction of 47.0 ± 3.5 ($n = 3$) arbitrary units (Fig. 4C), whereas IR in T_3-treated animals led to 34% increase in NF-κB activation over the T_3-sham group ($P < 0.05$, Fig. 4A and B) represented by a net enhancement of 17.1 ± 5.9 ($n = 5$) arbitrary units (Fig. 4C). In addition, $GdCl_3$ administration did not modify the decreasing effect of IR on NF-κB DNA binding compared with sham-operated animals (Fig. 4A and B) as evidenced by the net diminution of 44.7 ± 4.8 ($n = 6$) arbitrary units (Fig. 4C). IR in $GdCl_3$-pretreated T_3-treated animals also decreased liver NF-κB activation when compared with $GdCl_3$–T_3-sham rats (Fig. 4A and B), resulting in a net decrease of 7 ± 6 ($n = 6$) arbitrary units, which is significantly different to that found upon T_3 preconditioning without $GdCl_3$ pretreatment (Fig. 4C).

4. Discussion

Kupffer cells play key roles both in the homeostatic responses to liver damage and in those related to acute and chronic liver injury (Tsukamoto, 2002; Bilzer et al., 2006). Data reported in this work show that the IR protocol employed, involving 1 h ischemia followed by 20 h reperfusion, induces major changes in signal transduction and morphological parameters of the liver, including significant TNF-α response, loss of hepatic NF-κB DNA binding capacity, and substantial liver injury. Hepatic NF-κB is rapidly activated by IR, probably due to its higher sensitivity to ROS compared to others redox-sensitive transcription factors (Meyer et al., 1993), with a peak at 1–3 h mainly due to p50/p65 heterodimers triggering pro-inflammatory cytokine expression (Takahashi et al., 2001). Although the late phase of IR injury involves formation of p50 homodimers avoiding NF-κB p50/p65 binding to DNA, this protective response is abolished after 24 h reperfusion (Takahashi et al., 2001). Under these IR conditions, the drastic increase in the oxidative stress status of the liver and the concomitant TNF-α response observed may trigger various negative-feedback signaling mechanisms diminishing NF-κB activation, namely, binding of newly synthesized IκB proteins to nuclear NF-κB, inactivation of IκB kinase, and/or conjugation of IκB with the small ubiquitin-like protein SUMO-1 that prevents its degradation (Karin and Ben-Neriah, 2000). Contrarily to IR alone, administration of T_3 48 h prior to IR led to significant liver protection, which is related to the re-establishment of redox, TNF-α, and NF-κB homeostasis, in agreement with studies using ischemic hypothermia (Kuboki et al., 2007). These results point to the dual action of NF-κB in the induction of both pro-inflammatory and protective genes, which may be related to the degree of NF-κB activation achieved, and the mechanisms and type of hepatic cell populations involved (Luedde and Trautwein, 2006).

Data reported in this study demonstrate that T_3-induced liver preconditioning against IR injury is dependent upon Kupffer cell functioning. This was achieved by the administration of the Kupffer cell inactivator $GdCl_3$ 72 h prior to T_3, a protocol that achieved almost complete elimination of ED2(+) cells, without significantly

affecting ED1(+) cells or the morphological characteristics of liver parenchyma. Kupffer cells subpopulations were characterized with either a ED1 antibody recognizing a simple glycoprotein chain predominantly expressed on lysosomal membranes of macrophage populations, monocytes, and bone marrow precursors, or a ED2 antibody recognizing a membrane antigen of resident macrophages such as Kupffer cells (Damoiseaux et al., 1994; Ide et al., 2002). Liver ED2(+) cells are described as mature macrophages, often called large Kupffer cells (Armbrust and Ramadori, 1996), which are mainly located in periportal areas of the liver lobule (Bouwens et al., 1986). These mature liver macrophages have higher lysosomal enzyme activities, phagocytic capacity, and production of TNF-α, interleukin-1 and prostaglandin E_2 than smaller ED1(+) cells located in midzonal and central areas (Bilzer et al., 2006). TNF-α released from Kupffer cells exhibits paracrine actions interacting with specific receptors in target cells (Garg and Aggarwal, 2002). In hepatocytes, TNF-α–TNF-α receptor 1 coupling plays an important role in the homeostatic response to oxidative stress, triggering defense and reparative processes against injury under conditions of moderate prooxidant status, low levels of transient TNF-α expression and NF-κB activation (Diehl, 2000; Tsukamoto, 2002; Garg and Aggarwal, 2002), as those induced by T_3 administration (Videla et al., 2007). Thus, depletion of ED2(+) cells by $GdCl_3$ ensures diminution in Kupffer cell functioning, as evidenced by the significant reduction in colloidal carbon phagocytosis and abolishment of the carbon-induced respiratory burst activity observed in the perfused liver of $GdCl_3$-treated rats (Tapia et al., 1997). Under the experimental conditions used, T_3-induced liver preconditioning against IR injury was abolished by $GdCl_3$ pretreatment. This was shown by the re-establishment of both high levels of serum transaminases associated with substantial liver damage and TNF-α response, with partial diminution in liver NF-κB activation after IR, which were suppressed by T_3 in animals without Kupffer-cell inactivation. Loss of T_3 liver preconditioning by $GdCl_3$ pretreatment is related to deficient NF-κB DNA binding that may lead to derangement of the expression of protective NF-κB-dependent genes during IR. This response elicited by $GdCl_3$ could also be contributed by a sustained activation of hepatic AP-1 controlling TNF-α expression (Tsukamoto and Lin, 1997), considering that T_3 preconditioning reversed the substantial increase in AP-1 DNA binding and TNF-α response induced by IR (Fernández et al., 2008), which in the present study occurs under conditions of Kupffer-cell repopulation. This aspect is currently under investigation in our laboratory.

In conclusion, data presented suggest that Kupffer cell functioning is essential for the development of T_3 liver preconditioning, as shown by the re-establishment of liver injury after ED2(+) Kupffer cell elimination by $GdCl_3$ administration prior to T_3. T_3 liver preconditioning underlie the redox activation of signaling protective pathways by actions triggered at the Kupffer cell level, which may include Kupffer-cell-dependent hepatocyte proliferation through AP-1 activation (Fernández et al., 2007a) to cope with cell loss due to IR-induced hepatocelular necrosis (Fernández et al., 2007b) or partial hepatectomy (Malik et al., 2005). In addition to the liver, thyroid hormone preconditioning was also demonstrated in the heart, as evidenced by protection from IR injury by long-term thyroxine administration (Pantos et al., 2002), a response that is associated with attenuation of p38 mitogen-activated protein kinase activation and up-regulation of heat shock protein-70 (Pantos et al., 2001). Interestingly, decrease in cardiac performance resulting from brain death or a period of cardiopulmonary bypass, can be reversed by hormonal replacement therapy, in which T_3 plays a critical role (Cooper et al., 2009). Thus, studies addressing the molecular mechanisms underlying T_3 preconditioning are worthy of further investigation, to support its clinical application in preventing IR injury secondary to surgery or organ transplantation.

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