

# Causal role of oxidative stress in liver preconditioning by thyroid hormone in rats

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## Abstract

Hepatic ischemia–reperfusion (IR) injury, a major clinical drawback during surgery, is abolished by L-3,3',5-triiodothyronine (T<sub>3</sub>) administration. Considering that the triggering mechanisms are unknown, the aim of this study is to assess the role of oxidative stress in T<sub>3</sub> preconditioning using N-acetylcysteine (NAC) before T<sub>3</sub> administration. Male Sprague–Dawley rats given a single dose of 0.1 mg of T<sub>3</sub>/kg were subjected to 1 h ischemia followed by 20 h reperfusion, in groups of animals pretreated with 0.5 g of NAC/kg 0.5 h before T<sub>3</sub> or with the respective control vehicles. At the end of the reperfusion period, blood and liver samples were taken for analysis of serum aspartate aminotransferase (AST) and hepatic histology, glutathione (GSH) and protein carbonyl contents, and nuclear factor-κB (NF-κB) and activating protein 1 (AP-1) DNA binding. The IR protocol used led to a 4.5-fold increase in serum AST levels and drastic changes in liver histology, with significant GSH depletion and enhancement of protein carbonyl levels and of the protein carbonyl/GSH content ratio, whereas NF-κB and AP-1 DNA binding was decreased and enhanced, respectively. In a time window of 48 h, T<sub>3</sub> exerted protection against hepatic IR injury, with 88% reduction in the protein carbonyl/GSH ratio and normalization of NF-κB and AP-1 DNA binding, changes that were suppressed by NAC administration before T<sub>3</sub>. Data presented suggest that a transient increase in the oxidative stress status of the liver is an important trigger for T<sub>3</sub> preconditioning, evidenced in a warm IR injury model through antioxidant intervention.

**Keywords:** Oxidative stress; Thyroid hormone; Liver preconditioning; Ischemia–reperfusion injury; N-Acetylcysteine; Free radicals

Enhancement of the resistance of an organ to limit the detrimental effects of injurious stimuli constitutes the preconditioning phenomenon. This has been extensively studied in relation to ischemia–reperfusion (IR) injury, which in the liver is associated with transplantation, tissue resection under inflow occlusion (Pringle maneuver), and hypoperfusion shock [1–3]. Liver preconditioning strategies include the use of different additives

**Abbreviations:** AP-1, activator protein 1; AST, aspartate aminotransferase; GSH, reduced glutathione; IL, interleukin; IR, ischemia–reperfusion; JNK, c-Jun N-terminal kinase; NAC, N-acetylcysteine; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; T<sub>3</sub>, L-3,3',5-triiodothyronine; TNF-α, tumor necrosis factor-α.

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in University of Wisconsin preservation solution to minimize deleterious effects of cold ischemia in liver transplantation, as well as pharmacological treatments, gene therapy, and ischemic preconditioning [1,2,4,5]. In addition, strategies underlying sublethal oxidative stress that mimic the benefits of ischemic preconditioning have also been evaluated, such as hyperthermia [6], the model oxidants doxorubicin [7] and *tert*-butyl hydroperoxide [8], hyperbaric oxygen therapy [9], and ozone [10]. Under these conditions, the moderate increase in reactive oxygen (ROS) and nitrogen species in a defined time window may determine an imbalance capable of redox regulation [11,12], inducing cytoprotective responses against ischemia–reperfusion [4]. Cytoprotection by oxidative stress may involve signals regulating either protein function, through reversible oxidation or nitrosylation of protein sulfhydryls, or gene expression, through modulation of

specific kinases, phosphatases, and redox-sensitive transcription factors, or both [2,11,12].

Previous studies by our group revealed that thyroid hormone (L-3,3',5-triiodothyronine, T<sub>3</sub>) calorogenesis in the rat involves higher rates of O<sub>2</sub> consumption in the liver, with production of ROS in hepatocytes and Kupffer cells and antioxidant depletion [13]. This enhancement of the oxidative stress status of the liver, which is considered a mild redox alteration due to the lack of occurrence of morphologic changes in liver parenchyma, except for the hyperplasia and hypertrophy of Kupffer cells [14], was found to trigger the redox regulation of gene expression [13]. At the level of the Kupffer cells, hepatic macrophages that seem to be more responsive to ROS than hepatocytes [16,17], redox upregulation of cytokine expression (tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1, IL-6) is achieved [15]. This feature may be related to the presence and activation of NADPH oxidase, a multicomponent enzyme representing a major source of ROS in inflammatory cells [18], in Kupffer cells and to the fact that most of the antioxidant defenses of the liver are essentially confined to parenchymal cells [19]. T<sub>3</sub>-induced Kupffer cell-derived cytokines interact with surface receptors in target cells within the liver in order to mediate signaling from membrane to the nucleus, with the consequent upregulation of inducible nitric oxide synthase [20], manganese superoxide dismutase, Bcl-2 [21], acute-phase proteins [22], and proliferative response in hepatocytes [23]. The above responses, which represent adaptive mechanisms to reestablish redox homeostasis and promote cell survival, occur via NF- $\kappa$ B, STAT3, and AP-1 activation and afford protection against IR liver injury, thus representing an alternate preconditioning strategy [24]. The objective of this study was to test the hypothesis that T<sub>3</sub>-induced liver preconditioning is triggered by the oxidative

stress status associated with the calorogenic action of T<sub>3</sub>. For this purpose, T<sub>3</sub> liver preconditioning was assessed in a model of partial hepatic IR injury in the rat, either without or with pretreatment with the antioxidant *N*-acetylcysteine (NAC) [25], the results of which were correlated with parameters related to oxidative stress and cell death signaling, namely, NF- $\kappa$ B and AP-1 activation.

## Materials and methods

### *Animal treatments and model of partial hepatic ischemia–reperfusion injury*

Male Sprague–Dawley rats (Animal Facility of the Institute of Biomedical Sciences, Faculty of Medicine, University of Chile) weighing 180–200 g were housed on a 12-h light/dark cycle and were provided with rat chow and water ad libitum. Animals received a single intraperitoneal dose of 0.1 mg of T<sub>3</sub>/kg body weight or equivalent volumes of hormone vehicle (0.1 N NaOH, controls) at time 0 (Fig. 1). At 48 h after hormone 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), and 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, as previously described [24]. Control animals were subjected to anesthesia and sham laparotomy. Studies with NAC were carried out in the above-described groups receiving either 0.5 g/kg NAC or saline, 0.5 h before T<sub>3</sub> administration, thus comprising eight experimental groups (Fig. 1). At the end of the reperfusion

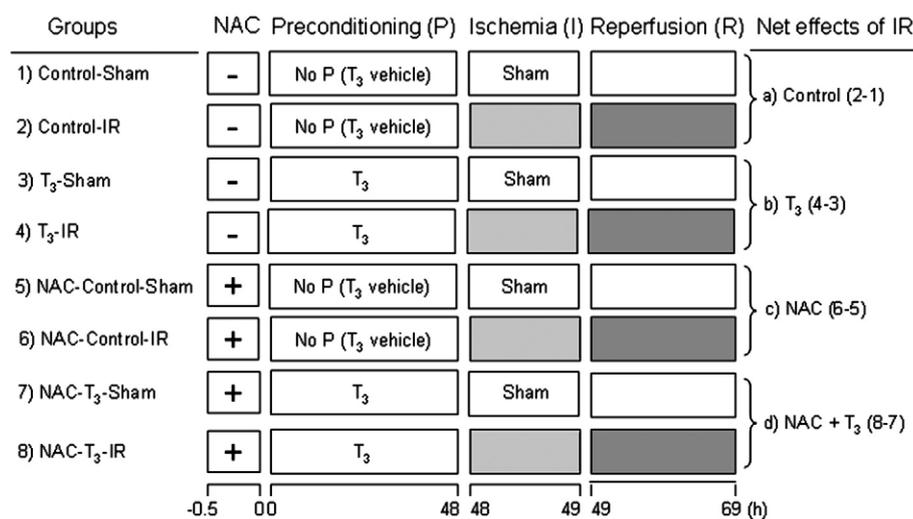


Fig. 1. Experimental protocol for the effects of *N*-acetylcysteine (NAC) on T<sub>3</sub> preconditioning. Animals were given either T<sub>3</sub> vehicle or a single dose of T<sub>3</sub> (0.1 mg · kg<sup>-1</sup>) at time 0. At 48 h after hormone administration, groups of control rats and T<sub>3</sub>-treated animals were subjected to sham operation or to 1 h ischemia (light gray bars) followed by 20 h reperfusion (dark gray bars). The effects of NAC were assessed by subjecting the above-described groups to either saline (0.9% NaCl (-)) or NAC (0.5 g · kg<sup>-1</sup> (+)) 0.5 h before T<sub>3</sub>, thus comprising eight experimental groups. Blood and liver samples were obtained at the end of the reperfusion period. Net changes induced by ischemia–reperfusion (IR) under conditions of no treatments (group (a) control) and after administration of T<sub>3</sub> (group (b)), NAC (group (c)), or NAC plus T<sub>3</sub> (group (d)) were calculated by subtracting individual values of control-IR minus the average value of control-sham, T<sub>3</sub>-IR minus T<sub>3</sub>-sham, NAC-IR minus NAC-sham, or NAC-T<sub>3</sub>-IR minus NAC-T<sub>3</sub>-sham, respectively.

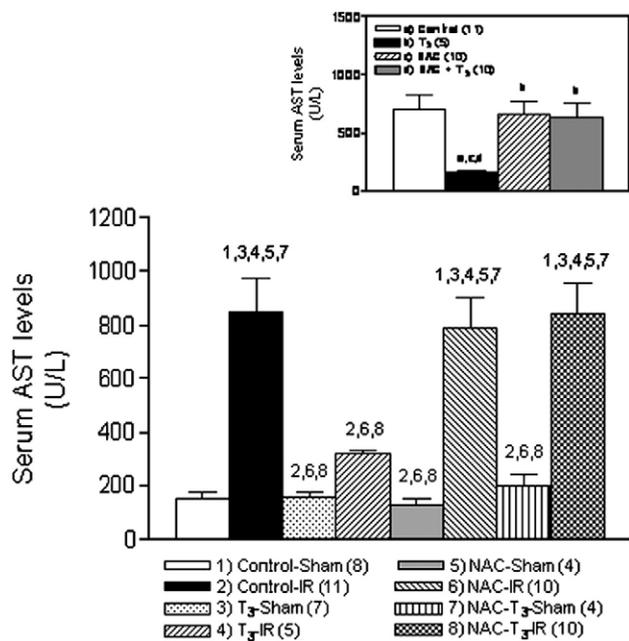


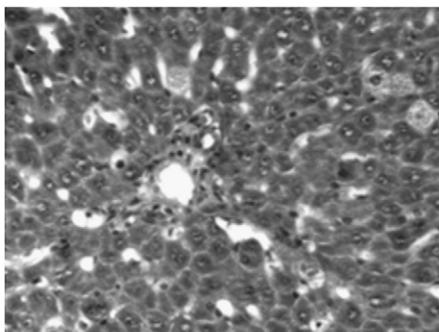
Fig. 2. Effect of NAC administration on the serum levels of AST after hepatic IR injury in unpreconditioned and T<sub>3</sub>-preconditioned rats. Inset: Net changes induced by IR under conditions of no treatment (group a) control and after administration of T<sub>3</sub> (group b), NAC (group c), or NAC plus T<sub>3</sub> (group d), calculated as described for Fig. 1. Values shown correspond to the means  $\pm$  SEM for the number of animals indicated in parentheses. Significance ( $p < 0.05$ ) is shown by the numbers (or letters in the inset) identifying each experimental group.

period (Fig. 1) blood samples were obtained by cardiac puncture for serum AST and TNF- $\alpha$  (ELISA; Biosource International, Camarillo, CA, USA) assessment, and liver samples taken from the medial lobes were processed immediately (glutathione and protein carbonyls measurements), frozen in liquid nitrogen (assessment of transcription factors), 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).

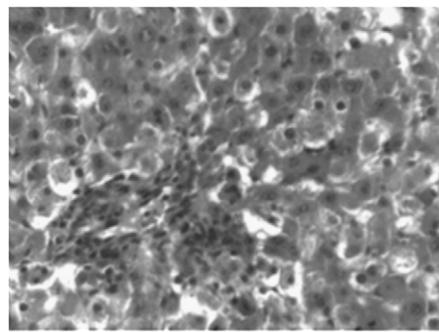
#### Plasma NAC levels

Blood was obtained by cardiac puncture in anesthetized (Zoletil 50) rats and plasma concentrations of total NAC were determined by the method of Jaworska et al. [26], with minor modifications. Briefly, plasma proteins were precipitated with perchloric acid and removed by centrifugation, and the supernatant was alkalinized with 1 M NaOH and mixed with borate buffer (100 mmol/L, pH 8.4). Capillary electrophoresis was performed in a fused-silica capillary, 72 cm (52 cm to detector)  $\times$  70  $\mu$ m i.d., by using a 270A-HT capillary electrophoresis system (Applied Biosystems, Foster City, CA, USA). A constant voltage of 25 kV, with the resulting current intensity of 10–20  $\mu$ A, was applied, and UV absorption was measured at 214 nm. Samples were introduced hydrodynamically with a

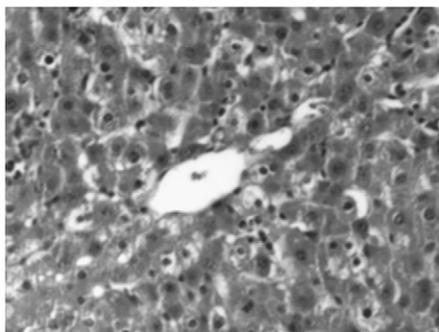
A. NAC-Control-Sham



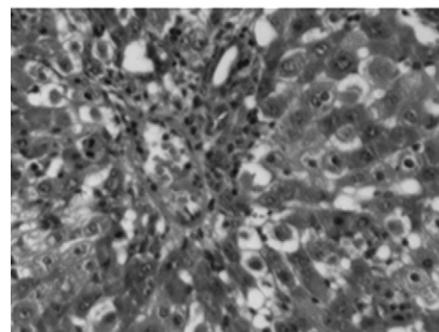
B. NAC-Control-IR



C. NAC-T<sub>3</sub>-Sham



D. NAC-T<sub>3</sub>-IR



vacuum of 5 in. of mercury. Standard calibration curves of increasing NAC concentrations against peak areas were constructed. The detection limit for NAC was 5  $\mu\text{g/ml}$ .

#### Parameters related to oxidative stress and liver injury

In anesthetized animals, livers were perfused in situ with a cold solution containing 159 mM KCl and 5 mM Tris (pH 7.4) to remove blood, and total reduced glutathione (GSH) [27], protein carbonyl [28], and total protein contents [29] were measured. In all experiments, the severity of liver damage was determined by measuring serum AST levels and by performing liver light microscopy.

#### NF- $\kappa\text{B}$ and AP-1 electromobility shift assay

Nuclear protein extracts from liver samples were prepared [30] and subjected to electromobility shift assay for assessment of NF- $\kappa\text{B}$  and AP-1 DNA binding, using the NF- $\kappa\text{B}$  probe 5'-GATCTCAGAGGGGACTTTCCGAG-3' or the AP-1 probe 5'-CGCTTGATGAGTCAGCCGAA-3' (Invitrogen Life Technologies, Carlsbad, CA, USA), labeled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP using the Klenow DNA Polymerase Fragment I (Invitrogen Life Technologies), as described previously [16,21,22,24]. 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- $\kappa\text{B}$  and AP-1 bands were detected by autoradiography and quantified by densitometry using Scion Image (Scion Corp., Frederick, MD, USA).

#### Statistical analyses

Values shown represent the means  $\pm$  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  $\text{T}_3$  (group b), NAC (group c), or NAC plus  $\text{T}_3$  (group d) were calculated by subtracting individual values of control-IR minus the average value of control-sham,  $\text{T}_3$ -IR minus  $\text{T}_3$ -sham, NAC-IR minus NAC-sham, or NAC- $\text{T}_3$ -IR minus NAC- $\text{T}_3$ -sham, respectively (Fig. 1). 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.

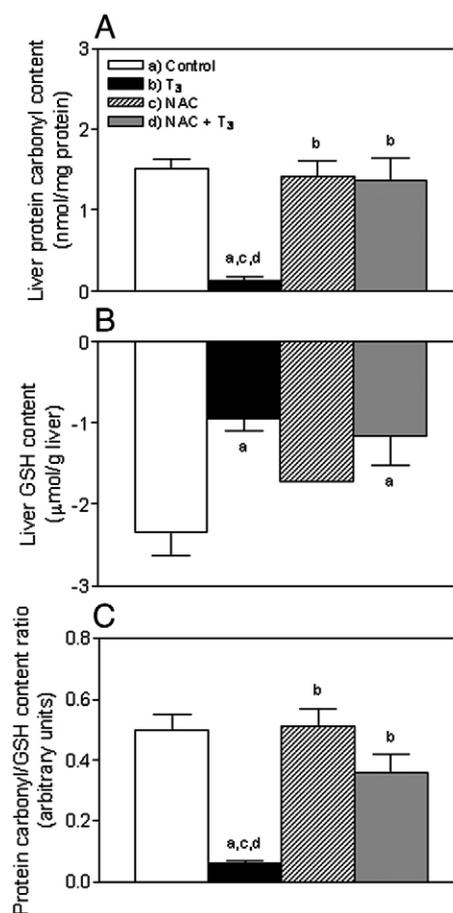
## Results

#### Plasma NAC levels

The administration of 0.5 g/kg NAC to fed animals led to a rapid enhancement of the circulating levels of the antioxidant, which amounted to  $1680 \pm 79$  ( $n=4$ )  $\mu\text{g/ml}$  after 0.5 h treatment. The plasma NAC levels were not detectable after 48.5 h of NAC administration, a time at which the hepatic GSH content was comparable to that in control animals given saline (controls,  $4.34 \pm 0.13$   $\mu\text{mol/g}$  liver(5); NAC-treated rats,  $4.17 \pm 0.26$  (3)).

#### Serum AST, liver histology, and serum TNF- $\alpha$

One hour of partial hepatic ischemia induced by vascular clamping followed by reperfusion for 20 h (Fig. 1) achieved minimal mortality but extensive liver injury, as shown by a significant 4.5-fold increase in serum AST levels, compared with sham-operated animals (Fig. 2). In  $\text{T}_3$ -preconditioned rats, IR led to a 1.05-fold enhancement of serum AST levels (Fig. 2), thus eliciting a net diminution of 77% in relation to the unpreconditioned group (Fig. 2, inset). After NAC administration, IR induced a 5.2-fold increase over the NAC-sham group and 3.2-fold enhancement over the NAC- $\text{T}_3$ -sham group (Fig. 2), thus abolishing the preconditioning effect of  $\text{T}_3$  (Fig. 2, inset). Similar results were observed when serum ALT levels were assessed (data not shown). In agreement with serum transaminase data, control animals subjected to NAC-sham or to NAC- $\text{T}_3$ -sham conditions exhibited normal liver histology (Figs. 3A and 3C), whereas the NAC-IR group showed mild to moderate congestion, scattered cellular necrosis (coagulation), and isolated sinusoidal neutrophil infiltration (Fig. 3B). Furthermore,



the NAC-T<sub>3</sub>-IR group exhibited substantial distortion of liver architecture, extensive necrosis in perivenular parenchyma reaching periportal zones, inflammation, and neutrophil infiltration, with scattered cellular necrosis and ballooning (Fig. 3D). IR led to a 130% enhancement of the serum levels of TNF- $\alpha$  ( $p < 0.05$ ) over control values in unpreconditioned rats, an effect that was abolished by T<sub>3</sub> preconditioning and reestablished in T<sub>3</sub>-treated animals pretreated with NAC ((1) control-sham,  $26 \pm 3$  pg/ml ( $n = 4$ ); (2) control-IR,  $60 \pm 5$  ( $n = 4$ )<sup>1,3,4,5,7</sup>; (3) T<sub>3</sub>-sham,  $35 \pm 2$  ( $n = 4$ )<sup>2,6,8</sup>; (4) T<sub>3</sub>-IR,  $34 \pm 3$  ( $n = 4$ )<sup>2,6,8</sup>; (5) NAC-control-sham,  $25 \pm 3$  ( $n = 9$ )<sup>2,6,8</sup>; (6) NAC-control-IR,  $58 \pm 5$  ( $n = 5$ )<sup>1,3,4,5,7</sup>; (7) NAC-T<sub>3</sub>-sham,  $26 \pm 3$  ( $n = 11$ )<sup>2,6,8</sup>; (8) NAC-T<sub>3</sub>-IR,  $60 \pm 4$  ( $n = 6$ )<sup>1,3,4,5,7</sup>;  $p < 0.05$  as shown by the numbers identifying each group).

#### Liver parameters related to oxidative stress

IR liver injury was found concomitant with a 122% increase in protein carbonylation compared with the control-sham group, with a net increase of  $1.52 \pm 0.12$  nmol/mg protein ( $n = 4$ ), an effect that was reduced by 91% by T<sub>3</sub> treatment but comparable to that elicited after NAC or NAC + T<sub>3</sub> administration (Fig. 4A). Also, IR induced a 37% depletion in liver GSH content over values in the control-sham group, with a net decrease of  $2.38 \pm$

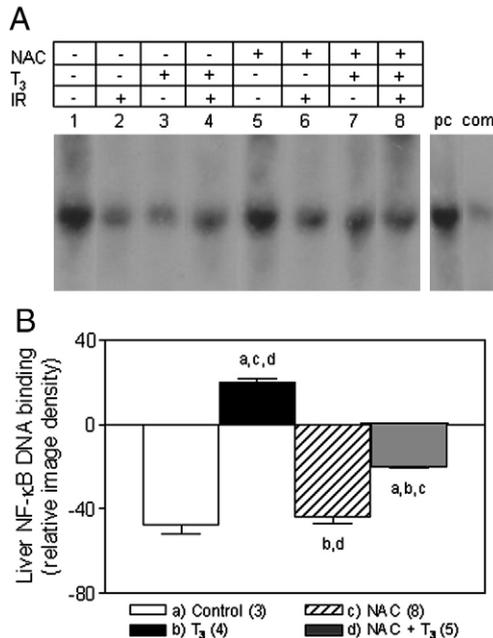


Fig. 5. Effect of NAC administration on liver NF- $\kappa$ B DNA binding on electromobility shift assay after hepatic IR injury in unpreconditioned and T<sub>3</sub>-preconditioned rats. (A) Autoradiographs representing lanes loaded with 8  $\mu$ g nuclear protein from an animal of each experimental group, as described for Fig. 1, and from a control-sham-operated rat (group 1) in competition experiments without (positive control, pc) and with 100-fold molar excess of the unlabeled DNA probe (com). (B) Bar graphs corresponding to densitometric quantification of relative NF- $\kappa$ B DNA binding, expressed as net changes induced by IR under conditions of no treatment (group (a) control) and after administration of T<sub>3</sub> (group b), NAC (group c), or NAC plus T<sub>3</sub> (group d), calculated as described for Fig. 1. Values shown are means  $\pm$  SEM for the number of animals indicated in parentheses. Significance ( $p < 0.05$ ) is shown by the letters identifying each experimental group.

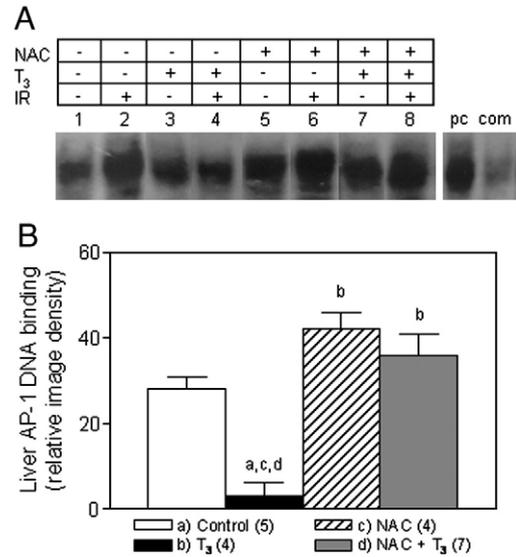


Fig. 6. Effect of NAC administration on liver AP-1 DNA binding on electromobility shift assay after hepatic IR injury in unpreconditioned and T<sub>3</sub>-preconditioned rats. (A) Autoradiographs representing lanes loaded with 8  $\mu$ g nuclear protein from an animal of each experimental group, as described for Fig. 1, and from a control-sham-operated rat (group 1) in competition experiments without (positive control, pc) and with 100-fold molar excess of the unlabeled DNA probe (com). (B) Bar graphs corresponding to densitometric quantification of relative AP-1 DNA binding, expressed as net changes induced by IR under conditions of no treatments (group (a) control) and after administration of T<sub>3</sub> (group b), NAC (group c), or NAC plus T<sub>3</sub> (group d), calculated as described for Fig. 1. Values shown are means  $\pm$  SEM for the number of animals indicated in parentheses. Significance ( $p < 0.05$ ) is shown by the letters identifying each experimental group.

$0.31 \mu\text{mol/g}$  liver ( $n = 5$ ), a change that exhibited a 59% reduction by T<sub>3</sub> administration and no significant changes after NAC treatment, being decreased by 33% upon NAC + T<sub>3</sub> administration (Fig. 4B). Accordingly, the calculated protein carbonylation/GSH content ratio exhibited a net increase of  $0.50 \pm 0.05$  arbitrary units ( $n = 4$ ) by IR over values in the control-sham group, which was diminished by 88% by T<sub>3</sub>, without significant changes being found after NAC and NAC + T<sub>3</sub> administration (Fig. 4C). These data show that T<sub>3</sub>-dependent diminution of the protein carbonyl/GSH content ratio, induced by IR in the liver, is suppressed by 68% by the administration of NAC before T<sub>3</sub> (Fig. 4C).

#### Liver DNA binding of NF- $\kappa$ B and AP-1

IR led to a significant 52% diminution of liver NF- $\kappa$ B DNA binding compared with control-sham-operated rats (Fig. 5A), with a net decrease of  $48 \pm 4$  arbitrary units ( $n = 3$ ) (Fig. 5B), whereas IR in T<sub>3</sub>-treated animals resulted in a 38% increase in NF- $\kappa$ B activation over the T<sub>3</sub>-sham group (Fig. 5A), leading to a net enhancement of  $20 \pm 2$  arbitrary units ( $n = 4$ ) (Fig. 5B). In addition, NAC administration did not modify the decreasing effect of IR on NF- $\kappa$ B DNA binding compared with sham-operated animals (Fig. 5A) as evidenced by the net diminution of  $44 \pm 3$  arbitrary units ( $n = 8$ ) (Fig. 5B). IR in NAC-pretreated T<sub>3</sub>-treated animals also decreased liver NF- $\kappa$ B activation

compared with NAC-T<sub>3</sub>-sham rats (Fig. 5A), resulting in a net decrease of 20±1 arbitrary units (*n*=5), which is significantly different from that observed upon T<sub>3</sub> preconditioning without NAC pretreatment (Fig. 5B).

Liver DNA binding of AP-1 was significantly augmented by IR over control-sham values (129%; *p*<0.05) (Fig. 6A), with a net increase of 28±3 arbitrary units (*n*=5) (Fig. 6B), an effect that was reduced by 89% in T<sub>3</sub>-preconditioned animals (Fig. 6A), leading to a net enhancement of 3±3 arbitrary units (*n*=4) (Fig. 6B). Furthermore, NAC administration did not modify the enhancing effect of IR on hepatic AP-1 DNA binding, compared with sham-operated animals (Fig. 6A), as evidenced by the net increase of 42±4 arbitrary units (*n*=4) (Fig. 6B). IR in NAC-pretreated T<sub>3</sub>-treated animals also increased liver AP-1 activation compared with NAC-T<sub>3</sub>-sham rats (Fig. 6A), resulting in a net enhancement of 36±5 (*n*=7) arbitrary units, which is significantly different from that observed upon T<sub>3</sub> preconditioning without NAC pretreatment (Fig. 6B).

Taking into account the net changes found under the studied experimental conditions, it can be estimated that T<sub>3</sub>-dependent enhancement of liver NF-κB DNA binding (Fig. 5B) and diminution of that of AP-1 (Fig. 6B) induced by IR are suppressed by 63 and 76%, respectively, by the administration of NAC before T<sub>3</sub>. Furthermore, T<sub>3</sub> preconditioning led to a net 67% diminution of the AP-1/NF-κB DNA binding ratio (IR, 1.12±0.08 (*n*=5); T<sub>3</sub>-IR, 0.37±0.04 (*n*=4); *p*<0.05), a change that was abolished by NAC administration before T<sub>3</sub> (NAC-IR, 1.78±0.06 (*n*=4); NAC-T<sub>3</sub>-IR, 1.80±0.06 (*n*=6); not significantly different).

## Discussion

The magnitude of the oxidative stress status developed under a given condition is a major factor determining the cellular responses achieved [11,12,31], being drastic in IR liver injury and moderate in T<sub>3</sub> preconditioning [24]. The data presented in this work demonstrate that reperfusion for 20 h after 1 h of warm ischemia induces significant liver injury and enhancement of oxidative stress-related parameters, as reported earlier [24]. Furthermore, IR was found to concomitantly alter the activation of redox-sensitive transcription factors that are related to liver injury, namely, reduction in DNA binding of NF-κB and enhancement of that of AP-1, with a significant TNF-α response. Although changes in NF-κB and AP-1 activation are presumed to exhibit a similar pattern after IR, the differential effects found may be due to differences in functional properties of the transcription factors: (i) NF-κB shows a higher sensitivity to activation by ROS than AP-1 and (ii) NF-κB preexists in the cells in a latent form, whereas AP-1 requires de novo synthesis of its components c-jun and c-fos for full activation of target genes [32]. These differences may explain the rapid activation of hepatic NF-κB, compared to AP-1, observed under IR conditions [33,34] or in Kupffer cells exposed to LPS [16], as NF-κB exhibits an early peak (1–3 h) mainly composed of p50/p65 heterodimers and a late peak (12 h) mainly composed of p50 homodimers that is suppressed by 24–48 h reperfusion [35]. It is proposed that the drastic

enhancement of the oxidative stress status and TNF-α response in liver IR assessed in the late phase of injury (20 h) may downregulate signal transduction and gene expression, in agreement with the diminution of NF-κB activation and related cytoprotective functions such as the acute-phase response [24]. Diminished DNA binding of hepatic NF-κB after IR injury may involve the induction of several negative-feedback mechanisms, namely: (i) inhibitor of κB (IκB) kinase inactivation, (ii) binding of newly synthesized IκB proteins to nuclear NF-κB, and/or (iii) formation of a modified form of IκBα conjugated with the small ubiquitin-like protein SUMO-1, which is resistant to signal-induced degradation [36,37]. In addition to loss of hepatic NF-κB activation, IR resulted in significant enhancement of AP-1 DNA binding. This observation agrees with the view that depression of NF-κB activation increases the susceptibility to TNF-α-induced injury, concurrent with sustained activation of c-Jun N-terminal kinase (JNK), a major contributor to the death response [38]. JNK phosphorylates and activates the c-Jun component of AP-1, and JNK, phosphorylated c-Jun, and AP-1 DNA binding are drastically augmented under IR conditions (Fig. 6) [33,34,39,40]. Sustained JNK activation was reported to depend on TNF-α-induced ROS generation at the mitochondrial level, due to oxidation and inhibition of JNK-inactivating phosphatases, specifically those of the MAP kinase phosphatase subfamily of dual-specificity phosphatases [38]. However, the exact role of these phosphatases in JNK activation and the mechanisms underlying reduction of NF-κB DNA binding remain to be established in liver IR injury.

The administration of a single dose of T<sub>3</sub> to rats elicited substantial protection against IR liver injury, when given 48 h before the IR protocol. Within this time interval, T<sub>3</sub> led to a temporary and reversible increase in the oxidative stress status of the liver in the absence of hepatotoxicity, concomitant with the transient enhancement of the activity of Kupffer cells [24], hepatic macrophages playing crucial roles in the homeostatic response to liver injury [18]. T<sub>3</sub> preconditioning in liver IR is related to the reestablishment of TNF-α homeostasis [24], with normalization of the oxidative stress status and recuperation of hepatocellular signaling functions altered during IR, namely, upregulation of NF-κB and downregulation of AP-1. Although these responses may protect the liver against IR-induced severe oxidative stress and TNF-α response, hepatocyte proliferation triggered by T<sub>3</sub> may also play a role [23], compensating for liver cell loss due to IR-dependent hepatocellular necrosis.

The present study demonstrates that T<sub>3</sub>-induced transient and reversible oxidative stress plays a causal role in the preconditioning mechanism of the hormone against liver IR injury. This was achieved by the use of NAC 0.5 h before T<sub>3</sub> administration, a protocol that results in significant circulating levels of the antioxidant, which are not detectable at the end of the preconditioning period of 48 h, in agreement with the elimination half-life of 1 to 4.3 h reported for total NAC in rats [41]. At this experimental time, animals treated with NAC and control rats exhibited comparable levels of hepatic GSH, thus ensuring the lack of interference of NAC with the oxidative stress mechanisms triggered by IR. Under these conditions, T<sub>3</sub> preconditioning was antagonized by NAC pretreatment, as evidenced by the

presence of liver injury, oxidative stress enhancement, and alteration in the DNA binding capacity of NF- $\kappa$ B and AP-1 induced by the IR protocol used with reestablishment of the TNF- $\alpha$  response, which were suppressed by T<sub>3</sub> in the absence of NAC treatment. NAC-dependent suppression of T<sub>3</sub> preconditioning may result from the abolishment of the oxidative stress component induced by T<sub>3</sub> considering the antioxidant properties of NAC, namely, direct free-radical scavenging action and stimulation of GSH biosynthesis [25]. In addition, NAC may also act by reducing relevant thiol groups in key signaling proteins, thereby altering signal-transduction pathways [17,42–44] that may be involved in T<sub>3</sub> preconditioning [13,15,20–23].

In conclusion, data presented here indicate that the development of transient and reversible oxidative stress in the liver of rats acutely treated with T<sub>3</sub> has a causal role in protection against IR injury, as evidenced by the reestablishment of liver damage after the administration of NAC before T<sub>3</sub>. Loss of T<sub>3</sub> preconditioning by NAC is associated with the recovery of two major factors drastically enhanced by IR and normalized by T<sub>3</sub>, namely, the mild pro-oxidant status of the liver and the AP-1/NF- $\kappa$ B DNA binding ratio. Under the IR conditions used, the enhancement of the AP-1/NF- $\kappa$ B ratio observed may be related to liver injury by determining either a deficient NF- $\kappa$ B DNA binding with loss of liver cell signaling functions, leading to cytoprotection, or a sustained AP-1 activation with exacerbation of cell death responses, or both. The results of this study support T<sub>3</sub> preconditioning as a novel strategy to protect the liver and other organs against IR injury. This is particularly important considering that, with the exception of ischemic preconditioning that may find application in human liver resections [45] and transplantation [46], all other preconditioning strategies studied have not gained access to clinical application [2,4]. Contrary to this view, T<sub>3</sub> preconditioning has clinical potential, considering that T<sub>3</sub> is an endobiotic substance, a widely used and well-tolerated therapeutic agent, which at low doses has either no important adverse effects or minimal side effects that can be readily controlled [47]. However, further studies are required to understand the molecular mechanisms underlying T<sub>3</sub> preconditioning in experimental animals and humans, to support its application in preventing IR injury during liver surgery or in liver transplantation using reduced-size grafts from living donors.

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