Ischemia and reperfusion (IR) of the liver lead to severe injury (IRI), a feature observed in liver transplantation and liver resection that is associated with oxygen and nutrient deprivation occurring during the procedure in the former case and with vascular occlusion to prevent hemorrhage in the latter case [1,2]. The mechanisms underlying liver IRI gathered in experimental models led to the development of numerous surgical and pharmacological protective strategies; however, few have reached clinical practice [1,2]. Development of an acute, nonlethal burst of oxidative stress constitutes a major pharmacological liver preconditioning (PC) strategy [3,4], which includes the administration of thyroid hormone calorigenesis (T3; triiodothyronine, T3) before an IR protocol [5], as evidenced by the reestablishment of liver injury after the administration of the antioxidant N-acetylcysteine (NAC) before T3 [6]. T3 is considered a hormetic agent that triggers biologically beneficial effects in the liver in the low-dose range, leading to cell proliferation and the induction of proteins affording cellular protection, namely, antioxidant, antiapoptotic, and acute-phase proteins [7]; phase II detoxication enzymes; and phase III transporters [8]; and downregulation of the mRNA and protein expression of cytokines and adhesion molecules related to liver inflammation [9]. Accordingly, liver PC is confronted with a major energy requirement to cope with ATP demands for operation of most of the PC mechanisms outlined above, plus those for repair and resynthesis of altered biomolecules during IR and repletion of ATP levels reduced in the ischemic phase [7]. In agreement with this view, T3 administration upregulates liver AMP-activated protein kinase (AMPK) [10], a sensor of energy status supporting cellular energy homeostasis [11]. Liver AMPK upregulation by T3 is associated with significant enhancements in (i) AMPK mRNA expression, (ii) AMPK phosphorylation coupled to the activation of the upstream kinases Ca2+/calmodulin-dependent protein kinase kinase-β and transforming growth factor-β-activated kinase-1, and (iii) AMP/ATP ratios promoting allosteric AMPK activation [10]. Consequently, AMPK signaling induced by T3 enhances hepatic fatty acid oxidation leading to a ketogenic response, representing a key molecular mechanism regulating energy dynamics to limit metabolic stresses such as IRI [12]. T3-induced liver O2 consumption is accompanied by an elevation in the rate of mitochondrial superoxide radical (O2•−) [13] and hydrogen peroxide (H2O2) [13,14] generation. Liver production of reactive oxygen species (ROS) induced by T3 is also elicited at microsomal, cytosolic, and peroxisomal levels in hepatocytes, with Kupffer cells playing a contributory role owing to enhancement of
the respiratory burst activity [15]. Under these conditions, T3 achieved a substantial enhancement in hepatic free radical-de-pendent protein oxidation, assayed by the reaction of protein carbonyls with 2,4-dinitrophenylhydrazine, in addition to a lipid peroxidation response [16]. Oxidized proteins undergo partial unfolding or misfolding due to the loss of secondary and tertiary structures within the domain of the oxidative impact, which results in proteome instability that may trigger endoplasmic reticulum (ER) stress [17]. The ER is primarily involved in the synthesis, folding, and maturation of secreted and transmembrane proteins, lipid biosynthesis, and Ca2+ homeostasis, and overload of these functions leads to ER stress and the unfolded protein re-
sponse (UPR) [18]. Upon ER stress, ER-located transmembrane sensors evoke diverse protective responses through exposure of hydrophobic domains in oxidized proteins, which may be re-
ognized by chaperones or by the proteasome, representing an important adaptive mechanism in secretory cells such as hepa-toocytes to attain homeostatic regulation [17,18]. Considering the role of T3 in liver PC [5,15] and that the cellular UPR program is aimed to alleviate ER stress promoting cell survival [19], the aim of this work was to test the hypothesis that in vivo T3 administration involving the development of oxidative stress with a protein oxida-
tion response triggers UPR development in the liver. For this purpose, rats were subjected to a T3 protocol after which para-
meters related to T3 calorigenesis, liver viability, and hepatic in-
dicators of oxidative stress (levels of reduced glutathione (GSH), 8-isoproteines, and protein carbonyls) and ER stress (components of the protein kinase RNA-like ER kinase (PERK) axis and protein disulfide isomerase (PDI))–ER oxidoreductin 1α (ERO1α) couple) were measured, followed by assessments in separate groups of animals upon antioxidant (NAC) intervention.

1. Materials and methods

1.1. Animal treatment

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 an intraperitoneal dose of 0.1 mg of T3/kg bw or equivalent volumes of hormone vehicle (0.1 N NaOH, controls) at time 0, 24, and 48 h, and studies were performed 24 h after hormone treat-
ment. At this experimental time, T3-induced calorigenesis was assessed by the rectal temperature of the animals by means of a thermocouple (Cole-Palmer Instrument Co., Chicago, IL, USA), and the rats were anesthetized with 1 ml/kg zolazepam chloride (25 mg/ml) and tiletamine chloride (25 mg/ml) ip (Zoletil 50; Virbac S/A, Carros, France) to obtain blood by cardiac puncture and liver samples. Blood serum was used to measure T3 levels via ELISA (Monobind, Lake Forest, CA, USA), aspartate amino transferase (AST) (international units/L), and albumin (Valtek Diagnostics, Santiago, Chile). Liver samples were fixed in 10% buffered formaldehyde and paraffin embedded, and sections were stained with hematoxylin–eosin for light-microscopy studies [20]. Sepa-
rate liver samples were taken, frozen in liquid nitrogen, and kept at −80 °C for protein carbonyl, 8-isoproteine, and GSH content measurements, in addition to those using Western blot and real-
time quantitative PCR analyses. The rate of O2 consumption was assessed polarographically in liver perfusion experiments in se-
parate groups of animals as previously described [21].

Studies with NAC were carried out in separate groups of rats receiving 0.5 g/kg or saline (controls) ip, 0.5 h before each dose of T3 administered, and studies were performed at 24 h after hor-
mone treatment in the resulting four experimental groups, namely, (a) controls, (b) T3, (c) NAC, and (d) NAC + T3. 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 the Ethics Committee of the Faculty of Medicine, University of Chile (Protocol CBA 0440 (FMUCH)).

1.2. Oxidative stress-related parameters

Liver samples frozen in liquid nitrogen and stored at −80 °C were used for 8-isoprostanes evaluation (ELISA kit, Cayman Che-

1.3. Western blot analysis

The cytosolic fraction was isolated from homogenized liver samples (300 mg) suspended in a buffer solution (pH 7.9), contain-
ing 10 mM Hepes, 1 mM EDTA, 0.6% NP-40, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitors (1 μg/
ml aprotinin, 1 μg/ml leupeptin), and phosphatase inhibitor (1 mM orthovanadate), followed by centrifugation (3000 g for 5 min). Protein concentration was determined using the BCA kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer’s protocols. A 60 μg protein sample was separated on 12% poly-
acrylamide gels using SDS–PAGE [24] and transferred to nit-
rocellulose membranes [25], which were blocked for 1 h at room temperature with Tris-buffered saline (TBS) containing 5% bovine serum albumin. The blots were washed with TBS containing 0.1% Tween 20, and the following primary antibodies were added: rabbit polyclonal primary antibodies for pPERK, PERK, phosph-
ylated eukaryotic translation initiation factor 2α (p-eIF2α), eIF2α, BiP, ATF-4, α-tubulin, and lamin A/C (1:1000; Cell Signaling Technology, Danvers, MA, USA). Then the blots were incubated at 4 °C overnight. In all determinations, rabbit monoclonal antibody for anti-α-tubulin was used as internal control. After extensive washing, the antigen–antibody complexes were detected using horseradish peroxidase goat anti-rabbit IgG or goat anti-mouse IgG and the SuperSignal West Pico chemiluminescence kit detec-
tion system (Pierce). Bands were quantified by densitometry using a gel documentation system, Biosens SC-750 (Shanghai Bio-Tech Co., Ltd., China). Results are expressed as relative units (individual protein/α-tubulin or phosphorylated protein/total protein). Lamin A/C was used to control the purity of cytosolic fractions (data not shown).

1.4. RNA isolation and cDNA synthesis

Total RNA was isolated from tissues with the RNeasy Lipid Tissue Mini Kit (Qiagen Sciences, Germantown, MD, USA) ac-
cording to the manufacturer’s instructions. Two micrograms of RNA was used for generation of cDNA using the ThermoScript RT-
PCR System (Life Technologies Corp., Carlsbad, CA, USA) according to the manufacturer’s protocols.

1.5. Real-time quantitative PCR

Real-time quantitative PCR was performed on a Stratagene Mx3005P thermocycler (Agilent Technologies, Santa Clara, CA, USA) using the Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) following the manufacturer’s protocols. The primer pair sequences used are shown in Table 1. Primers were optimized to yield 95–100% reaction efficiency with PCR products run on an
agarose gel to verify the correct amplification length (data not shown). Melt-curve analyses verified the formation of a single desired PCR product in each PCR. The expression levels of each sample were normalized against β-actin (internal control). The relative expression levels were calculated using the comparative CT method (\(\Delta\Delta CT\)).

1.6. Statistical analyses

Data showing Gaussian distribution according to the Kolmogorov–Smirnov test are expressed as means ± standard error of the mean (SEM) for the number of separate experiments indicated. As required, one-way ANOVA and the Newman–Keuls test or Student t test for unpaired data were used to assess the statistical significance (\(p < 0.05\)) of differences between mean values. To analyze the association between variables, the Pearson correlation coefficient was used. All statistical analyses were computed employing GraphPad Prism version 2.0 (GraphPad Software, San Diego, CA, USA).

2. Results

2.1. T3-induced calorogenesis is associated with liver oxidative stress involving protein oxidation

T3 administration according to the protocol used resulted in a significant sixfold increment in serum T3 levels over basal values (controls, 50 ± 10 ng/dl; T3-treated rats, 350 ± 20 ng/dl; \(p < 0.05\)), concomitant with a 10% diminution in those of albumin (controls, 3.96 ± 0.05 g/dl; T3-treated rats, 3.56 ± 0.14 g/dl; \(p < 0.05\)), in control rats (Fig. 1A) and T3-treated animals (Fig. 1B) exhibiting normal liver morphology and comparable (\(p > 0.05\)) serum AST levels (Fig. 1C). Under these conditions, the rectal temperature of the animals (Fig. 1D), liver oxygen consumption rate (Fig. 1E), and contents of protein carbonyls (Fig. 1F) and 8-isoprostanes (Fig. 1G) were significantly enhanced by 4, 39, and 140% and 25.9-fold, respectively, whereas hepatic GSH levels (Fig. 1H) were diminished by 59% (\(p < 0.05\)). The analysis of correlations between the studied parameters shown in Fig. 1 exhibited significant associations of the rectal temperature with liver O2 consumption \((r = 0.67; p < 0.04)\), protein carbonyls \((r = 0.96; p < 0.0001)\), and 8-isoprostanes \((r = 0.80; p < 0.02)\), whereas a

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat CHOP</td>
<td>ACCACACACCTGAAAGCAG</td>
<td>ACGTGGACACTGCTCAAG</td>
</tr>
<tr>
<td>Rat GADD34</td>
<td>TCCGGCTCTGAAAATCTCC</td>
<td>GAACTTGGGCTCCACATC</td>
</tr>
<tr>
<td>Rat PDI</td>
<td>TCCAAAGATTTCGGAGCTG</td>
<td>TGCCTAATTCCACATCTAG</td>
</tr>
<tr>
<td>Rat ERO1α</td>
<td>ATTECAAGGAAGCAACTCTC</td>
<td>TGCCTGCTCCTCAACTGAA</td>
</tr>
<tr>
<td>Rat β-actin</td>
<td>AGCCATGTACCTAGCCACTCC</td>
<td>CTTCAGCTGTGTTGTTGAA</td>
</tr>
</tbody>
</table>

Sequences are listed 5’→3’.

![Fig. 1](image-url)
negative correlation was obtained for the rectal temperature of the animals and liver GSH content ($r = -0.89; p < 0.03$).

2.2. T$_3$-induced liver UPR is dependent on oxidative stress development

T$_3$-dependent liver protein oxidation (Fig. 2A) was associated with activation of ER stress, as evidenced by the enhancement in the phosphorylation status of the ER stress sensor PERK [18,19], which showed 61% increase in pPERK/PERK ratios over control values (Fig. 2B). In addition, the downstream target of PERK, eIF2$\alpha$, exhibited 106% enhancement in peIF2$\alpha$/eIF2$\alpha$ ratios (Fig. 2C), with a concurrent increase in activating transcription factor 4 (ATF4) translation, as shown by the 95% increment in its protein levels compared to controls (Fig. 2D). Under these conditions, NAC pretreatment before T$_3$ administration eliminated the T$_3$-induced protein carbonylation response (Fig. 2A), with suppression of the enhancement in liver pPERK/PERK (Fig. 2B) and peIF2$\alpha$/eIF2$\alpha$ (Fig. 2C) ratios and ATF4 induction (Fig. 2D) elicited by T$_3$. In this experimental setting, liver protein carbonyl levels were significantly associated with pPERK/PERK ($r = 0.81; p < 0.0008$) and peIF2$\alpha$/eIF2$\alpha$ ($r = 0.94; p < 0.0001$) ratios and ATF4 protein levels ($r = 0.95; p < 0.0001$).

T$_3$-induced liver UPR involving the PERK-eIF2$\alpha$-ATF4 regulatory axis was characterized by 54% increase ($p < 0.05$) in the protein levels of the ER-located chaperone binding immunoglobulin protein (BiP) compared to control values (Fig. 3A). These findings were observed concomitant with upregulation of the ATF4-dependent mRNA expression of C/EBP homologous protein (CHOP) (Fig. 3B; 275% increase; $p < 0.05$) and the CHOP-dependent expression of growth arrest and DNA damage 34 (GADD34) (Fig. 3C; 555% increase; $p < 0.05$). In addition, T$_3$ administration also enhanced the mRNA expression of liver PDI (Fig. 3D; 118% increase, $p < 0.05$) and that of ERO1$\alpha$ (Fig. 3E; 100% increase, $p < 0.05$) over control levels. The enhancements in liver BiP protein content and in the mRNA levels of CHOP, GADD34, PDI, and ERO1$\alpha$ achieved by T$_3$ were abolished by the combined NAC-T$_3$ protocol studied (Fig. 3). Under these conditions, significant correlations were found between the levels of liver ATF4 protein and CHOP mRNA ($r = 0.96; p < 0.0001$), CHOP and GADD34 mRNA contents ($r = 0.77; p < 0.002$), and PDI and ERO1$\alpha$ mRNA levels ($r = 0.92; p < 0.0001$).
3. Discussion

Cell protection, survival, and functional recovery are elicited by the administration of low doses of T3 to experimental animals [7]. In the liver, these features are coupled to the activation of the redox-sensitive transcription factors nuclear factor-κB (NF-κB), signal transducer and activator of transcription 3 (STAT3), activating protein 1, or nuclear factor-erythroid 2-related factor 2 (Nrf2) upon oxidative stress development, with concurrence of AMPK upregulation for energy supply (Fig. 4). These mechanisms are effective in protecting the liver against IRI [5,6], the beneficial actions of thyroid hormones being also observed in ischemic...
injury of the heart [26], kidney [27], and brain [28] or in the repair of organs subjected to several types of injury other than IRI [29]. Data presented indicate that a protocol comprising the administration of single daily doses of 0.1 mg T3/kg to rats on three consecutive days underlies an enhanced liver ROS generation coupled to T3-induced increase in oxygen consumption as a result of the calorigenic effect achieved, leading to significant lipid peroxidation and protein oxidation responses and concomitant GSH depletion. These changes induced by T3 point to the development of an acute, nonlethal oxidative stress status, considering the lack of damaging effects on the liver as evidenced by the presence of normal liver morphology and serum AST levels.

Protein oxidation by ROS is a complex and irreversible process involving several oxidative modifications of amino acid residues in proteins, which favor protein unfolding, degradation [23,30,31], and UPR activation [17–19], a signaling mechanism that may be of importance in the PC and repairing effects of T3 (Fig. 4). Concomitant with the enhancement in liver protein oxidation induced by T3, the ER-localized signal transducer PERK and its downstream components are activated, responses that are abolished by the use of NAC (0.5 g/kg) previous to each dose of T3. This protocol of NAC administration was adopted considering the rapid onset and offset in the circulating levels of the antioxidant observed [6], which exhibits an elimination half-life of 1 to 4.5 h in the rat [32]. Abolishment of liver T3-induced UPR by NAC can be ascribed to the direct free radical-scavenging activity of NAC and its function as a precursor for GSH biosynthesis [33,34], thus establishing a causal role for ROS in UPR development in the hyperthyroid state.

T3-dependent activation of liver UPR involves the PERK regulatory axis, in which accumulation of oxidized, unfolded proteins in the ER lumen releases PERK bound to the ER chaperone BiP. PERK then dimerizes and autophosphorylates to achieve maximal activity [18,35]. This is evidenced by significant increases in the hepatic pPERK/PERK ratios and in BiP protein levels by T3 over control values, which correlate with the enhanced elf2α phosphorylation on Ser51. This response attenuates global mRNA translation [18,35,36], which is known to include that of the inositol-requiring enzyme-1 and/or activating transcription factor 6 [18,35,36], cannot be discarded, an aspect that is currently being evaluated in our laboratory. Thyroid hormone-induced liver UPR is in agreement with the enhanced synthesis and release of the positive acute-phase proteins (APPs) α1-acid glycoprotein [51], haptoglobin [51,52], and those involved in coagulation, inflammatory response, and lipoprotein metabolism [52]. Higher liver mRNA levels and serum activity of ceruloplasmin by thyroxine (T4) administration were also reported [53], which comply with the direct involvement of the ER in the biosynthesis and maturation of secreted proteins by hepatocytes [18]. This contention is further supported by the T3-induced upregulation of the hepatic expression of the APP ferritin posttranscriptionally [54] and that of haptoglobin and β-fibrinogen, which are associated with the redox activation of NF-κB and STAT3, respectively [55]. In agreement with the upregulation of positive APPs, T3 led to a decrease in the serum levels of albumin, a negative APP that under acute stress conditions is downregulated to achieve a redistribution of liver protein pools [56,57]. Considering that the UPR confers rapid adaptation to the increase in unfolded proteins in the ER lumen by expanding its protein-folding capacity, T3-induced liver UPR may represent a favorable mechanism against deleterious stimuli [5,6,9,29] in addition to those studied in experimental models (Fig. 4). In humans, readjusting thyroid hormones to normal levels is also beneficial in specific cases, as shown by (i) the improvement in mental, motor, and neurological outcomes in infants of <28 weeks’ gestation [58]; (ii) the lower cognitive side effects of lithium and electroconvulsive therapy in patients with bipolar disorders [59]; and (iii) the higher number of organs from brain-dead donors that are acceptable for transplantation and exhibit better graft survival [60]. Recently, low-dose T3 replacement was proposed as a therapy for diabetic vascular complications in humans, as it preserves coronary microvasculature and attenuates cardiac dysfunction in an experimental model [61], whereas T3 supplementation in rats substantially recovered hypothyroidism-induced liver apoptosis [62], supporting the role of T3 in cytoprotection and functional recovery.
Acknowledgment

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References
