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Thyroid hormone suppresses ischemia-reperfusion-induced liver NLRP3 inflammasome activation: Role of AMP-activated protein kinase

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ABSTRACT

Thyroid hormone (T_3) induces liver preconditioning (PC) against ischemia-reperfusion (IR), a response energetically supported by AMP-activated protein kinase (AMPK) upregulation. The aim of this work is to evaluate the influence of T_3 on IR-induced liver NLRP3 inflammasome activation and the relevance of AMPK activity on liver injury by the use of the AMPK inhibitor compound C (CC). Male Sprague-Dawley rats were given 0.1 mg T_3/kg (time zero) and 10 mg CC/kg (time zero and 24 h) or the respective vehicles, and subjected to 1 h ischemia–20 h reperfusion 48 h after hormone treatment. Measurements included parameters of liver injury, hepatic levels of mRNAs (qPCR) and proteins (Western Blot or ELISA). IR induced substantial distortion of liver architecture, hepatocyte necrosis, and neutrophil infiltration with increased serum aspartate aminotransferase (AST) levels. T_3 suppressed IR liver injury and AST enhancement, effects that were reverted by CC. Concomitantly, IR-induced liver mRNA and protein expression of NLRP3 and interleukin-1β (IL-1β) were restrained by T_3, whereas CC eliminated T_3-dependent PC. In conclusion, *in vivo* T_3 administration triggers liver PC against IR injury by suppressing the inflammatory response associated with hepatic NLRP3 and IL-1β upregulation, with AMPK playing a causal role regulating energy dynamics to upkeep PC.

1. Introduction

Liver injury by ischemia-reperfusion (IR) is characterized by massive loss of hepatocytes during reperfusion as the result of the concurrence of oxidative stress, endoplasmic reticulum stress, and inflammatory mechanisms [1]. In this setting, liver resident cells and cells recruited in response to injury initially generate pro-inflammatory signals, such as cytokines, chemokines, lipid messengers, and reactive oxygen species (ROS), which induce cell death [1,2]. Under these conditions, dying hepatocytes release damage associated molecular patterns (DAMPs) including ATP, nuclear DNA, and high mobility group Box 1 (HMGB1) among others, which aggravate the IR-induced inflammatory response [1,2], acting as inflammasome activators [3]. Inflammasomes are platforms for inflammatory signaling activation, the nucleotide-binding oligomerization domain (NOD), leucine-rich repeat containing family, pyrin domain containing 3 (NLRP3) inflammasome being a key mediator of pro-inflammatory cytokine release and liver damage in IR [4,5].

Liver preconditioning (PC) involves the enhancement of the tolerance to noxious stimuli such as IR, which is triggered by previous manoeuvres inducing beneficial molecular changes [6], such as 3,3′,5-triiodothyronine (T_3) administration [7,8]. As a PC strategy, T_3 increases the homeostatic potential of the liver through the redox activation of transcription factors inducing the expression of protective proteins. These include (i) antioxidant proteins (NF-κB, Nrf2) [9,10]; (ii) anti-apoptotic and acute-phase proteins (NF-κB, STAT3) [9,11]; (iii) phase II enzymes and phase III transporters of the xenobiotic biotransformation system (Nrf2) [12]; and (iv) cell proliferation (AP-1, STAT3) [13], with higher energy supply being met by upregulation of AMP-activated protein kinase (AMPK) [14]. AMPK is a component of a cascade sensing energy dynamics through reduction of anabolism, to avoid excessive ATP consumption, and enhancement of catabolism, to increase ATP generation [15]. Under conditions of T_3 administration, liver AMPK upregulation is achieved through higher AMPK mRNA expression and AMPK Thr 172 phosphorylation, with allosteric activation of the

Abbreviations: AMPK, AMP activated protein kinase; CC, compound C; DAMP, damage associated molecular pattern; IR, ischemia-reperfusion; NLRP3, nucleotide-bonding oligomerization domain (NOD) leucine-rich repeat containing family pyrin containing 3; ROS, reactive oxygen species.

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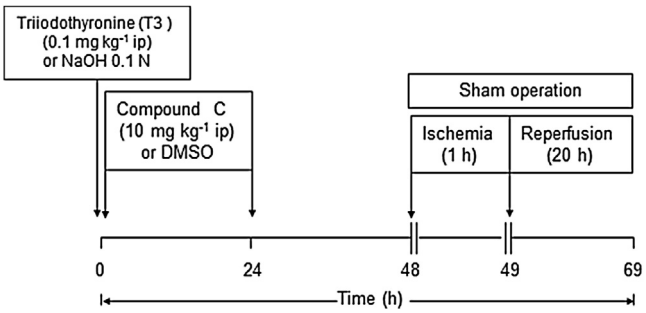

Fig. 1. Experimental design depicting the administration of thyroid hormone (T_3) and/or the AMP-activated protein kinase inhibitor (AMPK) compound C (CC) and their respective vehicles before applying the ischemia-reperfusion protocol.

enzyme being promoted by the concomitant increase in hepatic AMP/ATP ratios [16]. The Thr 172 phosphorylation mechanism of AMPK activation is associated with upregulation of the upstream kinases TAK1 and CaMKKβ [16], the operation of the latter kinase being supported by the enhancement in cytosolic Ca^{2+} concentrations elicited by T_3 [17]. In addition to the above mechanisms of T_3-induced liver AMPK signaling, production of reactive oxygen species (ROS) has a causal role, as evidenced by the suppressive effect of the antioxidant N- acetylcysteine on AMPK, CaMKKβ, and TAK1 induction, when given prior to T_3 [14]. These findings are in agreement with the activating effect of ROS on AMPK observed upon addition to cell cultures or under *in vitro* [18–20] and *in vivo* [21,22] conditions underlying ROS generation.

In view of these considerations, the aim of this study was to test the hypothesis that IR-induced liver NLRP3 activation [4,5] is abrogated by a PC dose of T_3. The relevance of T_3-dependent hepatic AMPK upregulation on liver injury and on mRNA and protein levels of NLRP3 and interleukin-1β (IL-1β) induced by IR was assessed by the *in vivo* administration of compound C (CC), a selective inhibitor of AMPK [23].

2. Materials and methods

2.1. Animal treatments

Male Sprague-Dawley rats (Rattus novergicus) (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 were treated with T_3 and/or CC and their respective vehicles and subjected to IR or sham laparotomy, as indicated in Fig. 1 [7]. Experimental groups described in Fig. 2A were anesthetized with Zoletil-50 (50 mg/kg zolazepam chlorhydrate, 50 mg/kg tiletamine chlorhydrate) (Virbac S/A, Carros, France), and IR was induced by temporarily interrupting the blood supply by means of a Schwartz clip (Fine Science Tools Inc., Vancouver, BC, Canada), as previously described (Fig. 1) [7]. Blood samples were obtained by cardiac puncture at the end of the reperfusion period (Fig. 1) to measure serum T_3 (ELISA, Monobind, Lake Forest, CA, USA), aspartate aminotransferase (AST) (IU/L), and plasma IL-1β (pg/mL) (Quantikine ELISA; R&D Systems Inc., Minneapolis, USA) according to manufacturer's instructions. At this time, liver samples were taken and frozen in liquid nitrogen or fixed in phosphate-buffered formalin, paraffin embedded, and stained with haematoxylin-eosin. 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 Bioethics Committee of the Faculty of Medicine, University of Chile (protocol CBA #0440 FMUCH).

2.2. RNA extraction and quantitative real-time PCR

For real-time quantitative PCR (qPCR) for NLRP3 and IL-1β, total RNA was extracted from liver (30 mg) using RNeasy® Lipid Tissue Mini Kit (Quiagen Sciences, Maryland, USA) and cDNA was synthesized using ThermoScriptTM RT-PCR System (Life Technologies Corporation, Carlsbad, California, USA) according to the manufacturer's instructions. qPCR was carried out in a Stratagene Mx3000P using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, California, USA) following the manufacturer's protocols. Gene specific primer sequences used are forward 5′-ttctctgcatgccgatatctg-3′, reverse 5′-gagccatggaagcaaagttc-3′ for NLRP3, forward 5′-aaagctctccacctcaatgg-3′, reverse 5′-tcgttgcttgtctctcctta-3′ for IL-1β, and forward 5′-gtaggggttgaagccaaaca-3′, reverse 5′-caccttaaagcggactccag-3′ for Ribosomal Protein S23 (Rps23). The expression levels of each sample were normalized against Rps23 (internal control). The relative expression levels were calculated using the comparative CT method (ΔΔCT), and expressed as individual mRNA/Rps23.

2.3. Western blot analysis

Protein levels of NLRP3 and IL-1β were analysed in liver samples (300 mg) frozen in liquid nitrogen, which were homogenized and suspended in 1.5 ml of a buffer solution (pH 7.9) containing 10 mM Hepes, 1 mM EDTA, 0.6% v/v NP-40, 150 mM NaCl, 0.5 mM PMSF, protease inhibitors (1 μg/ml aprotinin, leupeptin, and pepstanin), and phosphatase inhibitor (1 mM orthovanadate), followed by centrifugation (3000*g* for 5 min at 4 °C). Cytosolic soluble protein fractions (50 μg) were separated on 12% w/v polyacrylamide gels using sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDSPAGE) [24] and transferred to nitrocellulose membranes [25], which were blocked for 1 h at room temperature with Tris buffer saline (TBS) containing 5% w/v bovine serum albumin. The blots were washed with TBS containing 0.5% w/v Tween-20 and hybridized with rabbit polyclonal primary antibodies for NLRP3 (110 kDa), α-tubulin (52 kDa) (1:1000; Cell Signalling Technology Inc., MA, USA) and IL-1β (17 kDa) (1:1000; Santa Cruz Biotechnology, Inc., Texas, USA). In all determinations, rabbit polyclonal 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 and SuperSignalWest Pico Chemiluminescence kit detection system (Pierce, Rockford, IL, USA). Bands were run in two separate gels as indicated in Figs. 3C and 4C , and quantified by densitometry using Gel Documentation System Biosens SC-750 (Shanghai Bio-Tech Co., Ltd., China). Results are expressed as individual protein/α-tubulin.

2.4. Statistical analysis

Values shown are means ± SEM for the number of separate experiments indicated. One-way ANOVA and the Newman-Keuls' test assessed the statistical significance ($P < 0.05$) of differences between mean values and are represented by the letters (or numbers) identifying each experimental group. Associations between different variables were evaluated by the Pearson correlation test.

3. Results

3.1. Suppression of IR liver injury by T_3 is overcome by AMPK inhibition

Rats were treated as described in Fig. 1, and those given T_3 exhibited higher serum levels of T_3 than controls (Controls, 1.34 ± 0.12