T₃-induced liver AMP-activated protein kinase signaling: Redox dependency and upregulation of downstream targets

Luis A Videla, Virginia Fernández, Pamela Cornejo, Romina Vargas, Paula Morales, Juan Ceballo, Alvaro Fischer, Nicolás Escudero, Oscar Escobar

Luis A Videla, Virginia Fernández, Pamela Cornejo, Romina Vargas, Paula Morales, Juan Ceballo, Alvaro Fischer, Nicolás Escudero, Oscar Escobar, Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Independencia 1027, Santiago, Chile
Pamela Cornejo, School of Medical Technology, Faculty of Medicine, Diego Portales University, Ejército 141, Santiago, Chile
Author contributions: Videla LA designed the research and wrote the article with the input of Fernández V, Cornejo P and Vargas R; Biochemical and molecular analyses were performed by Vargas R, Morales P, Ceballo J, Fischer A, Escudero N and Escobar O; all authors have approved the final manuscript and declare that there is no conflict in interest that could be perceived as prejudicing the impartiality of the research reported.
Supported by National Commission for Scientific and Technological Research Grant No. 1120034
Correspondence to: Luis A Videla, Professor, Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Independencia Avenue 1027, Chile. lvidela@med.uchile.cl
Telephone: +56-2-29786256 Fax: +56-2-27372783
Received: April 22, 2014 Revised: June 6, 2014 Accepted: July 29, 2014 Published online: December 14, 2014

Abstract

AIM: To investigate the redox dependency and promotion of downstream targets in thyroid hormone (T₃)-induced AMP-activated protein kinase (AMPK) signaling as cellular energy sensor to limit metabolic stresses in the liver.

METHODS: Fed male Sprague-Dawley rats were given a single ip dose of 0.1 mg T₃/kg or T₃ vehicle (NaOH 0.1 N; controls) and studied at 8 or 24 h after treatment. Separate groups of animals received 500 mg N-acetylcysteine (NAC)/kg or saline ip 30 min prior T₃. Measurements included plasma and liver β-isoprostane and serum β-hydroxybutyrate levels (ELISA), hepatic levels of mRNAs (qPCR), proteins (Western blot), and phosphorylated AMPK (ELISA).

RESULTS: T₃ upregulates AMPK signaling, including the upstream kinases Ca²⁺-calmodulin-dependent protein kinase kinase-β and transforming growth factor-β-activated kinase-1, with T₃-induced reactive oxygen species having a causal role due to its suppression by pretreatment with the antioxidant NAC. Accordingly, AMPK targets acetyl-CoA carboxylase and cyclic AMP response element binding protein are phosphorylated, with the concomitant carnitine palmitoyltransferase-1α (CPT-1α) activation and higher expression of peroxisome proliferator-activated receptor-γ co-activator-1α and that of the fatty acid oxidation (FAO)-related enzymes CPT-1α, acyl-CoA oxidase 1, and acyl-CoA thioesterase 2. Under these conditions, T₃ induced a significant increase in the serum levels of β-hydroxybutyrate, a surrogate marker for hepatic FAO.

CONCLUSION: T₃ administration activates liver AMPK signaling in a redox-dependent manner, leading to FAO enhancement as evidenced by the consequent ketogenic response, which may constitute a key molecular mechanism regulating energy dynamics to support T₃ preconditioning against ischemia-reperfusion injury.

© 2014 Baishideng Publishing Group Inc. All rights reserved.

Key words: Liver; Thyroid hormone; N-acetylcysteine; AMP-activated protein kinase; Fatty acid oxidation

Core tip: This work investigated the redox dependency and promotion of downstream targets in thyroid hormone (T₃)-induced AMP-activated protein kinase (AMPK) signaling. T₃ upregulates AMPK with T₃-induced reactive oxygen species having a causal role due to its suppression by pretreatment with the antioxidant NAC. Accordingly, AMPK targets acetyl-CoA carboxylase and cyclic AMP response element binding protein...
are phosphorylated, with the concomitant carnitine palmitoyltransferase-1α activation and higher expression of peroxisome proliferator-activated receptor-γ co-activator-1α and that of the fatty acid oxidation (FAO)-related enzymes. This lead to enhancement in the serum levels of β-hydroxybutyrate, a surrogate marker for hepatic FAO, which represent a key molecular mechanism regulating energy dynamics to limit metabolic stresses.

Videla LA, Fernández V, Cornejo P, Vargas R, Morales P, Ceballo J, Fischer A, Escudero N, Escobar O. T3-induced liver AMP-activated protein kinase signaling: Redox dependency and up-regulation of downstream targets. World J Gastroenterol 2014; 20(46): 17416-17425 Available from: URL: http://www.wjgnet.com/1007-9327/full/v20/i46/ DOI: http://dx.doi.org/10.3748/wjg.v20.i46.17416

INTRODUCTION

Genomic[1] and nongenomic[10] mechanisms underlying thyroid hormone action are responsible for cellular responses such as O2 consumption of most tissues and the metabolic rate of individuals. The liver, a target organ for L-3,3,5-triiodothyronine (T3) calorigenesis, exhibits a concomitant enhancement in antioxidant capacity (ROS) production, which is due to acceleration of mitochondrial respiration and activation of other ROS-generating systems[9]. Under these conditions, activation of redox-sensitive transcription factors nuclear factor-κB, activating protein-1, signal transducer and activator of transcription-3, and nuclear factor (erythroid-derived 2)-like 2 is achieved, with promotion of cell proliferation and the expression of cytoprotective proteins including antioxidant, anti-apoptotic, and acute-phase proteins, phase-II detoxification enzymes, and phase-III transporters[3-5,9,10]. Thus, in vivo T3 administration re-establishes redox homeostasis, promotes cell survival, and protects the liver against ischemia-reperfusion injury (IRI), which constitute the basis for T3 liver preconditioning[9,10] and an important issue in post-stress recovery and repair[1].

Organ preconditioning (PC) refers to the development of an increased tolerance to noxious stimuli such as IRI due to previous maneuvers triggering beneficial molecular and functional changes. In the liver, numerous experimental PC and post-conditioning strategies have been proposed, however, difficulties with gene therapy and pharmacological maneuvers have hindered their transfer to clinical practice, whereas those based on surgical approaches are limited or controversial[9,10]. In this respect, the T3 PC strategy has potential clinical application, considering that T3 is a widely employed and well-tolerated therapeutic agent whose side effects are readily controlled, and that its mechanisms of PC action are beginning to be understood[9,10]. Among these, AMP-activated protein kinase (AMPK) may constitute the metabolic basis of T3 liver PC, as effective PC involves high ATP demands to power the expression of numerous protective proteins, oxidized biomolecules repair (phospholipids, DNA) or resynthesis (proteins), and hepatocyte and Kupffer-cell proliferation, in addition to energy requirements needed for normal hepatic metabolic, secretory, and excretory functions[9,10]. AMPK is considered a key energy sensor able to limit anabolic pathways, to reduce ATP consumption, and to facilitate catabolic pathways, to increase ATP production[9]. In the present study, we show that T3 administration to rats significantly enhances liver (1) AMPK signaling in a redox-sensitive manner; (2) the phosphorylation of downstream AMPK targets; and (3) the expression of components associated with fatty acid (FA) oxidation (FAO), leading to a ketogenic response.

MATERIALS AND METHODS

Animal treatments

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-hour light/dark cycle and were provided with rat chow and water ad libitum. Animals received a single intraperitoneal (i.p.) dose of 0.1 mg T3/kg body weight or equivalent volumes of hormone vehicle (0.1 mol/L NaOH, controls) at time zero and studies were done after 24 h of treatment. Studies with N-acetylcysteine (NAC) were carried out in the above described groups receiving either 0.5 g/kg or saline (controls) i.p., 0.5 h before T3 administration, and studies were performed at 8 or 24 h after treatment in four experimental groups, namely, (1) controls; (2) T3; (3) NAC; and (4) NAC + T3. T3-induced calorigenesis was assessed by the rectal temperature of the animals by means of a thermocouple (Cole-Palmer Instrument Co., Chicago, IL). Blood samples to measure 8-isoprostanes in plasma and β-hydroxybutyrate in serum were obtained by cardiac puncture in rats anesthetized (1 mL/kg) with zolazepan chlorhydrate (25 mg/mL) and tiletamine chlordihydrate (25 mg/mL) i.p (Zoletil 50; Virbac S/A, Carros, France), and liver samples were taken, frozen in liquid nitrogen, and kept at -80°C for measurements of mRNA and protein expression. All experiments and animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, NIH Publication 6-23, revised 1985) and were approved by the Bioethics Committee for Research in Animals, Faculty of Medicine, University of Chile (Protocol CBA 0381 FUMCH).

8-isoprostanes and β-hydroxybutyrate levels

Liver and plasma 8-isoprostane levels were measured in samples frozen in liquid nitrogen and kept at -80°C, using a commercial kit (ELISA; Cayman Chemical Co., Ann Arbor, MI, United States) according to the manufacturer’s instructions at 405-420 nm. Results were calculated from a standard curve and expressed as ng/g of liver or pg/mL of plasma, respectively. Serum β-hydroxybutyrate levels were assayed in samples frozen in liquid nitrogen and
Sequences are listed in the 5’ → 3’ direction. ACCα(β): Acetyl-CoA carboxylase α and β; ACOT2: Acyl-CoA thioesterase; ACOX1: acyl-CoA oxidase 1; AMPK: AMP-activated protein kinase; CaMKKβ: Ca2+-Calmodulin-dependent protein kinase β; CPT-1α: Carnitine palmitoyltransferase-1α; CREB: cAMP-response element-binding protein; PGC-1α: Peroxisome proliferator-activated receptor-γ coactivator-1α; RPS23: Ribosomal protein S23; TAK1: Transforming growth factor-β-activated kinase-1.

RNA isolation and cDNA synthesis
Total RNA was isolated using RNeasy® Lipid Tissue Mini Kit (QUIAGEN Sciences, Maryland, United States) according to the manufacturer’s instructions. cDNA was synthesized using ThermoScript™ RT-PCR System (Life Technologies Corporation, Carlsbad, California, United States) according to the manufacturer’s instructions.

Real-time quantitative PCR for AMPK, CaMKKβ, TAK1, ACCα(β), CREB, PGC-1α, CPT-1α, ACOX1, and ACOT2
Real-time quantitative PCR was carried out in a Stratagene Mx3005P (Agilent Technologies, California, United States) using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, California, United States) following the manufacturer’s protocols. Gene specific primer sequences used are shown in Table 1. Primers were optimized to yield 95%-100% reaction efficiency with PCR sequences used are shown in Table 1. Primers were optimized to yield 95%-100% reaction efficiency with PCR products run on agarose gel to verify the correct amplification length. Melting curve analyses verified the formation of a single desired PCR product in each PCR reaction. The expression levels of each sample were normalized against RPS23 and β-actin (internal controls). The relative expression levels were calculated using the comparative Ct method (ΔΔCt) and values were normalized to RPS23 level or β-actin level as internal control genes.

Western Blot Analysis for CaMKKβ, TAK1 and pTAK1, ACC and pACC, CREB and pCREB, CPT-1α, ACOX1, and ACOT2
Liver samples (100-500 mg) frozen in liquid nitrogen were homogenized and suspended in 1.5 mL of a buffer solution (pH 7.9), containing 10 mmol/L Hepes, 1 mmol/L EDTA, 0.6% NP-40, 150 mmol/L NaCl, 0.5 mmol/L PMSF, protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin) and fosfatase inhibitor (1 mmol/L orthovanadate) followed by centrifugation (3000 g for 5 min). Cytosolic soluble protein fractions (70 μg) were separated on 12% polyacrylamide gels using sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes which were blocked for 1 h at room temperature with Tris buffer saline (TBS) containing 5% bovine serum albumin. The blots were washed with TBS containing 0.5% Tween 20 and hybridized with rabbit polyclonal primary antibodies for CaMKKβ, TAK1, pTAK1, pACC, and CPT-1α (1:1000; Beyo, Cambridge, MA, United States), PGC1-α, ACC, α-tubulin, and lamin A/C (1:1000; Cell Signalling Technology, Inc, MA, United States), acyl-coenzyme A thioesterase 2 (ACOT2) and acyl-coenzyme A oxidase 1 (ACOX1) (1:1000, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, United States), pCREB and CREB (1:1000; EMD Millipore Corporation, Billerica, MA, United States); acyl-coenzyme A thioesterase-1 (ACOT2) and acyl-coenzyme A oxidase 1 (ACOX1) (1:1000, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, United States). In all determinations, rabbit monoclonal antibody for anti-α-tubulin was used as internal control and anti-lamin A/C was employed as control for purity. After extensive washing, the antigen-antibody complexes were detected using horseradish peroxidase goat anti-rabbit IgG or goat anti-mouse IgG and SuperSignal West Pico Chemiluminescence kit detection system (Pierce, Rockford, IL, United States). Bands were quantified by densitometry using Gel Documentation System Biosens SC-750 (Shanghai Bio-Tech Co., Ltd., China). Results are expressed as relative units (individual protein/α-tubulin) with further normalization by the average values obtained in the control groups.

AMPKα [pT172] ELISA kit
AMPKα [pT172] concentration was determined by AMPKα [pT172] ELISA kit (In Vitrogen Corporation, CA, United States) according to the manufacturer’s instructions and read the plate at a wavelength at 450 nm. Results were calculated from a standard curve.

Table 1  Primers for SYBR green based qRT-PCR assays

| mRNA     | Forward primer   | Reverse primer   |
|----------|------------------|------------------|
| Rat ACCα | GAC GTT CGC CAT AAC CAA GT | CTC GAG GTT CTC AAT GCA AA |
| Rat ACCβ | CCT GTA CAT GCC AGT CAG CA | AGT TCT CGG GAG GAA CAG GT |
| Rat ACOT2| TCA GGA TGA CCA CAACCTG GA | ATG TTA GCA CCC ACC AGG AG |
| Rat ACOX1| CTT ATG AAA TAC GCC CAG GT | GCT CCC ATA CGT CAG GCTT |
| Rat AMPK | TTT GCC TAG AAT CCC CAC AG | TAA GGA GCC CAG AAA ACA GC |
| Rat β-actin| AGC CAT GTA CGT AGC CAT CC | CTC TCA GCT GTG GTG AG |
| Rat CaMKKβ| GGA TGG TGG TGG CTG AAA TC | AGG CTG GAA ATG TGT TTG AC |
| Rat CPT-1α| CAG CTC GCA CAT TAC AAG GA | TGG ACA AAG GTG CAG GAC TC |
| Rat CREB | TCA GGC GGC TAC GTC CAT TC | CTC CTC TCT TTC CTG CT |
| Rat PGC-1α| TCC ACA GAT TCA AGC CAG TG | TGA CCA AAG TGC TTG TTC AG |
| Rat RPS23| GTA GGG GTT GAA GCC AAA CA | CAC CTG AAA GGG GAC TCC AG |
| Rat TAK1 | AAC AAG TCC CTG CCA CAA AC | CAT CCT GGC CTC AGA AG |

Real-time quantitative PCR for AMPK, CaMKKβ, TAK1, ACCα(β), CREB, PGC-1α, CPT-1α, ACOX1, and ACOT2
Real-time quantitative PCR was carried out in a Stratagene Mx3005P (Agilent Technologies, California, United States) using Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies, California, United States) following the manufacturer’s protocols. Gene specific primer sequences used are shown in Table 1. Primers were optimized to yield 95%-100% reaction efficiency with PCR products run on agarose gel to verify the correct amplification length. Melting curve analyses verified the formation of a single desired PCR product in each PCR reaction. The expression levels of each sample were normalized against RPS23 and β-actin (internal controls). The relative expression levels were calculated using the comparative Ct method (ΔΔCt) and values were normalized to RPS23 level or β-actin level as internal control genes.

Western Blot Analysis for CaMKKβ, TAK1 and pTAK1, ACC and pACC, CREB and pCREB, CPT-1α, ACOX1, and ACOT2
Liver samples (100-500 mg) frozen in liquid nitrogen were homogenized and suspended in 1.5 mL of a buffer solution (pH 7.9), containing 10 mmol/L Hepes, 1 mmol/L EDTA, 0.6% NP-40, 150 mmol/L NaCl, 0.5 mmol/L PMSF, protease inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin) and fosfatase inhibitor (1 mmol/L orthovanadate) followed by centrifugation (3000 g for 5 min). Cytosolic soluble protein fractions (70 μg) were separated on 12% polyacrylamide gels using sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes which were blocked for 1 h at room temperature with Tris buffer saline (TBS) containing 5% bovine serum albumin. The blots were washed with TBS containing 0.5% Tween 20 and hybridized with rabbit polyclonal primary antibodies for CaMKKβ, TAK1, pTAK1, pACC, and CPT-1α (1:1000; AbCam, Cambridge, MA, United States), PGC1-α, ACC, α-tubulin, and lamin A/C (1:1000; Cell Signalling Technology, Inc, MA, United States), acyl-coenzyme A thioesterase 2 (ACOT2) and acyl-coenzyme A oxidase 1 (ACOX1) (1:1000, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, United States). In all determinations, rabbit monoclonal antibody for anti-α-tubulin was used as internal control and anti-lamin A/C was employed as control for purity. After extensive washing, the antigen-antibody complexes were detected using horseradish peroxidase goat anti-rabbit IgG or goat anti-mouse IgG and SuperSignal West Pico Chemiluminescence kit detection system (Pierce, Rockford, IL, United States). Bands were quantified by densitometry using Gel Documentation System Biosens SC-750 (Shanghai Bio-Tech Co., Ltd., China). Results are expressed as relative units (individual protein/α-tubulin) with further normalization by the average values obtained in the control groups.

AMPKα [pT172] ELISA kit
AMPKα [pT172] concentration was determined by AMPKα [pT172] ELISA kit (In Vitrogen Corporation, CA, United States) according to the manufacturer’s instructions and read the plate at a wavelength at 450 nm. Results were calculated from a standard curve.
Statistical analysis
Data showing Gaussian distribution according to the Kolmogorov-Smirnov test are expressed as means ± SEM for the number of separate experiments indicated. As required, one-way ANOVA analysis of variance and the Newman-Keuls test or the Student’s t-test for unpaired data assessed the statistical significance (P < 0.05) of differences between mean values. To analyze the association between different variables, the Pearson correlation coefficient was used. All statistical analyses were computed employing GraphPad Prism™ version 2.0 (GraphPad Software, Inc., San Diego, CA, United States).

RESULTS
Liver AMPK upregulation by T3 administration is redox-sensitive
The administration of a single dose of T3 to fed rats elicited a calorigenic response, with (1) increases in the rectal temperature of the animals (8 h after treatment, controls, 36.5 ± 0.04 (n = 4) ℃; T3-treated rats, 38.0 ± 0.2 (n = 5); P < 0.05; 24 h after treatment, controls, 36.3 ± 0.06 (n = 13); T3-treated rats, 37.7 ± 0.17 (n = 13); P < 0.05); and (2) the associated development of liver oxidative stress as evidenced by increases in O2 consumption in the time range of 2-24 h,[14] with enhanced protein carbonylation at 12 h and glutathione depletion at 24 h after T3,[15], and higher levels of 8-isoprostanes in plasma (112% higher) and liver (41% higher) over control values at 24 h (Figure 1A). The enhancement in 8-isoprostane levels as index related of oxidative stress was suppressed by 0.5 g of NAC/kg given 0.5 h before T3 (Figure 1A). Under these conditions, NAC treatment did not modify the mRNA expression of AMPK (Figure 1B) or that of the upstream kinases Ca2+-calmodulin-dependent protein kinase-β (CaMKKβ) (Figure 1C) and transforming growth factor-β-activated kinase-1 (TAK1) (Figure 1D), or the protein levels of CaMKKβ (Figure 1E). TAK1 activation by phosphorylation was significantly increased by NAC over controls (Figure 1F). Consistent with a previous report showing liver T3-induced AMPK upregulation in the period of 8-36 h after treatment,[15], the enhancement in AMPK activation observed at 24 h was abolished by NAC (Figure 1B). Furthermore, early (8 h) CaMKKβ upregulation (Figure 1C and E) and late (24 h) enhancement in TAK1 mRNA expression and phosphorylation (Figure 1D and F) induced by T3 were either suppressed or significantly diminished by NAC pretreatment.

Liver AMPK phosphorylation targets are enhanced by T3 administration
T3-induced liver AMPK upregulation (Figure 1B and 2A) was found to trigger two major downstream signaling pathways, namely, acetyl-CoA carboxylase α and β (ACCα and ACCβ) and cyclic AMP response element binding protein (CREB), either through mRNA expression or protein phosphorylation (Figure 2C and D), parameters that were significantly correlated (Figure 2E). These changes occurred with the concomitant increase in the mRNA expression of hepatic peroxisome proliferator-activated receptor (PPAR)-γ co-activator-1α (PGC-1α) (Figure 2B).

Liver AMPK-dependent metabolically operative targets are increased by T3 administration
T3 administration enhanced hepatic mRNA expression of PGC-1α over control values by 2.15-fold, which correlated with the 37% increase in CPT-1α protein content (Figure 3A) (r = 0.86; P < 0.02). Under these conditions, PGC-1α upregulation by T3 was associated with increased expression of the FAO-related enzymes ACOX1 (Figure 3B and ACOT2 (Figure 3C), both at transcriptional and translational levels, with 72% enhancement in serum β-hydroxybutyrate levels over control values (Figure 3D).

DISCUSSION
Data presented indicate that T3-induced liver AMPK upregulation is accomplished through enhancement in AMPK transcription and AMPK phosphorylation associated with early CaMKKβ and late TAK1 activation, as upstream AMPK kinases,[9], mechanisms subjected to redox signaling due to their abrogation by the ROS suppressive action of NAC. The NAC protocol used results in significant circulating levels of the antioxidant,[16] which decline within 24 h due to the elimination half-life of 1.4-3 h,[17], an agent having a potent antioxidant activity due to its direct ROS scavenging action and the stimulation of hepaticcellular glutathione synthesis.[18] Although NAC did not alter the mRNA expression of CaMKKβ, TAK1, and AMPK, TAK1 phosphorylation was significantly increased, an effect that could be ascribed to the maintenance of relevant sulfhydryl groups in proteins in the reduced state,[19], which may lead to modifications of signal transduction pathways by favoring the phosphorylation process. In agreement with the causal role of ROS in AMPK upregulation induced by T3, ROS trigger cellular AMPK activation under different conditions including (1) in vitro hydrogen peroxide (H2O2) addition to cell cultures,[20-23], (2) in vitro and in vivo conditions underlying ROS production in hepatocytes, heart, or skeletal muscle,[24,25], and (3) ROS generation associated with chain reactions induced by polyphenols.[26] In this respect, H2O2 is considered as a major ROS able to achieve redox signaling that can be achieved by reversible oxidation of cysteine residues in signaling proteins into cysteine-sulfenylate derivatives, glutathionylation, or intramolecular disulfide formation.[27] H2O2 being significantly enhanced in hyperthyroid state[4].

Upregulation of liver AMPK by T3, as evidenced by the increase in hepatic AMPK mRNA levels, is in agreement with the observed higher Thr172-phosphorylated AMPK levels, which in turn correlate with the activation of CaMKKβ and TAK1 as upstream kinases. These data suggest that T3 substantially enhances the phosphorylating potential of hepatic AMPK, considering the higher...
Figure 1  Effect of N-acetylcysteine on T₃-induced liver CaMKKβ/TAK1-AMPK cascade. (A) Rat plasma and liver 8-isoprostane levels, liver mRNA levels of AMPK (B) and CaMKKβ (C, E) hepatic CaMKKβ protein content, and liver expression of TAK1 mRNA (D) and pTAK1/TAK1 ratios (F). Values shown are means ± SEM (n= 3-4). Statistical significance (one-way ANOVA and the Newman-Keuls test; P< 0.05) is indicated by the letters identifying each experimental group. AMPK: AMP-activated protein kinase; CaMKKβ: Ca²⁺-calmodulin-dependent protein kinase kinase-β; NAC: N-acetylcysteine; (p)TAK1, (phosphorylated) transforming growth factor-β-activated kinase-1.
transcriptional activity of the AMPK gene and the activation of the enzyme by both phosphorylation (Figure 2A) and by the concomitant increase in the hepatocellular AMP/ATP ratio previously observed\cite{15}. Phosphorylation of hepatic ACC by activated AMPK is known to result in ACC inhibition, with a substantial diminution in the levels of malonyl-CoA, the potent allosteric inhibitor of CPT-1\textsubscript{\alpha} that favors the entry of acyl-CoA into

**Figure 2 T\textsubscript{3}-induced changes in liver direct AMP-activated protein kinase targets.** A: pAMPK levels; B: PGC-1\textsubscript{\alpha} mRNA content; C: Contents of ACC\textsubscript{\alpha} mRNA and pACC\textsubscript{\alpha}; D: Levels of CREB mRNA and pCREB/CREB ratios; E: Correlations between pAMPK and pACC\textsubscript{\alpha}, pCREB/CREB ratios, and PGC-1\textsubscript{\alpha}. Values shown are means ± SEM (n = 3-6). Statistical significance was performed by one-way ANOVA and the Newman-Keuls test (C, D) or Student’s t-test for unpaired data (A, B) (P < 0.05). Associations between variables were analyzed by the Pearson correlation coefficient. pAMPK: Phosphorylated AMP-activated protein kinase; (p) ACC\textsubscript{\alpha}: (Phosphorylated) acetyl-CoA carboxylase-\alpha; (p)CREB: (phosphorylated) cAMP-response element-binding protein; PGC-1\textsubscript{\alpha}: Peroxisome proliferator-activated receptor-\gamma coactivator-1\textsubscript{\alpha}. 

Transcriptional activity of the AMPK gene and the activation of the enzyme by both phosphorylation (Figure 2A) and by the concomitant increase in the hepatocellular AMP/ATP ratio previously observed\cite{15}. Phosphorylation of hepatic ACC by activated AMPK is known to result in ACC inhibition, with a substantial diminution in the levels of malonyl-CoA, the potent allosteric inhibitor of CPT-1\textsubscript{\alpha} that favors the entry of acyl-CoA into.
mitochondria thus enhancing the FAO potential of the liver\[29\]. In addition to ACC, T₃-induced AMPK activation is associated with increased CREB mRNA expression and CREB phosphorylation, a transcription factor that is central to diverse cellular responses including those related to T₃ signaling. In fact, after inducing the dissociation of the repression complex, T₃ stimulates the recruitment of several nuclear co-activators including CREB-binding protein (CBP/p300), which facilitates T₃/thyroid hormone receptor-dependent transcription activation\[30\]. This is accomplished by AMPK-dependent CREB phosphorylation at Ser133\[31\], which is known to increase its association with co-activator CBP/p300\[32\]. Interestingly, T₃ administration significantly increased the mRNA expression of hepatic PGC-1α, which co-activates several transcription factors associated with mitochondrial biogenesis that may also enhance the capacity of the cell for FAO\[29\]. Furthermore, activated AMPK is able to elicit PGC-1α activation by direct phosphorylation\[33\], whereas control of PGC-1α transcription is partially mediated by CREB\[33\]. Taken as a whole, these data suggest that T₃ triggers liver AMPK upregulation confronting FAO, which appears to be associated with (1) phosphorylation of its target enzyme ACC; and (2) phosphorylation and transcriptional regulation of CREB and PGC-1α, signaling components that may undergo reinforcing mechanisms.

Transcriptional activation of nuclear receptor target genes is known to be triggered by co-activator molecules. These include PGC-1α that upon activation coordinates induction of hepatic FA oxidation via co-activation of transcription factors PPAR-α, nuclear respiratory factors 1 and 2, and estrogen receptor-related-α\[34\]. Accordingly, PGC-1α plays a role in the transduction of the T₃ stimulus to the transcriptional regulation of genes involved in liver energy metabolism, as proposed for cold exposure or fasting\[35\]. Thus, T₃-induced liver AMPK signaling may involve CREB-CBP/p300 interaction with PGC-1α upregulation leading to the PPAR-α-dependent expression of FAO-related enzymes (Figure 4). These include (1) CPT-1α facilitating the entry of FAs into mitochondria for FAO; (2) ACOX1, a peroxisomal enzyme catalyzing the FAD-dependent desaturation of long-chain acyl-coenzyme A derivatives; and (3) ACOT2, a mitochondrial

**Figure 3** T₃-induced changes in liver AMPK-dependent targets related to fatty acid oxidation. mRNA and protein levels of CPT-1α (A), ACOX1 (B), and ACOT2 (C), and circulating levels of β-hydroxybutyrate (D). Values shown are means ± SEM (n = 3-5). Statistical significance was performed by Student’s t-test for unpaired data (P < 0.05). AMPK: AMP-activated protein kinase; ACOT2: Acyl-CoA thioesterase 2; ACOX1: Acyl-CoA oxidase 1; CPT-1α: Carnitine palmitoyltransferase-1α.
phosphorylation, with CPT-1α activation, and CREB phosphorylation, with enhanced expression of co-activator PGC-1α and that of the FAO-related enzymes CPT-1α, ACOX1, and ACOT2 (Figure 4). Enhancement in FAO-related energy metabolism by T3 is in accordance with the enhanced liver ATP turnover reported, which may comply with high-energy requiring processes such as liver PC. In agreement with this contention, T3 was recently shown to stimulate hepatic FAO coupled with the induction of autophagy, a stress-related process degrading cellular components to produce FAs to generate ATP or amino acids to synthesize proteins for cell survival. Thyroid hormone PC is not restricted to the liver, considering that protective effects against IRI are also observed in the heart, kidney, and brain. In addition to ischemia-reperfusion injury, thyroid hormones have a critical role in the repair in several tissues subjected to other types of injury, namely, mechanical injury, nerve transection, chemotherapy-induced toxicity, hyperoxia injury, serum starvation, or wound.

**REFERENCES**

1. Harvey CB, Williams GR. Mechanism of thyroid hormone action. *Thyroid* 2002; 12: 441-446 [PMID: 12165104 DOI: 10.1097/01 THY.0000000000000001]

2. Arnold S, Goglia F, Kadenbach B. 3,5-Diodothyronine binds to subunit Va of cytochrome-c oxidase and abolishes the allosteric inhibition of respiration by ATP. *Eur J Clin Invest* 2002; 32: 89/105072502760143791

Figure 4  Schematic representation of T3 signaling related to AMPK up-regulation and consequent fatty acid oxidation enhancement. ACC: Acetyl-CoA carboxylase; ACOT2: acyl-CoA thioesterase 2; ACOX1: acyl-CoA oxidase 1; AMPK: AMP-activated protein kinase; CaMKKβ: Ca2+-calmodulin-dependent protein kinase kinase-β; CBP/p300: CREB-binding protein; CPT-1α: Carnitine palmitoyltransferase-1α; CREB: cAMP-response element-binding protein; Fa: Fatty acid; PGT-1: Peroxisome proliferator-activated receptor-γ coactivator-1α; QO: Rate of oxygen consumption; ROS: Reactive oxygen species; TAK1: Transforming growth factor-β-activated kinase-1.

In conclusion, data presented indicate that T3-induced ROS production, revealed by higher 8-isoprostane levels in liver and plasma, have a critical role in upregulating rat liver AMPK signaling, resulting in FAO enhancement to support energy-demanding processes such as T3-PC or tissue repair. This is evidenced by suppression of T3-induced hepatic AMPK mRNA levels and those of the upstream kinases CaMKKβ and TAK1, as well as their protein expression, by pretreatment with the antioxidant NAC previous to T3, findings that agree with higher pAMPK levels observed over control values. In addition, the higher AMPK phosphorylation potential elicited by T3 is associated with enhancement in hepatic pACC/ACC and pCREB/CREB ratios as direct targets of activated AMPK (Figure 4). The latter changes induced by T3 trigger FAO as assessed by the consequent ketogenic response, which may involve both ACC phospho-
Videla LA et al. T-induced liver AMPK signaling

Biochem 1998; 252: 325-330 [PMID: 9523704 DOI: 10.1046/j.1432-1327.1998.252032.x]

Videla LA. Hormetic responses of thyroid hormone calorigenesis in the liver: Association with oxidative stress. JUMBR Life Sci 2010; 62: 463-466 [PMID: 20913439 DOI: 10.1002/jub.2545]

Cornejo P, Vargas R, Videla LA. Nrf2-regulated phase-II de-toxification enzymes and phase-III transporters are induced by thyroid hormone in rat liver. Biofactors 2013; 39: 514-521 [PMID: 23554160 DOI: 10.1002/biof.1094]

Fernández V, Castillo I, Tapia G, Romanque P, Uribe-Echevarría S, Uribe M, Cartier-Ugarde G, Santander G, Vial MT, Videla LA. Thyroid hormone preconditioning: protection against ischemia-reperfusion liver injury in the rat. Hepatology 2007; 45: 170-177 [PMID: 17187421 DOI: 10.1002/hep.21476]

Taki-Eldin A, Zhou L, Xie HY, Chen KJ, Yu D, He Y, Zheng SS. Triiodothyronine attenuates hepatic ischemia/reperfusion injury in a partial hepatectomy model through inhibition of proinflammatory cytokines, transcription factors, and adhesion molecules. J Surg Res 2012; 178: 646-656 [PMID: 22729740 DOI: 10.1016/j.jss.2012.05.069]

Mourouzis I, Politi E, Pantos C. Thyroid hormone and tissue repair: new tricks for an old hormone? J Thyroid Res 2013; 2013: 312014 [PMID: 23539950]

Bahré R, Spiegel HU. Hepatic ischaemia-reperfusion injury from bench to bedside. Br J Surg 2010; 97: 1461-1475 [PMID: 20645395 DOI: 10.1002/bjs.2716]

Viiolet B, Guigas B, Leclerc J, Hébrard S, Lantier L, Mounier R, Andreelli F, Foretz M. AMP-activated protein kinase in the regulation of hepatic energy metabolism: from physiology to therapeutic perspectives. Acta Physiol (Oxf) 2009; 196: 81-98 [PMID: 19245566 DOI: 10.1111/j.1748-1716.2009.01970.x]

Fernández V, Tapia G, Videla LA. Recent advances in liver preconditioning: Thyroid hormone, 3-long-chain polyunsaturated fatty acids and iron. World J Hepatol 2012; 4: 119-128 [PMID: 22567184 DOI: 10.4254/wjh.v4.i11.19]

Hasenour CM, Berglund ED, Wasserman DH. Emerging role of AMP-activated protein kinase in endocrine control of metabolism in the liver. Mol Cell Endocrinol 2013; 366: 152-162 [PMID: 22796337 DOI: 10.1016/j.mce.2012.06.018]

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227: 680-685 [PMID: 5432603]

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 1979; 76: 4350-4354 [PMID: 388439]

Romanque P, Cornejo P, Valdés S, Videla LA. Thyroid hormone administration induces rat liver Nrf2 activation: suppression by N-acetylcysteine pretreatment. J Biol Regul Homeost Agents 2003; 4: 312104 [PMID: 23533950]

Vargas R, Ortega Y, Bozo V, Andrade M, Minuzzi G, Cornejo P, Fernandez V, Videla LA. Thyroid hormone activates rat liver adenosine 5',monophosphate-activated protein kinase: relation to CaMKKb, TAK1 and LKB1 expression and energy cellular signals. J Appl Physiol 2007; 104: 1052-1060 [PMID: 17481327 DOI: 10.1152/japplphysiol.00900.2007]

Echevarría S, Uribe M, Cartier-Ugarte D, Santander G, Vial MT, Videla LA. Causal role of oxidative stress in liver adenosine 5',monophosphate-activated protein kinase activity. J Biol Chem 2011; 286: 17513-17520 [PMID: 21765556 DOI: 10.1074/jbc.M702390200]

Winder WW, Hardie DG. Inactivation of acetyl-CoA carboxylase and activation of AMP-activated protein kinase in muscle during exercise. Am J Physiol Endocrinol Metab 1996; 270: E299-E304

Collins QF, Liu HY, Pi J, Liu Z, Quon MJ, Cao W. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, suppresses hepatic gluconeogenesis in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase. J Biol Chem 2005; 280: 17515-17520 [PMID: 16255556 DOI: 10.1074/jbc.M702390200]

Forman HJ, Maierino M, Ursini F. Signaling functions of reactive oxygen species. Biochemistry 2010; 49: 835-842 [PMID: 20505630 DOI: 10.1021/bi9020578]

Thomson DM, Winder WW. AMP-activated protein kinase control of fat metabolism in skeletal muscle. Acta Physiol (Oxf) 2009; 196: 147-154 [PMID: 19245563 DOI: 10.1111/j.1748-1716.2009.01970.x]

Zhang X, Grand RJ, McCabe CJ, Franklyn JA, Gallimore PH, Turnbull AS. Transcriptional regulation of the human glycoprotein hormone alpha subunit gene by cAMP-response-element-binding protein (CREB)-binding protein (CBP)/p300 and p53. Biochem J 2002; 368: 191-201 [PMID: 12164786 DOI: 10.1042/bj02003634]

Thomson DM, Herway ST, Fillmore N, Kim H, Brown JD, Barrow JR, Winder WW. AMP-activated protein kinase phosphorylates transcription factors of the CREB family. J Appl Physiol 2008; 104: 429-438 [PMID: 18063805 DOI: 10.1152/japplphysiol.00900.2007]

Shaywitz AJ, Greenberg ME. CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals. Annu Rev Biochem 1999; 68: 821-861 [PMID: 10872467 DOI: 10.1146/annurev.biochem.68.1.821]

Jäger S, Handschin C, St-Pierre J, Spiegelman BM. AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1alpha. Proc Natl Acad Sci USA 2007; 104: 12017-12022 [PMID: 17693638 DOI: 10.1073/pnas.070507104]

Schmidt SF, Mandrup S. Gene program-specific regulation of PGC-1alpha activity. Genes Dev 2011; 25: 1453-1458 [PMID: 21764849 DOI: 10.1101/gad.2076411]

Vega RB, Huss JM, Kelly DP. The coactivator PGC-1 coop...
erates with peroxisome proliferator-activator receptor α in transcriptional control of nuclear genes encoding mitochondrial fatty acid oxidation enzymes. *Mol Cell Biol* 2000; 20: 1868-1876 [DOI: 10.1128/MCB.20.5.1868-1876.2000]

36 Aoyama T, Sourri M, Kamijo T, Ushikubo S, Hashimoto T. Peroxisomal acyl-coenzyme A oxidase is a rate-limiting enzyme in a very-long-chain fatty acid beta-oxidation system. *Biochem Biophys Res Commun* 1994; 201: 1541-1547 [PMID: 8024599 DOI: 10.1006/bbrc.1994.1879]

37 Kirkby B, Roman N, Kobe B, Kellie S, Forwood JK. Functional and structural properties of mammalian acyl-coenzyme A thioesters. *Prog Lipid Res* 2010; 49: 366-377 [PMID: 20478824 DOI: 10.1016/j.plipres.2010.04.001]

38 Ismail-Beigi F, Salibian A, Kirsten E, Edelman IS, White A. Effects of thyroid hormone on adenine nucleotide content of rat liver. *Proc Soc Exp Biol Med* 1973; 144: 471-474 [PMID: 4746919]

39 Sinha RA, You SH, Zhou J, Siddique MM, Bay BH, Zhu X, Privalsky ML, Cheng SY, Stevens RD, Summers SA, Newgard CB, Lazar MA, Yen PM. Thyroid hormone stimulates hepatic lipid catabolism via activation of autophagy. *J Clin Invest* 2012; 122: 2428-2438 [PMID: 22684107 DOI: 10.1172/JCI60580]

40 Pantos CI, Malliopoulou VA, Mourouzis IS, Karamanoli EP, Paizis IA, Steinberg N, Varonos DD, Cokkinos DV. Long-term thyroxine administration protects the heart in a pattern similar to ischemic preconditioning. *Thyroid* 2002; 12: 325-329 [PMID: 12094058 DOI: 10.1089/10507250252949469]

41 Kumar A, Taliyan R, Sharma PL. Evaluation of thyroid hormone induced pharmacological preconditioning on cardiomyocyte protection against ischemic-reperfusion injury. *Indian J Pharmacol* 2012; 44: 68-72 [PMID: 22345873 DOI: 10.4103/0019-5049.98307]

42 Li F, Lu S, Zhu R, Zhou Z, Ma L, Cai L, Liu Z. Heme oxygenase-1 is induced by thyroid hormone and involved in thyroid hormone preconditioning-induced protection against renal warm ischemia in rat. *Mol Cell Endocrinol* 2011; 339: 54-62 [PMID: 21458530 DOI: 10.1016/j.mce.2011.03.019]

43 Ferreyra C, O’Valle F, Osorio JM, Moreno JM, Rodríguez I, Vargas F, Osuna A. Effect of preconditioning with triiodothyronine on renal ischemia/reperfusion injury and poly(ADP-ribose) polymerase expression in rats. *Transplant Proc* 2009; 41: 2073-2075 [PMID: 19715835 DOI: 10.1016/j.transproceed.2009.06.060]

44 Genovese T, Impellizzeri D, Ahmad A, Cornelius C, Campolo M, Cuzzocrea S, Esposito E. Post-ischaemic thyroid hormone treatment in a rat model of acute stroke. *Brain Res* 2013; 1513: 92-102 [PMID: 2350606 DOI: 10.1016/j.brainres.2013.03.001]
