Endocr (2009) 36:198–204
DOI 10.1007/s12020-009-9210-2

ORIGINAL ARTICLE

Thyroid hormones alterations during acute liver failure: possible underlying mechanisms and consequences

Georgia Kostopanagiotou · Konstantinos Kalimeris · Iordanis Mourouzis · Constantinos Costopanagiotou · Nikolaos Arkadopoulos · Dimitrios Panagopoulos · Nikolaos Papoutsidakis · Aikaterini Chranioti · Agatha Pafiti · Danai Spanou · Vassilios Smyrniotis · Constantinos Pantos

Received: 25 July 2008 / Accepted: 2 June 2009 / Published online: 19 June 2009

Abstract Thyroid hormones are now recognized to change in different disease states with important consequences on severity and prognosis of disease. However, little is known about thyroid hormones' alterations in acute liver failure (ALF). To study the changes in thyroid hormones and cardiac thyroid receptors during ALF, we subjected seven female pigs to surgical liver devascularization. Liver function biochemical markers, thyroid hormones, endogenous opioids, malondialdehyde (MDA), and interleukins 1 and 6 were measured in serum for 24 h postoperatively. Heart biopsies were harvested at the end of the experiment. Baseline heart biopsies were taken from five additional animals. Serum thyroxin (T_4) and triiodothyronine (T_3) levels markedly decreased, whereas free-triiodothyronine and thyroxin-stimulating hormone levels did not change. T_4 and T_3 levels correlated with the degree of liver failure and with MDA and interleukin-6 levels. Beta-endorphin levels initially increased, whereas levels of leucine-enkephalin did not change. Thyroid hormone receptor-α1 protein expression in the heart decreased 1.6-fold after ALF, whereas myocardial myosin isoform expression remained unchanged. The downregulation of T_4 and T_3 levels during ALF seems to correlate well with the severity of disease. This downregulation related to inflammation and oxidative stress and resulted in changes in myocardial thyroid receptors.

Keywords Acute liver failure · Thyroid hormone receptor α1 · Non-thyroidal illness syndrome · Lipid peroxidation · Endogenous opioids

G. Kostopanagiotou · K. Kalimeris (✉) · A. Chranioti
2nd Department of Anesthesiology, Attikon Hospital, University of Athens School of Medicine, Rimini 1 Str., Chaidari, Athens, Greece
e-mail: k_kalimeris@hotmail.com

I. Mourouzis · D. Spanou · C. Pantos
Department of Pharmacology, University of Athens School of Medicine, Athens, Greece

C. Costopanagiotou · N. Arkadopoulos · D. Panagopoulos · N. Papoutsidakis · V. Smyrniotis
2nd Department of Surgery, Aretaieion Hospital, University of Athens School of Medicine, Athens, Greece

A. Pafiti
Department of Pathology, Aretaieion Hospital, University of Athens School of Medicine, Athens, Greece

Introduction

Thyroid hormones are now recognized to change in various disease states such as heart failure, acute myocardial infarction, cardiopulmonary bypass surgery, and sepsis, and these alterations seem to be related with the severity and prognosis of disease [1–4]. Acute liver failure (ALF) is accompanied by hormonal implications, as has been recently shown for the hepatoadrenal syndrome [5]. Thyroid function alterations have been described during chronic liver failure [6–8], but much less clear data are available for ALF [9, 10]. In cirrhotic and also in acutely ill patients from various etiologies, derangements of thyroid hormones are common (up to 79% in the latter group) [11]. This syndrome, called non-thyroidal illness syndrome (NTIS), usually manifests itself as low triidothyronine (T_3) levels, with low thyroxine (T_4) levels in severe cases as well, but normal free-triidothyronine (FT_3), and normal or even decreased thyroxin-stimulating hormone (TSH) levels.

The NTIS has been proposed to represent an adaptive mechanism to energy expenditure and catabolism during critical illness [12]. Some studies have proposed cytokines,

mainly interleukin-6 (IL-6), as possible mediators of NTIS [13, 14]. A role for endogenous opioids was also suggested by few studies, but their alterations during ALF and their clinical significance are still unclear [15, 16]. Peripheral mechanisms, normal tissue activity despite decreased serum levels, and the presence of a circulating inhibitor have also been proposed, but no single mechanism has proven satisfactory [17, 18].

In this study, we investigated changes in thyroid hormones in a surgical model of ALF and the possible correlations of these changes to the severity of ALF. Our aim was to document if surgical devascularization of the liver induces the NTIS and whether the thyroid hormones alterations correlate with severity of ALF. In an effort to uncover possible mediators, we also determined the levels of interleukins 1 and 6, malondialdehyde (MDA), and endogenous opioids in systemic circulation. We also examined if ALF apart from changes in the thyroid hormones levels in plasma could also result in changes in thyroid hormone signaling at the tissue level. In order to address this issue we examined thyroid hormone receptors expression in the heart, which is known to be an organ highly responsive to thyroid hormone.

Results

Biochemical parameters, thyroid hormone axis, MDA, interleukins, and endogenous opioids after ALF

Time-dependent changes of serum biochemical parameters after ALF are shown in Table 1. After ALF, serum albumin and total protein levels decreased, whereas ammonia, lactate dehydrogenase (LDH), MDA, total, and direct bilirubin levels increased.

Time-dependent changes after ALF in thyroid hormone levels, interleukins, and endogenous opioids are shown in Tables 2 and 3. Marked decreases were observed in T_3 and T_4 levels during the 24-h observation period, whereas no changes were found in FT_3 or TSH levels. Serum IL-6 levels markedly increased. On the other hand, serum interleukin-1 (IL-1) levels did not change.

Table 2 Serum interleukins 1 and 6 at 0, 12 and 24 h after completion of surgery

Interleukin	0 h	12 h	24 h
IL-6 (pg/ml)	0.8 ± 0.2	537 ± 266^{I}	502 ± 404
IL-1 (pg/ml)	34 ± 37	36 ± 16	71 ± 33

Data are expressed as mean ± SD

I $P < 0.05$ compared to 0 h (I)

Beta-endorphin levels increased at 6 h and then decreased toward baseline levels. Leucine-enkephalin levels showed a slight decrease which did not reach statistical significance.

Correlations of thyroid hormones with biochemical markers of severity of ALF

T_4 levels showed a positive correlation to total protein ($r = +0.554$, $P = 0.006$) and albumin ($r = +0.551$, $P = 0.004$) and a negative correlation to NH_3 ($r = -0.760$, $P < 0.001$), LDH ($r = -0.698$, $P < 0.001$), total bilirubin ($r = -0.625$, $P = 0.001$) and direct bilirubin levels ($r = -0.479$, $P = 0.015$). In addition, total T_3 levels negatively correlated to NH_3 ($r = -0.637$, $P = 0.001$), LDH ($r = -0.786$, $P < 0.001$), total bilirubin ($r = -0.475$, $P = 0.017$) and direct bilirubin ($r = -0.557$, $P = 0.004$). No correlations of FT_3 levels were found with the above biochemical markers.

Correlations of thyroid hormones with MDA levels, interleukins, and endogenous opioids

T_4 and total T_3 levels correlated to serum IL-6 levels ($r = -0.600$, $P = 0.018$; $r = -0.643$, $P = 0.010$, respectively)

Table 1 Biochemical parameters at 0, 6, 12, 18, and 24 h after completion of the surgical procedure

Parameter	0 h (I)	6 h (II)	12 h (III)	18 h (IV)	24 h (V)
Albumin (g/dl)	1.2 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	0.7 ± 0.4	0.6 ± 0.1^{I,II,III}
Total protein (g/dl)	4.9 ± 0.9	4.1 ± 0.4	4.6 ± 0.6	3.2 ± 1.1	2.1 ± 1.0^{I}
NH_3 (μmol/l)	89 ± 59	329 ± 36^{I}	455 ± 115	793 ± 130^{i,II,iii}	759 ± 301
LDH (U/l)	489 ± 232	1386 ± 383	2282 ± 154^{i,II}	2127 ± 411^{I}	1892 ± 563
Total Bil (mg/dl)	0.08 ± 0.06	0.36 ± 0.22	0.89 ± 0.32	1.17 ± 0.51^{I,II}	1.31 ± 0.49^{I,II}
Direct Bil (mg/dl)	0.04 ± 0.04	0.17 ± 0.09	0.27 ± 0.25	0.31 ± 0.15	0.40 ± 0.13^{I,II}
MDA (nmol/ml)	1.2 ± 0.3	3.1 ± 0.1^{i}	5.3 ± 4.2	5.5 ± 2.0	6.0 ± 1.9^{II}

Data are expressed as mean ± SD

Bil bilirubin, *LDH* lactate dehydrogenase, *MDA* plasma MDA, *I* $P < 0.05$ compared to 0 h (I), *II* $P < 0.05$ compared to 6 h (II), *III* $P < 0.05$ compared to 12 h (III), *i* $P < 0.01$ compared to 0 h (I), *ii* $P < 0.01$ compared to 6 h (II), *iii* $P < 0.01$ compared to 12 h (III)

Table 3 T_4, T_3, FT_3, TSH, β-endorphin, and leu-enkephalin concentrations

Parameter	0 h (I)	6 h (II)	12 h (III)	18 h (IV)	24 h (V)
T4 (μg/dl)	6.82 ± 2.13	3.45 ± 1.04	2.54 ± 0.82	1.75 ± 0.64^{II}	1.03 ± 0.56^{I,ii,III}
T3 (ng/dl)	79.86 ± 14.95	48.95 ± 6.09^{I}	37.88 ± 4.46^{I}	41.68 ± 19.90	37.39 ± 3.74^{I}
FT3 (pg/ml)	0.07 ± 0.15	0.25 ± 0.27	0.16 ± 0.13	0.16 ± 0.14	0.17 ± 0.16
TSH (μIU/ml)	0.29 ± 0.12	0.19 ± 0.09	0.19 ± 0.11	0.16 ± 0.06	0.12 ± 0.03
β-END (ng/ml)	0.69 ± 0.09	10.51 ± 2.74^{I}	2.77 ± 2.01^{II}	1.26 ± 2.27^{ii}	0.96 ± 0.62^{II}
L-ENK (ng/ml)	0.90 ± 0.49	0.34 ± 0.26	0.35 ± 0.45	0.16 ± 0.25	0.17 ± 0.15

Data are expressed as mean ± SD

β-END β-endorphin, *L-ENK* leucine-enkephalin, *I* $P < 0.05$ compared to 0 h (I), *II* $P < 0.05$ compared to 6 h (II), *III* $P < 0.05$ compared to 12 h (III), *i* $P < 0.01$ compared to 0 h (I), *ii* $P < 0.01$ compared to 6 h (II), *iii* $P < 0.01$ compared to 12 h (III)

and plasma MDA levels ($r = -0.624$, $P = 0.001$; $r = -0.477$, $P = 0.016$, respectively), but not to IL-1 or β-endorphin levels. In addition, leucine-enkephalin correlated to T_4 ($r = +0.665$, $P < 0.001$) but not to T_3. No correlations were found among FT_3 concentrations and interleukins, MDA levels, or endogenous opioids.

Thyroid hormone receptor a1 and myosin isoform expression in the myocardium at baseline and after ALF

Thyroid hormone receptor α1 (TRα1) nuclear protein expression in the heart was found to decrease 1.6-fold after ALF ($P < 0.05$), whereas myocardial myosin heavy chain (MHC) isoform expression remained unchanged. Only β-MHC was found to be expressed in pig heart (Fig. 1).

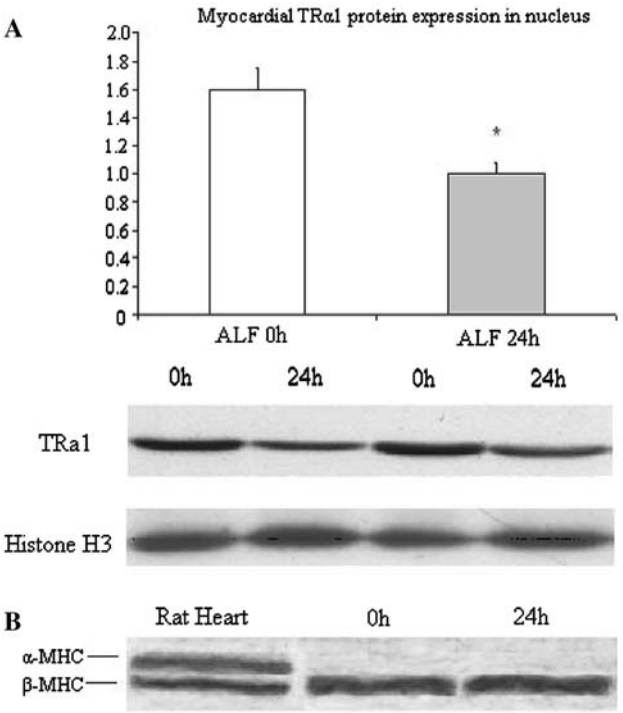

Fig. 1 **a** Densitometric assessment in arbitrary units and representative Western blots of thyroid hormone receptor α1 (TRα1) protein expression in hearts from animals subjected to ALF at 0 and 24 h (columns are means of optical ratios, bar = SEM). **b** Representative figure showing myosin isoforms expression in hearts from animals subjected to ALF at 0 and 24 h. * $P < 0.05$ vs. 0 h

Discussion

In this study, we induced ALF by surgical devascularization of the liver and we studied thyroid hormones changes. T_3 and T_4 levels were clearly found to decrease without being accompanied by changes in FT_3 or TSH concentrations. The reduction of T_3 and T_4 levels closely correlated to markers of severity of liver failure, namely total protein, albumin, ammonia and total and direct bilirubin levels. The progressive increases in MDA and IL-6 levels in systemic circulation showed significant negative correlations to both T_3 and T_4 levels. Beta-endorphin levels showed an initial increase and then decreased without correlating to thyroid hormones' levels, whereas leucine-enkephalin levels did not change significantly. TRα1 protein expression in the heart decreased significantly during ALF, without being accompanied by changes in MHC expression.

NTIS has been repeatedly documented in critically ill patients and has been related to severity of critical illness and higher mortality rates [11, 12, 19]. Although some studies on NTIS in critical care patients had also enrolled patients with ALF, no single study has addressed this issue clearly in ALF [9, 10]. In this model of ALF, significant derangements of liver function indices were noted, including hypoproteinemia, hyperbilirubinemia, and hyperammonemia. These changes were accompanied by progressive decreases in T_3 and T_4 levels, which significantly correlated to the above markers of liver failure. This has been previously shown for chronic liver insufficiency but not for ALF [6, 7]. In our study, the decrease in T_3 and T_4 levels was not accompanied by changes in FT_3 or TSH concentrations. The observed hypoproteinemia due to impaired hepatic

synthesis could account for the decrease in both T_3 and T_4 levels, despite the normal levels of FT_3. Although only T_4, but not T_3 levels correlated to total protein and albumin levels, this could be due to the more tight relation of T_4 to albumin and other binding proteins, such as transthyretin and thyroid-binding globulin. Therefore, reduced protein synthesis by the liver could be a significant determinant of thyroid hormones reduction in ALF. Hyperammonemia has also been shown to impair pituitary function and alter TSH responses, possibly due to alterations in brain neurotransmitters [20]. However, correlations of other biochemical indices except for albumin seem more likely to be indirect, following liver function deterioration and the resultant reduction in protein synthesis.

Cytokines have been implicated in the pathogenesis both of ALF and NTIS [14, 21]. The production of IL-6 in ALF is considered part of the acute phase response, playing a pivotal role in the pathogenesis of ALF [13]. In our study, the dramatic increase in serum IL-6 levels correlated closely to both T_3 and T_4 levels, thereby relating the acute inflammatory response in ALF with the thyroid hormones alterations. Interleukin-6 when given to both rats and humans induced the NTIS, but IL-1 did not [22, 23]. Mechanisms that have been suggested are suppression of thyroid hormone formation and inhibition of TSH production by IL-6 [24, 25]. The latter is unlikely to be the case in our experiment, since TSH levels did not change. In addition, because liver circulation was interrupted, the effect of IL-6 cannot be attributed to an inhibition of type I 5′-deiodinase in the liver. Nonetheless, the possibility that IL-6 contributed to the T_3 and T_4 decrease during ALF by altering TSH-induced responses of the thyroid gland, as has been suggested by in vitro studies [26], cannot be ruled out. The contribution of IL-6 in the observed changes in ALF deserves further evaluation.

Except for IL-6, other mechanisms may have also contributed to NTIS. Plasma MDA levels increased early in the course of ALF in our model and significantly correlated to both T_3 and T_4 concentrations. Lipid peroxidation has been implicated in the development of ALF [27] and has also been proposed as a mechanism of NTIS during critical illness [28]. However, conclusive data on oxidative stress in NTIS are lacking and the possibility that they represent independent events during ALF cannot be excluded.

A role for endogenous opioids has been suggested in the central regulation of the thyroidal axis, since opioid receptors blockade can reduce TSH secretion [26]. However, the clinical impact of endogenous opioids in stimulating TSH secretion has been considered minor [29]. In addition, endogenous opioids have been found either to increase or remain stable in liver failure [30, 31]. During ALF in our animals β-endorphin initially increased at 6 h and then returned to baseline levels, without correlating to thyroidal hormones. On the other hand, leucine-enkephalin concentrations were not significantly altered. These changes in the endogenous opioids are unlikely to have affected the progressive and intense decreases in the thyroid hormones, possibly arguing against a significant in vivo role of endogenous opioids in the NTIS.

The heart is a main target organ of the thyroid hormones, since the latter modulate the expression of several enzymes, functional, and structural proteins, such as phospholamban and MHCs in the heart [32]. Most of the physiological actions of thyroid hormone are mediated through chromatin-associated nuclear receptors, which bind thyroid hormone regulatory elements in the promoter region of target genes. TRα1 is the predominant thyroid receptor isoform in the heart and plays an important role in determining cardiac function by regulating the transcription of MHC isoforms expression and ion channel genes [33–36]. The alterations of cardiac thyroid receptors during the NTIS have not been examined before. The significant decrease in TRα1 expression in our animals despite the low circulating thyroid hormones concentrations is not in accordance with a compensatory response of the cardiac receptors, as could have been perhaps expected. Instead, the decrease in both thyroid hormone levels and TRα1 could be conceived as a protective adaptation of the heart during the severe systemic stress induced by ALF. It is known that low thyroid hormone levels preserve a low metabolic status of oxygen demands [2, 37, 38]. The downwards altered equilibrium between thyroid hormones and TRα1 could therefore represent an adaptation in a state of decreased oxygen and energy consumption, as has been elsewhere suggested [12, 36]. Whether the downregulation of the cardiac thyroid receptors in ALF is cardioprotective remains to be answered.

It has been shown that hypothyroidism results in increased expression of β-MHC and leads to decreased speed of systolic contraction [2, 34]. Despite the downregulation of thyroid hormones and TRα1, β-MHC protein expression was not altered in our model. Besides, the questions whether acute changes in thyroid hormones metabolism impair cardiac function and whether thyroid hormones' replacement in NTIS provides long-term beneficial effects in outcome still remain to be elucidated [1].

Our findings are limited by several factors. First, not all correlations of liver dysfunction markers with thyroid hormones decline during the progression of ALF are due to causative relationships. However, if a parameter does not correlate to the thyroid hormones levels, then it would be more unlikely to etiologically relate to NTIS. Although central acquired hypothyroidism may not be reflected by the correlations of liver dysfunction with thyroid hormones, some conclusions can be drawn from the changes in

peripheral hormones. For instance, in a paper by Wadwekar and Kabadi [39] replacement with levothyroxine did not prevent thyroid hormones' decrease during acute illness, indicating that peripheral mechanisms and thyroid hormones metabolism may be the crucial element during NTIS. Therefore, hypoalbuminemia and IL-6 could represent peripheral mechanisms that could relate, but not necessarily etiologically, with NTIS during ALF. Appropriate future studies should focus on thyroid gland function and specific thyroid binding proteins during ALF.

In conclusion, in this study we showed that (1) ALF clearly induces the NTIS, by significantly decreasing T_3 and T_4, without changing TSH; (2) hypoalbuminemia may be a significant cause of the derangement of the thyroid hormones. Interestingly, endogenous opioids alterations during ALF do not seem to significantly affect NTIS, but IL-6 production and lipid peroxidation may contribute to the ALF-induced NTIS; and (3) during NTIS, thyroid hormone receptor-α1 in the heart is down-regulated. Overall, induction of ALF with surgical liver devascularization reproducibly produced the NTIS and its severity correlated to markers of liver dysfunction. Whether highlighted mechanisms such as reduction of binding proteins and induction of interleukin-6 and lipid peroxidation contributes to NTIS remain to be clarified. It should be further examined whether downregulation of cardiac thyroid receptors during NTIS represents a cardioprotective adaptation during severe systemic stress.

Materials and methods

Animal preparation and anesthesia

The protocol was approved by the Animal Research Committee of the University of Athens. Care and handling of the animals was in accordance with European guidelines for ethical animal research. Twelve female pigs with body weight ~24 kg were used. After 24 h deprivation of food and ad libitum access to water, premedication with 3–5 mg/kg ketamine, 0.5 mg/kg midazolam, and 0.5 mg atropine was administered intramuscularly. Endotracheal intubation was facilitated with iv thiopental sodium 4–6 mg/kg and ketamine 2–4 mg/kg. The animals were mechanically ventilated with a fraction of inspired oxygen of 0.6 and tidal volumes of ~10 ml/kg, so that normocapnia was maintained.

After intravenous administration of fentanyl 10–20 μg/kg and vecuronium bromide 0.5 mg/kg, the right external jugular vein was exposed and a central vein catheter 6.5 Fr (G986, Arrow International, Reading, PA, USA) was inserted to obtain central venous pressure measurements and blood samples. The right carotid artery was also cannulated for arterial pressure monitoring. Temperature was kept stable by means of a heating pad. Electrocardiography, arterial blood pressure, end-tidal CO_2, and pulse oxymetry were monitored continuously. Blood samples were obtained at 0, 6, 12, 18, and 24 h after completion of operation. An aliquot of 750 mg intravenous cefuroxime was given every 6 h beginning from incision. Anesthesia was maintained with continuous intravenous infusion of fentanyl 15–20 μg/kg/h, ketamine 5–8 mg/kg/h, and vecuronium bromide 0.5 mg/kg/h. Dextrose 5% and Ringer's lactated 100–200 ml/h were given in order to maintain normoglycemia, normal electrolytic values, and central venous pressure between 4 and 9 mmHg.

Surgical procedure and postoperative care

In seven animals, ALF was induced using our previously described modification of the liver devascularization model [40]. In brief, an end-to-side porta-caval anastomosis was fashioned first in order to prevent bowel congestion and then the hepatoduodenal ligament with its contents (hepatic artery, bile duct) was ligated. All other ligamentous attachments of the liver were also transected in order to disrupt blood inflow from small collaterals. The procedure lasted approximately 60 min and was accompanied by minimal blood loss. At the end of the experiment animals were euthanized with intravenous infusion of KCl 2 g, thiopental sodium 20 mg/kg, and vecuronium bromide 20 mg. Heart biopsies were taken from the left ventricle for myocardial protein expression analysis. Autopsies verified the absence of surgical complications and histological examination of the liver confirmed total liver necrosis in each animal (Fig. 2).

Baseline heart biopsies from the left ventricle were taken from five additional animals after endotracheal

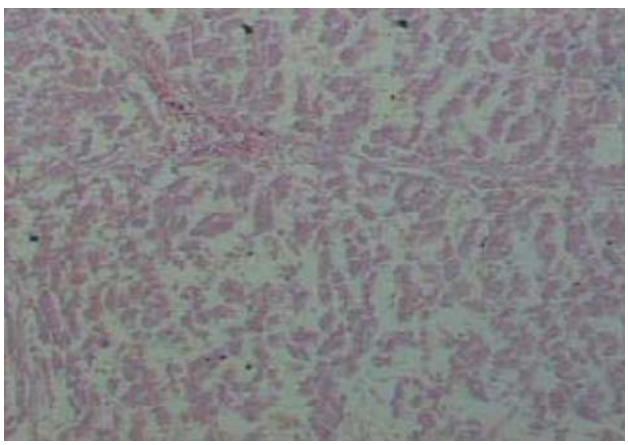

Fig. 2 Histological examination of the liver at 24 h confirms extensive necrosis (hematoxylin-eosin stain, original magnification ×25)

intubation and euthanasia as above, for baseline measurement of TRα1 and β-MHC.

Thyroid hormones and opioid peptides measurement

Levels of FT_3, total T_3, and T_4 in serum were determined by the ACTIVE® Free T_3 (DSL-10-41100), the ACTIVE® Total T_3 (DSL-10-3100S), and the ACTIVE® T_4 (DSL-10-3200) Enzyme Immunoassay (EIA) kits, respectively, by Diagnostic Systems Laboratories Inc. (Webster, TX, USA). Levels of TSH in serum were determined by the ACTIVE® TSH (DSL-10-5300) enzyme-linked immunosorbent assay (ELISA) kit by Diagnostic Systems Laboratories Inc. Serum levels of β-endorphin and leucine-enkephalin were determined by the β-endorphin (EK-022-32) and the leucine-enkephalin (EK-024-21) EIA porcine kits by Phoenix Europe GmbH (Karlsruhe, Germany). The ELISA scanner for the above measurements was Model A3 by Das (Rome, Italy) and the software used was ACTION 1.8.

MDA measurement

Plasma MDA was measured by adding 0.65 ml of 10.3 mM *N*-methyl-2-phenyl-indole in acetonitrile to 0.2 ml of plasma. After vortexing for 3–4 s and adding 0.15 ml of HCl 37%, samples were mixed well, closed with a tight stopper and incubated at 45°C for 60 min. The samples were then cooled on ice, centrifuged and the absorbance was measured spectrophotometrically at 586 nm. A calibration curve of an accurately prepared standard MDA solution (from 2 to 20 nmol/ml) was also run for quantitation. All measurements were performed in triplicate.

Interleukins determination

Serum IL-1 and IL-6 were measured using commercially available porcine cytokine sets (R&D Systems, Minneapolis, USA) according to manufacturer's instructions.

Protein isolation, sodium dodecyl sulfate-protein polyacrylamide gel electrophoresis, and immunodetection

Approximately 0.2 g of left ventricular tissue was homogenized in ice-cold buffer (A) containing 10 mM Hepes (pH: 7.8), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5 mM PMSF, 1 mM DTT, and 10 μg/ml leupeptin. An aliquot of 200 μl of 10% Igepal was added and samples were left in ice for 30 min. Homogenization was repeated and a small fraction of total lysis was kept for MHC isoform analysis. The rest of the homogenate was centrifuged at $1000\times g$ for 5 min, at 4°C, and the pellet containing the nuclear fraction was washed again in buffer (A) with 1% Igepal. The final pellet was resuspended in 300 μl buffer (B) containing 20 mM Hepes (pH: 7.8), 420 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1 mM DTT, 10 μg/ml leupeptin, and 10% glycerol and samples were incubated at 4°C for 60 min (under agitation) followed by centrifugation at $10000\times g$ for 5 min, at 4°C. The supernatant containing the nuclear fraction was separated and stored at −80°C, whereas the pellet containing cellular debris and cytoskeleton was discarded. TRα1 protein expression was determined in nuclear fraction as previously described [3, 41]. Protein concentrations were determined by the BCA method.

Samples were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by boiling for 5 min in Laemmli sample buffer containing 5% 2-mercaptoethanol. An aliquot of 20 μg (nuclear fraction) of total protein was loaded onto 10% (w/v) acrylamide gels and subjected to SDS-PAGE in a Bio-Rad Mini Protean gel apparatus. For Western blotting, following SDS-PAGE, proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond ECL) at 100 V and 4°C, for 1.5 h using Towbin buffer. After Western blotting, filters were probed with specific antibodies against TRα1 (Affinity Bioreagents, Golden, CO, USA, PA1-211A, dilution 1:1000, 2 h at 37°C), and histone H3 (Cell Signaling Technology, Beverly, MA, USA, #9715, dilution 1:1000, o/n at 4°C). Filters were incubated with appropriate anti-rabbit (Cell Signaling Technology, Beverly, MA, USA) HRP secondary antibodies and immunoreactivity was detected by enhanced chemiluminescence using Lumiglo reagents (New England Biolabs, Beverly, MA, USA) and exposed to Hyperfilm paper (Amersham, Amersham, UK). Five samples from each group were loaded on the same gel. Histone H3 protein expression was used in order to normalize slight variations in nuclear protein loading. Immunoblots were quantified using the AlphaScan Imaging Densitometer (Alpha Innotech Corporation, San Leandro, CA, USA).

Measurement of MHC isoform content

Homogenates of all samples were diluted 40-fold with Laemmli sample buffer containing 5% 2-mercaptoethanol. The composition and preparation of the gels was carried out as previously described [30, 42]. In brief, the stacking and separating gels consisted of 4 and 8% acrylamide (w/v), respectively, with Acryl:bis-Acryl in the ratio of 50:1. The stacking and separating gels included 5% (v/v) glycerol. The upper running buffer consisted of 0.1 M Tris (base), 150 mM glycine, 0.1% SDS, and 2-mercaptoethanol at a final concentration of 10 mM. The lower running buffer consisted of 0.05 M Tris (base), 75 mM glycine, and 0.05% SDS. The gels were run in Biorad Protean II xi

electrophoresis unit at a constant voltage of 240 V for 22 h at 4°C. The gels were fixed and silver-stained (Biorad silver stain kit). Gels were scanned and quantified using the AlphaScan Imaging Densitometer (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical analysis

Time-dependent changes were analyzed using one-way repeated measures ANOVA followed by the Bonferroni post hoc test. Differences in TRα1 and β-MHC between baseline values and values after ALF were analyzed with ANOVA. Correlations were carried out by calculating Pearson's correlation coefficient. All statistical analyses were made with SPSS v.15.0 software; P-values < 0.05 were considered statistically significant.

References

1. G. Iervasi, A. Pingitore, P. Landi, M. Raciti, A. Ripoli, M. Scarlattini, A. L'Abbate, L. Donato, Circulation **107**, 708–713 (2003)
2. C. Pantos, V. Malliopoulou, D.D. Varonos, D.V. Cokkinos, Basic Res. Cardiol. **99**, 101–120 (2004)
3. C. Pantos, I. Mourouzis, T. Saranteas, I. Paizis, C. Xinaris, V. Malliopoulou, D.V. Cokkinos, Basic Res. Cardiol. **100**, 422–432 (2005)
4. A.G. Cerillo, L. Sabatino, S. Bevilacqua, P.A. Farneti, M. Scarlattini, F. Forini, M. Glauber, Ann. Thorlac. Surg. **75**, 82–87 (2003)
5. P.E. Marik, T. Gayowski, T.E. Starzl, Crit. Care Med. **33**, 1254–1259 (2005)
6. L. Caregaro, F. Alberino, P. Amodio, C. Merkel, P. Angeli, M. Plebani, A. Gatta, J. Hepatol. **28**, 115–121 (1998)
7. B. Zietz, G. Lock, B. Plach, W. Drobnik, J. Grossmann, J. Schölmerich, R.H. Straub, Eur. J. Gastroenterol. Hepatol. **15**, 495–501 (2003)
8. S. Kayacetin, G. Kisakol, A. Kaya, Swiss Med. Wkly **133**, 210–213 (2003)
9. P. Bratusch-Marrain, H. Vierhapper, B. Grubeck-Loebenstein, W. Waldhäusl, G. Kleinberger, Endokrinologie **80**, 207–212 (1982)
10. E.M. Kaptein, W.J. Robinson, D.A. Grieb, J.T. Nicoloff, J. Clin. Invest. **69**, 526–535 (1982)
11. R. Arem, G.J. Wiener, S.G. Kaplan, H.S. Kim, S. Reichlin, M.M. Kaplan, Metabolism **42**, 1102–1108 (1993)
12. E. Fliers, A. Alkemade, W.M. Wiersinga, Best Pract. Res. Clin. Endocrinol. Metab. **15**, 453–464 (2001)
13. P.H. Davies, E.G. Black, M.C. Sheppard, J.A. Franklyn, Clin. Endocrinol. **44**, 199–205 (1996)
14. K. Yamazaki, E. Yamada, Y. Kanaji, K. Shizume, D.S. Wang, N. Maruo, T. Obara, K. Sato, Endocrinology **137**, 4857–4863 (1996)
15. P. Rauhala, R.K. Tuominen, P.T. Männistö, Acta Endocrinol. **114**, 383–388 (1987)
16. J. Hangaard, M. Andersen, E. Grodum, O. KoldjÆr, C. Hagen, J. Clin. Endocrinol. Metab. **84**, 1595–1601 (1999)
17. M. Borzio, R. Caldara, F. Borzio, V. Piepoli, P. Rampini, C. Ferrari, Gut **24**, 631–636 (1983)
18. D.F. Gardner, R.L. Carithers Jr., R.D. Utiger, Ann. Intern. Med. **96**, 450–452 (1982)
19. B. McIver, C.A. Gorman, Thyroid **7**, 125–132 (1997)
20. J.L. Schlienger, M. Hasselmann, M. Imler, Acta Endocrinol. **89**, 284–295 (1978)
21. K.D. Sekiyama, M. Yoshiba, A.W. Thomson, Clin. Exp. Immunol. **98**, 71–77 (1994)
22. J.M. Stouthard, T. van der Poll, E. Endert, P.J. Bakker, C.H. Veenhof, H.P. Sauerwein, J.A. Romijn, J. Clin. Endocrinol. Metab. **79**, 1342–1346 (1994)
23. L. Bartalena, L. Grasso, S. Brogioni, E. Martino, Eur. J. Endocrinol. **131**, 302–306 (1994)
24. A. Boelen, M.C. Platvoet-Ter Schiphorst, W.M. Wiersinga, J. Clin. Endocrinol. Metab. **77**, 1695–1699 (1993)
25. T. Enomoto, H. Sugawa, S. Kosugi, D. Inoue, T. Mori, H. Imura, Endocrinology **127**, 2322–2327 (1990)
26. T. Tominaga, S. Yamashita, Y. Nagayama, S. Morita, N. Yokoyama, M. Izumi, S. Nagataki, Acta Endocrinol. **124**, 290–294 (1991)
27. C. Kusmic, U. Boggi, R. Bellini, F. Vistoli, M. Castellari, G. Taddei, A. Minervini, F. Filipponi, F. Mosca, R. Barsacchi, Hepatogastroenterology **48**, 762–769 (2001)
28. M. Karbownik-Lewińska, A. Kokoszko, M. Jozefiak, A. Lewiński, Neuro Endocrinol. Lett. **28**, 367–381 (2007)
29. M.H. Samuels, P. Kramer, D. Wilson, G. Sexton, J. Clin. Endocrinol. Metab. **78**, 1249–1252 (1994)
30. J.R. Thornton, M.S. Losowksy, Gut **30**, 1392–1395 (1989)
31. C. Yurdaydin, D. Karaveliogluo, O. Onaran, T. Celik, M.H. Yaşa, O. Uzunalimoglu, J. Hepatol. **29**, 796–801 (1998)
32. G.J. Kahaly, W.H. Dillmann, Endocr. Rev. **6**, 704–728 (2005)
33. C. Pantos, I. Mourouzis, V. Malliopoulou, I. Paizis, S. Tzeis, P. Moraitis, K. Sfakianoudis, D.D. Varonos, D.V. Cokkinos, Thyroid **15**, 16–23 (2005)
34. B. Gloss, S. Trost, W. Bluhm, E. Swanson, R. Clark, R. Winkfein, K. Janzen, W. Giles, O. Chassande, J. Samarut, W. Dillmann, Endocrinology **142**, 544–550 (2001)
35. L. Wikstrom, C. Johansson, C. Salto, C. Barlow, A. Campos Barros, F. Baas, D. Forrest, P. Thoren, B. Vennstrom, EMBO J. **17**, 455–461 (1998)
36. K. Kinugawa, K. Yonekura, R.C. Ribeiro, Y. Eto, T. Aoyagi, J.D. Baxter, S.A. Camacho, M.R. Bristow, C.S. Long, P.C. Simpson, Circ. Res. **89**, 591–598 (2001)
37. C. Pantos, V. Malliopoulou, I. Mourouzis, K. Sfakianoudis, S. Tzeis, P. Doumba, C. Xinaris, A.D. Cokkinos, H. Carageorgiou, D.D. Varonos, D.V. Cokkinos, J. Endocrinol. **178**, 427–435 (2003)
38. R. Bruck, R. Oren, H. Shirin, H. Aeed, M. Papa, Z. Matas, L. Zaidel, Y. Avni, Z. Halpern, Hepatology **27**, 1013–1020 (1998)
39. D. Wadwekar, U.M. Kabadi, Exp. Clin. Endocrinol. Diabetes **112**, 373–377 (2004)
40. G. Kostopanagiotou, C. Routsi, V. Smyrniotis, M.E. Lekka, E. Kitsiouli, N. Arkadopoulos, G. Nakos, Hepatology **37**, 1130–1138 (2003)
41. C. Pantos, I. Mourouzis, K. Markakis, A. Dimopoulos, C. Xinaris, A.D. Kokkinos, M. Panagiotou, D.V. Cokkinos, Eur. J. Cardiothorac. Surg. **32**, 333–339 (2007)
42. P.J. Reiser, W.O. Kline, Am. J. Physiol. **274**, H1048–H1053 (1998)