Title: Thyroid hormone receptors are down-regulated in skeletal muscle of patients with Non-Thyroidal Illness Syndrome secondary to non-septic shock.

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ABSTRACT

Non-thyroidal illness syndrome (NTIS) is related to changes in thyroid hormone (TH) physiology. Skeletal muscle (SM) plays a major role in metabolism and TH regulate SM phenotype and metabolism. We aimed to characterize the SM of non-septic shock NTIS patients in terms of: 1) expression of genes and proteins involved in TH metabolism and actions; and 2) NF-kB pathway activation, a responsible for some of the phenotypic changes in NTIS. We also investigated whether the patient’s serum can induce in vitro the effects observed in vivo.

Methods. Serum samples and SM biopsies from 14 patients with non-septic shock NTIS and 11 controls. Gene and protein expression and NF-kB activation were analyzed by QPCR and immunoblotting. Human skeletal muscle cell (HSkMC) cultures to investigate the effects of patients serum on TH action mediators.

Results. Patients with non-septic shock NTIS showed higher levels of pro-inflammatory cytokines than controls. Expression of TRβ, TRα1 and RXRγ was decreased in NTIS patients. RXRA gene expression was higher but its protein lower in NTIS than controls, suggesting the existence of a post-transcriptional mechanism that down-regulates protein levels. NF-kB pathway activation was not different between NTIS and control patients. HSkMC incubated with patients serum increased TH receptor and RXRG gene expression after 48h.

Conclusions. Patients with non-septic shock NTIS showed decreased expression of TH receptors and RXRs, which were not related to increased activation of the NF-kB pathway. These findings could not be replicated in cultures of human skeletal muscle cells incubated in the patients serum.
INTRODUCTION

Non-thyroidal Illness Syndrome (NTIS) forms a part of the neuroendocrine response to metabolic stress [1], and is characterised by a low 3,5,3’-triiodothyronine (T3) and normal-high 3,3’,5’-triiodothyronine (rT3) in serum and tissues, normal or low serum thyroxine (T4) and inappropriately normal or low serum thyroid-stimulating hormone (TSH) in relation to serum levels of T4 and T3 [2]. Whether NTIS is a beneficial adaptive response to reduce energy consumption, or a form of secondary hypothyroidism that requires thyroid hormone (TH) treatment is controversial [3-6].

The inappropriately normal or low serum TSH levels in NTIS patients is due to an impairment of hypothalamic-pituitary function [7,8], a situation where TH replacement could be beneficial. However, the molecular changes observed in some peripheral tissues from patients with NTIS seem to be directed at decreasing TH action. In humans, T3 is mainly produced by extrathyroidal enzymatic deiodination of T4, mainly in the liver and skeletal muscle (SM) [9]. Low T3 and elevated rT3 levels observed in NTIS patients are related to a decrease in liver type 1 iodothyronine deiodinase (DIO1) and SM type 2 iodothyronine deiodinase (DIO2) activities, and to an increase in type 3 iodothyronine deiodinase (DIO3) activity in the liver and SM [9-11]. Patients with fatal NTIS have decreased T4 and T3 in most tissues [12,13] caused in part by reduced tissue uptake, although thyroid hormone bioavailability is not limited if appropriate replacement therapy is given [2]. A decrease in mRNA expression of the thyroid hormone receptors: thyroid hormone receptor, alpha (THRA) and thyroid hormone receptor, beta (THRB); and their nuclear partner retinoid X receptor, gamma (RXRG), have been reported in SM and subcutaneous adipose tissue of patients with NTIS caused by septic shock [11]. The
functional consequences of decreased TH receptors and RXRG expression remains unknown, but a priori these findings suggest that TH replacement might be less effective in NTIS patients than in patients with hypothyroidism.

Many of the molecular mechanisms responsible for the above described changes remain to be characterized. Increased levels of pro-inflammatory cytokines, endogenous glucocorticoids and glucagon, typically seen in critically ill patients, together with the administration of glucocorticoids and dopaminergic drugs, could directly suppress TRH secretion, the pituitary response to TRH, and TSH secretion [7,8,14]. Patients with NTIS have altered TSH glycosylation which is associated with reduced biological activity [15], and lethal NTIS is associated with major morphological changes in the thyroid gland, including loss of colloid and reductions in follicular size and thyroid weight [16]. Cytokines affect thyroid cell function in several ways [14,17], including a decrease in basal and TSH-stimulated iodide uptake due to interleukin 1 (IL1) and tumor necrosis factor alpha (TNF-α), inhibition of thyroglobulin synthesis by IL1, TNF-α and interferon gamma (IFN-γ), a decrease in thyroperoxidase expression by IL1, interleukin 6 (IL6) and IFN-α, and a decrease in T3 secretion by IL1, TNF-α and IFN-γ.

NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a family of transcription factors that plays a pivotal role in immune and inflammatory responses. Activated NF-kB is a potential molecular factor at the root of NTIS in patients with septic shock and increased serum cytokines level [18]; in vitro studies using HepG2 cells have shown that NF-kB activation attenuates the induction of DIO1 by T3 [18], and that the decrease in DIO1 mRNA expression induced by IL1-β is mediated by the simultaneous activation of NF-kB and activator protein-1 (AP-1) pathways [19].
Skeletal muscle is a major contributor to resting metabolic rate and plays a crucial role in protein metabolism [20]. TH controls expression of more than 50% of SM genes [21] and, together with neuronal activity and physical exercise, regulates the cytoarchitecture and metabolic characteristics of SM. TH stimulates the development of fast muscle fiber characteristics in SM: T3 induces a shift from myosin heavy chain 1 (MYH1), mainly expressed in slow oxidative muscle fibers, to myosin heavy chain 4 (MYH4), typically expressed in the fast glycolytic muscle fibers [22]. T3 also regulates Ca\(^{2+}\)-cycling by the sarcoplasmic reticulum (SR), a source of heat generation; expression of SERCA1, a SR Ca\(^{2+}\)-ATPase predominantly expressed in fast muscle fibers, is increased by T3 in slow muscle fibers simultaneously with the shift from MYH1 to MYH4 in these fibers [23]. T3, through genomic and non-genomic actions, increases lipid oxidation and glycolysis in SM [24], although overall ATP production remains constant since TH increases energy uncoupling and expenditure. Finally, TH increases protein breakdown and the efflux of branched chain amino acids from the muscle [25].

Injurious stimuli increase adipose tissue lipolysis that increases availability of fatty acids to different tissues as a source of energy [26]. Peroxisome proliferator-activated receptor gamma (PPAR\(\gamma\)) plays a central role in lipid and glucose metabolism and is important for maintaining insulin sensitivity. PPAR\(\gamma\) expression is positively regulated by T3 [27], and it forms heterodimers with RXRs, nuclear receptor co-repressor 1 (NCOR1), and nuclear receptor co-repressor 2 (NCOR2=SMART) [28], competing with TH receptors for these proteins. In mice, lipopolysaccharides (LPS) causes a decrease in expression levels of TH receptors and PPAR\(\gamma\) in several tissues [26], and several members of the multiprotein complex that regulate the transcriptional activity of PPAR\(\gamma\) are affected during NTIS.
However, the changes and involvement of PPARγ in human NTIS remains unknown.

To gain further insights into human NTIS pathophysiology, we investigated some of the molecular changes related to thyroid hormone metabolism and actions in skeletal muscle samples from a group of patients with NTIS admitted onto an intensive care unit (ICU) for non-septic shock. Specifically, we quantified the following: 1) mRNA expression of thyroid hormone transporter monocarboxylate transporter 8 (MCT8=SLC16A2), DIO2, THRA, THRBI, RXRA, RXRB, RXRG, PPARG, NCOR1 SMART, and coactivator nuclear receptor coactivator 1 (NCOA1), glucocorticoid receptor alpha, nuclear receptor subfamily 3, group C, member 1 (NR3C1=GCR), and the thyroid hormone responsive genes, solute carrier family 2, member 4 (GLUT4=SLC2A4), uncoupling protein 3 (UCP3), lipoprotein lipase (LPL), the myosin heavy chain genes myosin heavy chain 1 skeletal muscle adult (MYH1) and myosin heavy chain 4 skeletal muscle (MYH4=MyHC-IIb), the SR calcium ATPases ATPase Ca++ transporting cardiac muscle fast twitch 1 (SERCA1) and ATPase Ca++ transporting, cardiac muscle slow twitch 2 (SERCA2); 2) protein expression levels of TRα, TRβ, RXRγ, RXRβ and PPARγ; and 3) mRNA expression of nuclear factor NF-kappa-B p50 subunit (Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells 1 or NFKB1) and p65 (V-rel Reticuloendotheliosis Viral Oncogene Homolog A [avian] or RELA), together with activation of the NF-kB pathway. Finally, we sought to determine whether the changes observed in mRNA expression of MCT8, THRA, THRBI and RXRG in SM in vivo could be reproduced in vitro by incubating cells from a human skeletal muscle cell line in serum from patients with NTIS.
Our results showed that patients with non-septic shock NTIS have a decreased expression of thyroid hormone receptors in the skeletal muscle, not mediated by an activation of the NF-kB pathway.

MATERIALS AND METHODS

Subjects
The study was conducted on two groups of patients with no previous history of thyroid disease. The control group comprised 11 patients (7 males and 4 females), median age 62 years (range 37-77), selected from among those referred to our hospital for knee or hip replacement. The non-septic NTIS group comprised 14 ICU patients (11 males and 3 females), median age 57 years (range 45-89), hospitalized for multiple traumatic injuries (n=4), intracranial hemorrhage (n=8), cardiac arrest during hemodialysis (n=1) and food asphyxia (n=1). NTIS patients were transferred to ICU for ventilatory and circulatory support. Low-medium dosage of noradrenaline was administered to 6 patients. Sedation and analgesia were maintained by continuous IV infusion of propofol and morphine, and nutrients were given by continuous total parenteral nutrition. Eight patients (57.14%) recovered after a median stay in ICU of 5.37 days (range 1-11 days) and 6 patients (42.86) died in ICU.

Skeletal muscle biopsies were obtained from the vastus lateralis muscle as previously described [11]. The biopsies were obtained during surgery in control patients and in the first 48 hours after ICU admission in NTIS patients.

In all cases informed consent was obtained, from the control patients or the closest family member for the ICU patients. The study protocol was approved by the Ethical Review Board of the Medical School of the University of Santiago de Compostela.
Serum analysis

Blood samples were obtained immediately before surgery in the control group and before the muscle biopsy in the non-septic NTIS group. Chemiluminescence was used to measure serum TSH (normal 0.41-4.94 mU/L), free T4 (FT4, normal 0.85-1.60 ng/dL, 10.94-21.75 pmol/L), and free T3 (FT3, normal 2.53-4.29 pg/mL, 0.039-0.066 pmol/L) (ADVIA Centaur, Bayer Diagnostics, Spain), total cortisol (ug/dl), tumor necrosis factor α (TNFα, ng/L), IL-2 receptor (IL-2R, U/ml), IL-6 (ng/l), and IL-8 (ng/l) (Immune 2000, DPC,Gwynedd,UK). Reverse of T3 (rT3, normal 0.15-0.35 ng/ml, 0.23-0.54 nmol/L) was measured by radioimmunoassay (Biocode Hycel, Massy, France). Thyroperoxidase antibodies (TPOAb) and thyroglobulin antibodies (TgAb) were measured by an ELISA (Orgentec Diagnostika, Mainz, Germany).

Human skeletal muscle cells culture for in vitro studies

Primary human skeletal muscle cells, HSkMC (ScienCell Research Laboratories, San Diego, USA) were incubated in 12-well plates using media recommended by the manufacturer. At 80-90% of confluence, growth medium was changed for a basal medium of DMEM plus 20% of a pool of human serum from control subjects or non-septic NTIS patients supplemented with gentamycin (50 µg/ml). Incubation was stopped at 0, 6, 12, 24, 48 and 72 h and cells were harvested for RNA extraction and quantification of gene expression as described below.

Total RNA isolation and real-time quantitative PCR (QPCR)

Total RNA from SM biopsies and HSkMC cell cultures was extracted with Trizol reagent (Invitrogen, Spain) according to the manufacturer’s instructions. cDNA synthesis was
generated from 1µg of total RNA using 5µM random hexamers, 2mM deoxynucleotides (Ecogen, Spain) and M-MLV reverse transcriptase (Invitrogen, Spain).

Gene expression changes were quantified by QPCR. mRNA expression of MCT8, THRA, THRBI and RXRG were evaluated in both SM biopsies and HSkMC cells; mRNA expression of DIO2, RXRA, RXRB, PPARG, SRC1, NCOR1, SMART, NCOA1, GLUT4, UCP3, LPL, NR3C1, MYH1, MYH4, SERCA1, SERCA2, NFKB1 and RELA were measured in SM biopsies. QPCRs were performed in a Light Cycler 2.0 (Roche, Spain) using specific probes and oligonucleotide primers designed by Universal ProbeLibrary (Roche, Spain) as previously described [11]. Gene expression was normalized to RNA polymerase II following the 2^ΔΔct method [29].

**Immunoblotting**

Due to the limited amount of SM tissue available from biopsies and the high protein concentration required for western blotting (25-75µg/lane), total protein extracts were used instead of cytoplasm and nucleus protein. Total protein extraction was obtained after sonication of SM biopsies in 150µl RIPA buffer pH7.4 (50mM Tris-HCl pH8, 150mM NaCl, 1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS) containing protein inhibitors (phenylmethylsulphonyl fluoride, PMSF and sodium orthovanadate, Na3VO4; each at 1µM final concentration). The lysate was centrifuged for 30min at maximum speed (14,000 rpm) and the supernatant collected. Protein was quantified using the Bradford method and equal amounts of protein were run on an SDS-PAGE (10% polyacrylamide gel). After electrophoresis, the proteins were transferred to a nitrocellulose membrane and blocked for at least 1h with 5% dried milk powder in TBS. The membrane was then incubated overnight with the first antibodies (dilution 1:1000 in blocking solution),
against TRβ (Acris Antibodies, AntibodyBcn SL, Spain, SM5022), TRα1 (Santa Cruz, Heidelberg, Germany, sc-772), PPARγ (Santa Cruz, sc-772), RXRγ (Santa Cruz, sc-555), RXRα (Santa Cruz, sc-553), and β-tubulin (Sigma, Spain, T-5168). After three washes of 10 minutes each with 0.2%TBS Tween 20 (TBS-T), the membrane was incubated with the appropriate HPR-conjugated secondary antibody (Sigma, Spain,). Proteins were detected using ECL (Amersham, Spain). Quantification was performed using alphaEaseFC software and normalised with β-tubulin.

**NF-kB pathway activation**

The status of activation of the NF-kB pathway was determined by analysis of cytoplasmic IkBα phosphorylation. After homogenization in PBS, samples were centrifuged for 5min at 500g at 4°C and resuspended in a sucrose buffer (0.32M Sucrose, 10mM Tris, HCl pH8.0, 3mM CaCl₂, 2mM MgOAc, 0.1mM EDTA, 0.5% NP-40, 1mM DTT and 0.5mM PMSF); cytoplasmic fractions were recovered after centrifugation of the samples for 5min at 500g at 4°C to precipitate the nuclei. Cytoplasmic samples (40μg/lane) were run and transferred as described above, blocked for 1h in TBS-T and 3% BSA, and incubated overnight at 4°C with the primary antibody (anti-IkBα Ab [pS32/pS36]; Biomol International, Grupo Taper SA, Spain, SA-412) diluted to 1:1000 in blocking solution; anti-GADPH (AbCam ref Ab9485) was used as a loading control. Membranes washing incubation with HPR-conjugated secondary antibody and proteins quantification were performed as described above. Quantification was normalised with GADPH.
To demonstrate the specificity of the phosphorylation-site specific antibody, a peptide competition experiment was performed using a IkBα (pS32/pS36) control peptide pair (Biomol International, Spain, SP-412).

**Statistical analysis**

Data were analysed using SPSS 12·0 for Windows (SPSS, Chicago, IL). Results are expressed as mean ± SD, median and range. The groups were compared by Student’s t-test or the Mann–Whitney non-parametric test, as appropriate.

**RESULTS**

Age and body mass index (BMI) were not significantly different between non-septic NTIS and control patients (Table 1). Non-septic NTIS patients had significantly lower serum TSH and FT3, and higher rT3 levels than controls, while no differences were found in serum FT4 (Table 1). TNFα, IL-6, IL-8 and IL-2R were all significantly higher in non-septic NTIS patients than in controls (Table 1). Although non-septic NTIS presented higher serum cortisol levels than controls, no statistically significant differences were observed. TPOAb and TgAb were negative in all patients.

**mRNA expression in skeletal muscle biopsies**

In controls SERCA and MYH1 genes showed the highest, and MCT8 and MYH4 the lowest expression levels (Figure 1). Glucocorticoid receptor alpha, NR3C1, RXRA, GLUT4, UCP3 and LPL genes were all highly expressed in SM (Figure 1).

Non-septic NTIS patients had lower expression levels of THRBI, THRA, RXRG and DIO2, but higher expression of RXRA, RXRB, UCP3 and GLUT4 than control patients (Figure 1). No differences between NTIS and control patients were observed for MCT8,
**Protein expression in skeletal muscle biopsies**

To evaluate whether changes in mRNA expression were accompanied by changes in protein levels, we analyzed the protein expression of TRα1 (55kDa), TRβ (52-55kDa), RXRγ (~51kDa), RXRα (54kDa), PPARγ (67kDa) normalized with β-tubulin (50kDa). RXRs immunoblotting showed the expected 51kDa (RXRγ) and 54kDa (RXRα) weights (Figure 2). A band that did not correspond to the size predicted was also observed, which could represent an alternative and unknown RXR isoform, reflecting the molecular complexity of RXRs. The TRβ antibody turned out to be relatively unspecific and only the band expected from the control test (nuclear protein extract from placenta) is shown (figure 2). TRβ, RXRγ and RXRα expression levels were significantly lower in non-septic NTIS than in control patients (Figure 2); although TRα1 expression was lower in non-septic NTIS than controls, statistical significance was not reached. No differences were observed in PPRAγ expression between the two groups (Figure 2).

**Expression of p50 and p65 subunits and NF-KB activation status in skeletal muscle.**

mRNA expression of *NFKB1* (p50) and *RELA* (p65) were significantly elevated in non-septic NTIS vs controls (Figure 3A).

IkBα immunoblotting showed two bands, a 40kDa product corresponding to phosphorylated IkBα and a 36kDa product that corresponds to non-phosphorylated IkBα (Figure 3B). No statistically significant differences were observed in IkBα phosphorylation between non-septic NTIS and control patients.
Effects of patient serum on mRNA expression of *MCT8*, *THRB1*, *THRA* and *RXRG* in human skeletal muscle cell cultures

HSkMC cultured in serum from control patients showed a significant increase in mRNA expression of *THRA*, *THRB1* and *RXRG* at 48h and 72h. By contrast, MCT8 expression increased at 6h and 12h, but by 24h had returned to basal expression levels with no further changes observed at 48h or 72h (Figure 4). HSkMC cultured in serum from NTIS patients showed a similar response although the expression of *THRB1* was higher at 48h and 72h with respect to controls (Figure 4).

**DISCUSSION**

NTIS is caused by an impairment of hypothalamic-pituitary function and of the physiological mechanisms producing T3 in peripheral tissues in response to moderate-severe illness and starvation. Depending on the severity of the noxious agent, extent of damage and time taken to recover, several molecular changes affecting the transport, tissue uptake, metabolism and action of thyroid hormones can be observed [30]. Although the molecular mechanisms responsible for NTIS remain speculative, the changes are tissue-specific [11].

Skeletal muscle has a crucial role in protein metabolism and contributes to the resting metabolic rate of the organism. Since TH regulates a large number of SM genes [21] we aimed to investigate some of the molecular changes in TH metabolism and action occurring in the SM of patients with NTIS. Previously we have shown that SM from patients with NTIS secondary to septic shock caused by gram-negative bacteria shows a decrease in the thyroid hormone receptors, *THRA* and *THRB*, as well as in *RXRG* [11]. LPS plays a central role in the pathophysiology of septic shock caused by gram-negative
bacteria. LPS binds to TLR-4, activating the NF-kB pathway which in turn increases transcription of a range of genes associated with inflammation and immunity, thus causing a strong inflammatory response. TLR-4 is present in skeletal muscle [31] and LPS binding to TLR-4 could therefore underlie the changes observed in the SM of patients with septic-shock-associated NTIS; indeed, mice deficient in TLR-4 do not develop NTIS in response to LPS and bacterial-related NTIS requires the activation of TLR-MyD88 pathway which leads to the production of inflammatory cytokines [32]. However the patients with non-septic shock NTIS also had decreased levels of TH receptors and RXRG mRNA, and protein expression in SM, suggesting that this pattern constitutes a common response of skeletal muscle during NTIS.

Thyroid hormone promotes proteolysis and a shift from the more energetically efficient slow oxidative muscle fibers to fast glycolitic muscle fibers, increasing energy uncoupling and thus the expenditure of energy as heat. The decreases in TH receptor levels and RXRG could therefore comprise a mechanism to prevent energy expenditure as well as to protect proteins from being broken down [25] under situations of low skeletal muscle activity. Interestingly, non-septic NTIS patients showed an increase in mRNA RXRA expression but a decrease in protein levels, suggesting the existence of a post-transcriptional abnormality in the synthesis or degradation of RXRα. Under catabolic conditions there is a ubiquitin-proteasome-dependent protein degradation [33] and RXRα suffers post-translational regulation by ubiquitination resulting in its rapid degradation [34,35]. This mechanism could explain the decrease in RXRα protein levels in NTIS patients. Thyroid-hormone receptor binding to myosin heavy chain is increased by RXRα [36], so a decrease in both transcription factors could constitute a mechanism by which to
prevent TH altering the skeletal muscle phenotype. Moreover, a decrease in TH receptors, RXRα, and RXRγ would reduce uptake and oxidation of fatty acids by SM, an effect that could be counteracted however by an increase in PPARγ transcriptional activity. The RXRA gene is highly expressed in SM suggesting an important regulatory role. In addition, proteasomal degradation of its protein product could affect PPARγ activity as shown in obese human adipose tissue [35]; PPARγ action depends on its dimerization with different RXR isotypes and a decrease in RXRα has been shown to increase transcriptional activity of PPARγ [35]. Finally, a decrease in RXRs could protect skeletal muscle cells from apoptosis during the stressful conditions of NTIS [37].

Unexpectedly no changes were observed in mRNA expression levels of the thyroid-hormone response genes MYH1, MYH4, SERCA1 and SERCA, and the expression of UCP3 and GLUT4, two genes up-regulated by TH, was higher in non-septic shock NTIS than in control patients. T3 content of SM biopsies was not measured, due to the small amount of tissue available, although it is reasonable to assume that it was low in NTIS SM since not only serum levels of T3 but also mRNA expression of DIO2 were lower in non-septic shock NTIS than in control SM. In mice under TH deprivation, the response of TH positively regulated genes is variable and some of these genes do not show basal repression [38]. This has also been observed in NTIS patients in our study, although the mechanisms responsible for these changes are unknown. It is possible that the non-repressed genes recruit co-repressors weakly or not at all in the absence of TH, or that the repressive effect of the unliganded TH receptors is overcome by the action of other transcription factors. UCP3 gene transcription is up-regulated by TH through both TRα and TRβ1 receptors; UCP3 expression is also increased under circumstances where fatty-
acids are elevated like during starvation (39), which is a cause of NTIS. Although not measured in the present study, the serum levels of fatty-acids were probably elevated in ICU patients because they frequently receive intravenous heparine, and lypolisis is increased under severe stressful situations. Thyroid hormone increases GLUT4 expression in skeletal muscle, causing an increase in basal and insulin-stimulated glucose transport (40,41). GLUT4 mRNA expression is higher in slow oxidative muscle fibers than in fast glycolytic muscle fibers (42), due in part to a muscle specific GLUT4 promoter enhancer that contributes to these differences (43). This enhancer contains three co-operative elements, a myocyte enhancer factor 2 (MEF2) binding site, a low-affinity binding site for thyroid hormone receptors and a MyoD-binding site next to the MEF2 site (43). Interestingly, under normal conditions (non damaged muscle) the thyroid response element binding site blocks the promoter activity. The greater activity of the muscle-specific GLUT4 enhancer in oxidative muscles than in glycolytic muscles is a consequence of a greater activity of the MEF2-binding site (43). A decrease in thyroid hormone and thyroid hormone receptor expression levels could increase the activity of MEF2 and the ratio of oxidative to fast muscle fibers, explaining in part why NTIS patients showed higher GLUT4 expression levels than controls. A higher expression of skeletal muscle MEF2 isoforms that operate in the enhancer could also contribute to the higher level of GLUT4 expression in NTIS patients. Overall, these unexpected changes seem to be the result of compensatory mechanisms that remain to be elucidated.

The NTIS patients have significantly increased serum levels of the pro-inflammatory cytokines IL6, IL8, IL2R and TNF which have been shown to decrease expression levels of TRα, TRβ and RXRα in liver cells [19, 44-46]. Since pro-inflammatory cytokines
activate NF-KB pathway, we investigated whether NF-kB was activated in SM from patients with non-septic shock NTIS. The NF-kB family is composed of five members that include *NFKB1* or p50 and *RELA* or p65 subunits that are responsible for many of the actions of NF-kB. NF-kB members form heterodimers that are sequestered into the cytoplasm bound to *Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells inhibitors* or IkB proteins that inhibit dimer migration to the nucleus; p50-p65 dimers are retained in the cytoplasm by IkBα. NF-kB is activated in response to a variety of stimuli that binds to the Toll-like receptor 4 (TLR4); the classical NF-kB pathway is activated by cytokines and bacterial lipopolysaccharide (LPS) through activation of inhibitory kB kinases (IKKs). IKKs phosphorylate IkBα at serines 32 and 36, causing the release of p50-p65 heterodimers that are free to migrate to the nucleus where they regulate gene transcription. *NFKB1* (p50) and *RELA* (p65) mRNA expression levels were increased in non-septic shock NTIS SM, suggesting that the classical NF-KB pathway might have been activated. Phosphorylation-based signal transduction is often transient, but an increased steady state level of IkBα phosphorylation has been reported under several conditions that activate the NF-kB pathway in lymphocytes from hyperthyroid rats [47], acute lymphoblastic leukemia cells [48], or melanoma cells [49]. No differences were observed in IkBα phosphorylation levels between non-septic shock NTIS and control patients, indicating that the SM of patients with NTIS did not have a more highly activated NF-KB pathway. Thus the changes observed in gene and protein expression between the two groups could not be attributed to changes in activation of the NF-kB pathway.
Human skeletal muscle cells incubated in serum from patients with NTIS did not show decreased mRNA expression of *MCT8, THRBI, THRA* or *RXRG*. Indeed, not only the expression of TH receptors but also of RXRG increased in cells incubated either in serum obtained from NTIS patients or in serum from control patients; *THRBI* expression was higher in cells incubated in NTIS serum than in control serum at 48h and 72h. These unexpected results might be attributed to the fact that cells were incubated in 20% serum that has lower concentration of interleukins than 100% serum. On the other hand, the fact that *THRBI* expression increased in cells incubated in NTIS serum argues against the presence of inhibitory factors in serum *in vivo* being responsible for changes observed in patients. Patients with critical illness may have an over-activated sympathetic nervous system that can lead to adverse consequences [50]; however the likely role of a sympathetic nervous system response in the pathophysiology of NTIS remains unknown.

In conclusion, skeletal muscle from patients with non-septic shock NTIS showed decreased gene and protein expression levels of thyroid hormone receptors and RXRs. The decrease in thyroid hormone receptors did not significantly affect the expression of genes important to SM physiology. No differences in IκBα phosphorylation were observed in the SM of NTIS and control patients, suggesting that activation of the classical NF-KB pathway seems not responsible for the decreases found in TH receptor and RXR expression levels. Finally, TH-receptor and RXRs expression in primary cultures of human skeletal muscle cells did not decrease when exposed to serum from NTIS patients, suggesting that the changes observed *in vivo* could be mediated by serum-independent factors or mechanisms.
Declaration of interest: The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Authors' contributions

JL-A conceived of the study, participated in its design and coordination, got the research funds, and wrote the manuscript.

AR carried out the gene and protein expression analyses and the cell culture studies.

IC-P carried out the NF-KB studies.

AR-P and JA-E participated in the study design, selected the patients, got the consents, and performed the biochemical analyses and the muscle biopsies.
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Table 1. Anthropometric and biochemical data for the control and non-septic shock NTIS patients. Data are shown as mean ± SD (upper) median and range (lower).

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<th>Control</th>
<th>NTIS</th>
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<tr>
<td>Age (years)</td>
<td>61.33±6.26</td>
<td>62.29±4.04</td>
<td>NS</td>
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<td></td>
<td>62 (37-77)</td>
<td>57 (45-89)</td>
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<td>BMI (kg/m2)</td>
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<td>27.57±0.90</td>
<td>NS</td>
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<td>29.14 (25.51-40.70)</td>
<td>26.30 (24.08-35.62)</td>
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<td>TSH (mU/L)</td>
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<td>0.90±0.32</td>
<td>p&lt;0.01</td>
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<td>2.37 (0.5-4.12)</td>
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<td>FT4 (ng/dL)</td>
<td>1.19±0.06</td>
<td>1.22±0.11</td>
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<td></td>
<td>1.19 (0.92-1.61)</td>
<td>1.26 (0.64-1.90)</td>
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<tr>
<td>FT3 (pg/mL)</td>
<td>2.81±0.13</td>
<td>1.84±0.99</td>
<td>p&lt;0.001</td>
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<tr>
<td></td>
<td>2.74 (2.18-3.48)</td>
<td>1.84 (0.92-2.35)</td>
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<tr>
<td>rT3 (ng/mL)</td>
<td>0.22±0.02</td>
<td>0.69±0.17</td>
<td>p&lt;0.01</td>
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<td>0.22 (0.12-0.33)</td>
<td>0.49 (0.19-2.36)</td>
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<tr>
<td>Cortisol (µg/dL)</td>
<td>15.50±2.27</td>
<td>25.93±6.18</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>15 (4-28)</td>
<td>21.50 (6-98)</td>
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<tr>
<td>TNFα (ng/L)</td>
<td>5.84±0.18</td>
<td>27.1±5.94</td>
<td>p&lt;0.001</td>
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<td></td>
<td>5.8 (5.4-6.25)</td>
<td>22 (12-70)</td>
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<tr>
<td>IL6 (ng/L)</td>
<td>4.70±0.82</td>
<td>311±132</td>
<td>p&lt;0.001</td>
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<td>4.1 (2.5-9.8)</td>
<td>164 (20-1277)</td>
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<tr>
<td>IL8 (ng/L)</td>
<td>11.56±1.31</td>
<td>143.2±106.7</td>
<td>p&lt;0.001</td>
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<td>11 (7-17)</td>
<td>25 (9-993)</td>
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<tr>
<td>IL2R (U/mL)</td>
<td>440±44</td>
<td>2032±795</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>416 (270-750)</td>
<td>976 (548-8022)</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1.
254x190mm (96 x 96 DPI)
Figure 2.
254x190mm (96 x 96 DPI)
Figure 3.
254x190mm (96 x 96 DPI)
Figure 4.
254x190mm (96 x 96 DPI)