CLINICAL STUDY

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

Aim: 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 regulates SM phenotype and metabolism. We aimed to characterize the SM of non-septic shock NTIS patients in terms of: i) expression of genes and proteins involved in TH metabolism and actions; and ii) NFκB's pathway activation, a responsible factor 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 NFκB1 activation were analyzed by quantitative PCR and immunoblotting. Human SM cell (HskMC) cultures to investigate the effects of patient's serum on TH action mediators.

Results: Patients with non-septic shock NTIS showed higher levels of pro-inflammatory cytokines than controls. Expression of TRβ (THRB), TRα1 (THRA), and retinoid X receptor γ (RXRG) was decreased in NTIS patients. RXRA gene expression was higher, but its protein was lower in NTIS than controls, suggesting the existence of a post-transcriptional mechanism that down-regulates protein levels. NFκB1 pathway activation was not different between NTIS and control patients. HskMC incubated with patient's serum increased TH receptor and RXRG gene expression after 48 h.

Conclusions: Patients with non-septic shock NTIS showed decreased expression of TH receptors and RXRs, which were not related to increased activation of the NFκB1 pathway. These findings could not be replicated in cultures of HskMCs incubated in the patient's serum.

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Introduction

Non-thyroidal illness syndrome (NTIS) forms a part of the neuroendocrine response to metabolic stress (1), and is characterized by a low 3,5,3'-triiodothyronine (T₃) and normal-high 3,3',5'-triiodothyronine (reverse of T₃, rT₃) in serum and tissues, normal or low serum thyroxine (T₄), and inappropriately normal or low serum 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 are 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 TH bioavailability is

not limited if appropriate replacement therapy is given (2). A decrease in mRNA expression of the TH receptors such as TH receptor α (*THRA*) and TH receptor β (*THRB*) and their nuclear partner retinoid X receptor γ (*RXRG*) has 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 remain 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- α (TNF- α), inhibition of thyroglobulin synthesis by IL1, TNF- α (TNF), and interferon- γ (IFN- γ), a decrease in thyroperoxidase expression by IL1, IL6, and IFN- α (IFNA1), and a decrease in T_3 secretion by IL1, TNF- α and IFN- γ (IFNG).

Nuclear factor of kappa light chain enhancer of activated B cells (NFKB) is a family of transcription factors that plays a pivotal role in immune and inflammatory responses. Activated NFKB1 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 NFKB1 activation attenuates the induction of *DIO1* by T_3 (18), and that the decrease in *DIO1* mRNA expression induced by IL1 β (IL1B) is mediated by the simultaneous activation of NFKB1 and AP-1 pathways (19).

SM 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: T_3 induces a shift from myosin heavy chain 1 (MYH1), mainly expressed in slow oxidative muscle fibers, to MYH4, typically expressed in the fast glycolytic muscle fibers (22). T_3 also regulates Ca^{2+} cycling by the sarcoplasmic reticulum (SR), a source of heat generation; expression of SERCA1 (ATP2A1), a SR Ca^{2+} -ATPase predominantly expressed in fast muscle fibers, is increased by T_3

in slow muscle fibers simultaneously with the shift from MYH1 to MYH4 in these fibers (23). T_3 , 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 γ (PPARG) plays a central role in lipid and glucose metabolism and is important for maintaining insulin sensitivity. PPARG expression is positively regulated by T_3 (27), and it forms heterodimers with RXRs, nuclear receptor co-repressor 1 (NCOR1), and NCOR2 (NCOR2 or *SMRT*) (28), competing with TH receptors for these proteins. In mice, lipopolysaccharides (LPS) cause a decrease in expression levels of TH receptors and PPARG in several tissues (26), and several members of the multiprotein complex that regulate the transcriptional activity of PPARG are affected during NTIS (11). However, the changes and involvement of PPARG in human NTIS remain unknown.

To gain further insights into human NTIS pathophysiology, we investigated some of the molecular changes related to TH metabolism and actions in SM samples from a group of patients with NTIS admitted into an intensive care unit (ICU) for non-septic shock. Specifically, we quantified the following: i) mRNA expression of TH transporter monocarboxylate transporter 8 (*MCT8* or *SLC16A2*), *DIO2*, *THRA*, *THRB1*, *RXRA*, *RXRB*, *RXRG*, *PPARG*, *NCOR1*, *NCOR2*, and nuclear receptor co-activator 1 (*NCOA1*), glucocorticoid receptor α , nuclear receptor subfamily 3, group C, member 1 (*NR3C1* or *GCR*), and the TH-responsive genes, solute carrier family 2, member 4 (*GLUT4* or *SLC2A4*), uncoupling protein 3 (*UCP3*), lipoprotein lipase (*LPL*), the MYH genes MYH1 SM adult (*MYH1*) and MYH4 SM (*MYH4* or *MyHC-IIb*), and the SR calcium ATPases ATPase Ca + + transporting, cardiac muscle, fast twitch 1 (*ATP2A1* or *SERCA1*) and ATPase Ca + + transporting, cardiac muscle, slow twitch 2 (*ATP2A2* or *SERCA2*); ii) protein expression levels of *THRA*, *THRB1*, *RXRG*, *RXRB*, and *PPARG*; and iii) 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 NFKB1 pathway. Finally, we sought to determine whether the changes observed in mRNA expression of *MCT8*, *THRA*, *THRB1*, and *RXRG* in SM *in vivo* could be reproduced *in vitro* by incubating cells from a human SM cell line (HskMC) in serum from patients with NTIS.

Our results showed that patients with non-septic shock NTIS have a decreased expression of TH receptors in the SM, which is not mediated by an activation of the NFKB1 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 six patients. Sedation and analgesia were maintained by continuous i.v. 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 six patients (42.86) died in ICU.

SM 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 h after ICU admission in NTIS patients.

In all cases, informed consent was obtained from the control patients or the closest family member of 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 T_4 (FT₄, normal 0.85–1.60 ng/dl, 10.94–21.75 pmol/l), free T_3 (FT₃, normal 2.53–4.29 pg/ml, 0.039–0.066 pmol/l) (ADVIA Centaur, Bayer Diagnostics), total cortisol (µg/dl), TNF- α (ng/l), IL2 receptor (IL2R, U/ml), IL6 (ng/l), and IL8 (ng/l) (Immulite 2000, DPC, Gwynedd, UK). rT₃ (normal 0.15–0.35 ng/ml, 0.23–0.54 nmol/l) was measured by RIA (Biocode Hycel, Massy, France). Thyroperoxidase antibodies (TPOAb) and thyroglobulin antibodies (TgAb) were measured by an ELISA (Orgentec Diagnostika, Mainz, Germany).

HSkMCs culture for in vitro studies

Primary HSkMCs (ScienCell Research Laboratories, San Diego, CA, 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 gentamicin (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

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

Gene expression changes were quantified by quantitative PCR (QPCR). mRNA expression of *MCT8*, *THRA*, *THRB1*, and *RXRG* was 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* was measured in SM biopsies. QPCRs were performed in a LightCycler 2.0 (Roche) using specific probes and oligonucleotide primers designed by Universal ProbeLibrary (Roche) as previously described (11). Gene expression was normalized to RNA polymerase II following the $2^{-\Delta\Delta C_t}$ method (29).

Immunoblotting

Owing to the limited amount of SM tissue available from biopsies and the high protein concentration required for western blotting (25–75 µg/lane), total protein extracts were used instead of cytoplasm and nucleus protein. Total protein extraction was obtained after sonication of SM biopsies in 150 µl RIPA buffer, pH 7.4 (50 mM Tris-HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS), containing protein inhibitors (phenylmethylsulfonyl fluoride (PMSF), and sodium orthovanadate (Na₃VO₄); each at 1 µM final concentration). The lysate was centrifuged for 30 min at maximum speed (16 000 g), and the supernatant was 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 1 h with 5% dried milk powder in TBS. The membrane was then incubated overnight with the first antibodies (dilution 1:1000 in blocking solution) against *THRB1* (Acris Antibodies, AntibodyBcn SL, Barcelona, Spain, SM5022), *THRA* (Santa Cruz, Heidelberg, Germany, sc-772), *PPARG* (Santa Cruz, sc-772), *RXRG* (Santa Cruz, sc-555), *RXRA* (Santa Cruz, sc-553), and β -tubulin (Sigma, T-5168). After three washes of 10 min each with 0.2% TBS Tween 20 (TBS-T), the membrane was incubated with the appropriate HRP-conjugated

secondary antibody (Sigma). Proteins were detected using ECL (Amersham). Quantification was performed using AlphaEaseFC software and normalized with β -tubulin.

NFKB1 pathway activation

The status of activation of the NFKB1 pathway was determined by analysis of cytoplasmic I κ B α (NFKB1A) phosphorylation. After homogenization in PBS, samples were centrifuged for 5 min at 500 *g* at 4 °C and resuspended in a sucrose buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 8.0, 3 mM CaCl₂, 2 mM MgOAc, 0.1 mM EDTA, 0.5% NP-40, 1 mM dithiothreitol, and 0.5 mM PMSF); cytoplasmic fractions were recovered after centrifugation of the samples for 5 min at 500 *g* at 4 °C to precipitate the nuclei. Cytoplasmic samples (40 μ g/lane) were run and transferred as described above, blocked for 1 h in TBS-T and 3% BSA, and incubated overnight at 4 °C with the primary antibody (anti-I κ B α Ab (pS32/pS36); Biomol International, Hamburg, Germany, Grupo Taper SA, Madrid, Spain, SA-412) diluted to 1:1000 in blocking solution; anti-GAPDH (AbCam, Cambridge, MA, USA ref Ab9485) was used as a loading control. Membranes washing incubation with HRP-conjugated secondary antibody and quantification of proteins were performed as described above. Quantification was normalized with GAPDH.

To demonstrate the specificity of the phosphorylation site-specific antibody, a peptide competition experiment was performed using an I κ B α (pS32/pS36) control peptide pair (Biomol International, SP-412).

Statistical analysis

Data were analyzed using SPSS 12.0 for Windows (SPSS, Chicago, IL, USA). Results are expressed as mean $\pm$ s.d., 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). TNF- α , IL6, IL8, and IL2R were all significantly higher in non-septic NTIS patients than in controls (Table 1). Although non-septic NTIS patients presented higher serum cortisol levels than controls, no statistically significant differences were observed. TPOAb and TgAb were negative in all patients.

mRNA expression in SM biopsies

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

Non-septic NTIS patients had lower expression levels of *THRB1*, *THRA*, *RXRG*, and *DIO2*, but higher

Table 1 Anthropometric and biochemical data for the control and non-septic shock non-thyroidal illness syndrome (NTIS) patients.

	Control	NTIS	P
Age (years)	61.33 $\pm$ 6.26 62 (37–77)	62.29 $\pm$ 4.04 57 (45–89)	NS
BMI (kg/m ²)	30.04 $\pm$ 1.24 29.14 (25.51–40.70)	27.57 $\pm$ 0.90 26.30 (24.08–35.62)	NS
TSH (mU/l)	2.21 $\pm$ 0.39 2.37 (0.5–4.12)	0.90 $\pm$ 0.32 0.47 (0.04–4.5)	P < 0.01
FT ₄ (ng/dl)	1.19 $\pm$ 0.06 1.19 (0.92–1.61)	1.22 $\pm$ 0.11 1.26 (0.64–1.90)	NS
FT ₃ (pg/ml)	2.81 $\pm$ 0.13 2.74 (2.18–3.48)	1.84 $\pm$ 0.09 1.84 (0.92–2.35)	P < 0.001
rT ₃ (ng/ml)	0.22 $\pm$ 0.02 0.22 (0.12–0.33)	0.69 $\pm$ 0.17 0.49 (0.19–2.36)	P < 0.01
Cortisol (μ g/dl)	15.50 $\pm$ 2.27 15 (4–28)	25.93 $\pm$ 6.18 21.50 (6–98)	NS
TNF- α (ng/l)	5.84 $\pm$ 0.18 5.8 (5.4–6.25)	27.1 $\pm$ 5.94 22 (12–70)	P < 0.001
IL6 (ng/l)	4.70 $\pm$ 0.82 4.1 (2.5–9.8)	311 $\pm$ 132 164 (20–1277)	P < 0.001
IL8 (ng/l)	11.56 $\pm$ 1.31 11 (7–17)	143.2 $\pm$ 106.7 25 (9–993)	P < 0.001
IL2R (U/ml)	440 $\pm$ 44 416 (270–750)	2032 $\pm$ 795 976 (548–8022)	P < 0.001

Data are shown as mean $\pm$ s.d. (upper), median, and range (lower).

Figure 1 Relative mRNA gene expression in the skeletal muscle biopsies from control and non-septic shock NTIS patients. Gene expression is represented as the difference in expression between a reference gene, RNA polymerase II, and the genes of interest. In control biopsies, *SERCA* and *MYH1* had the highest expression levels, and *MCT8* and *MYH4* had the lowest expression levels. NTIS patients had lower levels of *THRA*, *THRB1*, and *RXRG*, and higher levels of *RXRA*, *UCP3*, and *GLUT4* gene expression than control patients. * $P < 0.05$.

expression levels of *RXRA*, *RXRB*, *UCP3*, and *GLUT4* than control patients (Fig. 1). No differences between NTIS and control patients were observed for *MCT8*, *NCOR1*, *SMART*, *NCOA1*, *LPL*, *PPARG*, *NR3C1*, *MYH1*, *MYH4*, *SERCA1*, or *SERCA2* (Fig. 1).

Protein expression in SM biopsies

To evaluate whether changes in mRNA expression were accompanied by changes in protein levels, we analyzed the protein expression of *THRA* (55 kDa), *THRB1* (52–55 kDa), *RXRG* (~51 kDa), *RXRA* (54 kDa), and *PPARG* (67 kDa) normalized with β -tubulin (50 kDa). Immunoblotting of RXRs showed the expected 51 kDa (*RXRG*) and 54 kDa (*RXRA*) weights (Fig. 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 *THRB1* antibody turned out to be relatively unspecific, and only the band expected from the control test (nuclear protein extract from placenta) is shown (Fig. 2). *THRB1*, *RXRG*, and *RXRA* expression levels were significantly lower in non-septic NTIS patients than in control patients (Fig. 2); although *THRA* expression was lower in non-septic NTIS patients than in controls, statistical significance was not reached. No differences were observed in *PPARG* expression between the two groups (Fig. 2).

Expression of p50 and p65 subunits and NFKB1 activation status in SM

mRNA expression of *NFKB1* (p50) and *RELA* (p65) was significantly elevated in non-septic NTIS patients versus controls (Fig. 3A).

I κ B α immunoblotting showed two bands, a 40 kDa product corresponding to phosphorylated I κ B α and a 36 kDa product that corresponds to non-phosphorylated I κ B α (Fig. 3B). No statistically significant differences were observed in I κ B α phosphorylation between non-septic NTIS and control patients.

Effects of patient's serum on mRNA expression of MCT8, THRB1, THRA, and RXRG in HSkMC cultures

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

Discussion

NTIS is caused by an impairment of hypothalamic–pituitary function and of the physiological mechanisms producing T_3 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 THs can be observed (30). Although the molecular mechanisms responsible for NTIS remain speculative, the changes are tissue-specific (11).

SM 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

Figure 2 *THRA*, *THRB1*, *RXRG*, *RXRA*, and *PPARG* protein levels were evaluated by immunoblotting. All these proteins were decreased in SM samples from NTIS patients, although differences in *THRA* and *PPARG* were not statistically significant. * $P < 0.05$, NS means non-specific.

Figure 3 (A) mRNA expression of *NFKB1* (p50) and *RELA* (p65), two major components of the NFKB1 pathway, was increased in SM biopsies from NTIS patients. (B) No differences in SM IκBα phosphorylation levels were observed between control and NTIS patients. * $P < 0.05$, ** $P < 0.01$.

secondary to septic shock caused by Gram-negative bacteria shows a decrease in the TH receptors, *THRA* and *THRB1*, 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 Toll-like receptor 4 (TLR4), activating the NFKB1 pathway that in turn increases transcription of a range of genes associated with inflammation and immunity, thus causing a strong inflammatory response. TLR4 is present in SM (31), and LPS binding to TLR4 could therefore underlie the changes observed in the SM of patients with septic shock-associated NTIS; indeed, mice deficient in TLR4 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 SM during NTIS.

TH promotes proteolysis and a shift from the more energetically efficient slow oxidative muscle fibers to fast glycolytic muscle fibers, increasing energy uncoupling and thus the expenditure of energy as heat. The decreases in TH receptor levels and *RXRG* mRNA could therefore comprise a mechanism to prevent energy expenditure as well as to protect proteins from being broken down (25) under situations of low SM activity. Interestingly, non-septic NTIS patients showed an increase in *RXRA* mRNA expression but a decrease in protein levels, suggesting the existence of a post-transcriptional abnormality in the synthesis or degradation of *RXRA*. Under catabolic conditions,

there is an ubiquitin–proteasome-dependent protein degradation (33), and *RXRA* suffers post-translational regulation by ubiquitination resulting in its rapid degradation (34, 35). This mechanism could explain the decrease in *RXRA* protein levels in NTIS patients. TH receptor binding to MYH is increased by *RXRA* (36), so a decrease in both transcription factors could constitute a mechanism to prevent TH altering the SM phenotype. Moreover, a decrease in TH receptors, *RXRA*, and *RXRG*, would reduce uptake and oxidation of fatty acids by SM, an effect that could be counteracted, however, by an increase in *PPARG* 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 *PPARG* activity as shown in obese human adipose tissue (35); *PPARG* action depends on its dimerization with different RXR isotypes, and a decrease in *RXRA* has been shown to increase transcriptional activity of *PPARG* (35). Finally, a decrease in RXRs could protect SM cells from apoptosis during the stressful conditions of NTIS (37).

Unexpectedly, no changes were observed in mRNA expression levels of the TH-responsive 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 patients than in control patients. T_3 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 T_3 but also mRNA expression of

Figure 4 Cells from a primary human skeletal muscle cell line, HSKMC, were incubated for 72 h in serum from control or NTIS patients to evaluate changes in *THRA*, *THRB1*, *RXRG*, and *MCT8* gene expression. *THRA*, *THRB1*, and *RXRG* expression did not change from 0 to 24 h, but had increased significantly at 48 and 72 h. No differences were observed between cells incubated in control or NTIS serum with respect to *THRA* and *RXRG* expression, but at 48 and 72 h, *THRB1* expression was significantly higher in cells incubated in serum from NTIS patients. By contrast, *MCT8* expression had not increased at 48 and 72 h in either groups. * $P < 0.05$ NTIS versus controls.

DIO2 was lower in non-septic shock NTIS SM 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 *THRA* and *THRB1* receptors; *UCP3* expression is also increased under circumstances where fatty acids are elevated likely 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 i.v. heparin, and lipolysis is increased under severe stressful situations. TH increases *GLUT4* expression in SM, 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 TH 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 TH and TH 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 SM 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 *THRA*, *THRB1*, and *RXRA* in liver cells (19, 44–46). Since pro-inflammatory cytokines activate NFKB1 pathway, we investigated whether NFKB1 was activated in SM from patients with non-septic shock NTIS. The NFKB1 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 NFKB1. NFKB1 members form heterodimers that are sequestered into the cytoplasm bound to nuclear factor of kappa light polypeptide gene enhancer in B-cell inhibitors or IκB proteins that inhibit dimer migration to the nucleus;

p50–p65 dimers are retained in the cytoplasm by IκBα. NFKB1 is activated in response to a variety of stimuli that bind to the TLR4; the classical NFKB1 pathway is activated by cytokines and bacterial LPS through activation of inhibitory κB kinases (IKKs). IKKs phosphorylate IκBα 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 NFKB1 pathway might have been activated. Phosphorylation-based signal transduction is often transient, but an increased steady-state level of IκBα phosphorylation has been reported under several conditions that activate the NFKB1 pathway in lymphocytes from hyperthyroid rats (47), acute lymphoblastic leukemia cells (48), or melanoma cells (49). No differences were observed in IκBα 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 NFKB1 pathway. Thus, the changes observed in gene and protein expression between the two groups could not be attributed to changes in activation of the NFKB1 pathway.

HSkMCs incubated in serum from patients with NTIS did not show decreased mRNA expression of *MCT8*, *THRB1*, *THRA*, or *RXRG*. Indeed, not only the expression of TH receptors but also the expression of *RXRG* increased in cells incubated either in serum obtained from NTIS patients or in serum obtained from control patients; *THRB1* expression was higher in cells incubated in NTIS serum than in control serum at 48 and 72 h. These unexpected results might be attributed to the fact that cells were incubated in 20% serum that has lower concentration of ILs than 100% serum. On the other hand, the fact that *THRB1* 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, SM from patients with non-septic shock NTIS showed decreased gene and protein expression levels of TH receptors and RXRs. The decrease in TH 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 NFKB1 pathway seems to be not responsible for the decreases found in TH receptor and RXR expression levels. Finally, TH receptor and RXRs expression in primary cultures of HSkMCs 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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Author contribution statement

J Lado-Abeal conceived the study, participated in its design and coordination, got the research funds, and wrote the manuscript. A Romero carried out the gene and protein expression analyses and the cell culture studies. I Castro-Piedras carried out the NFkB1 studies. A Rodríguez-Pérez and J Álvarez-Escudero participated in the study design, selected the patients, got the consents, and performed the biochemical analyses and the muscle biopsies.

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