

# Septic shock non-thyroidal illness syndrome causes hypothyroidism and conditions for reduced sensitivity to thyroid hormone

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## Abstract

Non-thyroidal illness syndrome (NTIS) is part of the neuroendocrine response to stress, but the significance of this syndrome remains uncertain. The aim of this study was to investigate the effect of lipopolysaccharide (LPS)-induced NTIS on thyroid hormone (TH) levels and TH molecular targets, as well as the relationship between septic shock nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) activation and TH receptor  $\beta$  (*THRB*) gene expression at a multi-tissue level in a pig model. Prepubertal domestic pigs were given i.v. saline or LPS for 48 h. Serum and tissue TH was measured by chemiluminescence and RIA. Expression of THRs and cofactors was measured by real-time PCR, and deiodinase (DIO) activity was measured by enzyme assays. Tissue NF- $\kappa$ B nuclear binding activity was evaluated by EMSA. LPS-treated pigs had decreased TH levels in serum and most tissues. *DIO1* expression in liver and kidney and DIO1 activity in kidney decreased after LPS. No changes in DIO2 activity were observed between groups. LPS induced an increase in hypothalamus, thyroid, and liver DIO3 activity. Among the other studied genes, monocarboxylate transporter 8 and *THRB* were the most commonly repressed in endotoxemic pigs. LPS-induced NF- $\kappa$ B activation was associated with a decrease in *THRB* gene expression only in frontal lobe, adrenal gland, and kidney cortex. We conclude that LPS-induced NTIS in pigs is characterized by hypothyroidism and tissue-specific reduced TH sensitivity. The role of NF- $\kappa$ B in regulating *THRB* expression during endotoxemia, if any, is restricted to a limited number of tissues.

## Key Words

- ▶ non-thyroidal illness syndrome
- ▶ septic shock
- ▶ deiodinases
- ▶ thyroid hormone receptors

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## Introduction

Non-thyroidal illness syndrome (NTIS) is a component of the neuroendocrine and metabolic response to severe stress and starvation, characterized by low serum triiodothyronine ( $T_3$ ) and inappropriately normal or low

TSH; in severe cases, serum  $rT_3$  increases and thyroxine ( $T_4$ ) decreases (Wartofsky & Burman 1982, DeGroot 1999). As thyroid hormone (TH) increases energy expenditure (Silva 2006), NTIS can be considered an adaptive response

to counteract catabolism during fasting and illness (Wartofsky & Burman 1982, DeGroot 1999, Boelen *et al.* 2011). However, during prolonged illness, it is unclear whether NTIS is beneficial (DeGroot 2003, Stathatos & Wartofsky 2003) and, in fact, patients with low serum  $T_4$  have an increased probability of death (Slag *et al.* 1981).

NTIS results from a central hypothyroidism together with tissue-specific changes resembling consumptive hypothyroidism and reduced sensitivity to TH action. Studies on humans have shown that central hypothyroidism in NTIS is a consequence of decreased expression of *TRH* mRNA in the hypothalamus (Fliers *et al.* 1997, Van den Berghe *et al.* 1998). Also, studies on humans have shown a tissue-specific increase in type 3 deiodinase (DIO3) activity (Rodriguez-Perez *et al.* 2008), like that observed in consumptive hypothyroidism, which, combined with decreased activity of type 1 deiodinase (DIO1; Peeters *et al.* 2003, Debaveye *et al.* 2005), results in inactivation of  $T_4$  into  $rT_3$  and reduced synthesis and increased degradation of  $T_3$ . In both humans and rodents, NTIS is associated with decreased expression of TH receptors (*THRs*) and their nuclear partners retinoid X receptors (*RXR*) in some tissues (Beigneux *et al.* 2003, Feingold *et al.* 2004, Rodriguez-Perez *et al.* 2008, Boelen *et al.* 2011), which may create a situation of reduced sensitivity to the action of TH, the relevance of which has not yet been explored.

The molecular mechanisms at the root of NTIS have yet to be characterized. In rodents, the fasting-related increase in DIO3 activity may be a consequence of low leptin levels (Boelen *et al.* 2012), and leptin prevents starvation-related central hypothyroidism (Legradi *et al.* 1997). High levels of pro-inflammatory cytokines, as observed during moderate-to-severe illness (Rodriguez-Perez *et al.* 2008), block the synthesis and release of TRH and TSH (van Haasteren *et al.* 1994) and decrease DIO1 activity (Jakobs *et al.* 2002), an effect involving nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) activation (Nagaya *et al.* 2000) and a competition for limiting amounts of nuclear receptor co-activator 1 between the *DIO1* promoter and the promoters of cytokine-induced genes (Yu & Koenig 2000). TH increases expression and activity of DIO1, and as the fasting-related decrease in DIO1 activity in rodents is restored by TH replacement, it is believed that low  $T_3$  levels during NTIS are responsible for and not the consequence of this decrease (O'Mara *et al.* 1993, Boelen *et al.* 2012). *THRs* also decrease during starvation and illness. Although mechanisms responsible for inhibition of *THR* expression during fasting remain unknown, NF- $\kappa$ B activation has

been suggested to be responsible for the decrease in TH receptor  $\beta 1$  (*THR $\beta$ 1*) expression during inflammation in rodents (Kwakkel *et al.* 2006).

Limited access to human tissues is an obstacle for understanding the effects of NTIS on patients and uncovering the molecular mechanisms involved in NTIS. Restrictions inherent to human research can be resolved in part by establishing large animal models of NTIS. Domestic pigs are readily available and affordable large animals that have been proven to be a useful model for human TH metabolism (Wassen *et al.* 2004). Our aim was to investigate in pigs the effects of lipopolysaccharide (LPS)-induced septic shock NTIS on i) TH levels in serum and tissues, ii) changes in gene expression of TH molecular targets and iodothyronine DIOs as well as on the activity of DIO enzymes at a multi-tissue level, and iii) NF- $\kappa$ B nuclear binding activity and its correlation with the tissue-specific decrease in *THR $\beta$*  gene expression observed during endotoxemia.

## Materials and methods

### Animals

The study was approved by Texas Tech University Institutional Animal Care and Use Committee. Seventeen prepubertal female pigs (*Sus scrofa domesticus*) weighing 9–11 kg were randomly assigned to control ( $n=8$ ) or LPS-treated ( $n=9$ ) groups. Pigs were housed under controlled temperature ( $22 \pm 2$  °C), humidity ( $55 \pm 5\%$ ), and light–dark cycling (on 0700–1900 h, off 1900–0700 h).

Forty-eight hours before the infusion period, two catheters were placed into jugular veins through a neck incision under light sedation, subcutaneously passed to the back of the neck, and heparinized to maintain patency. Catheters were used for blood sampling and continuous infusion of saline or LPS.

Experiments were always performed in pairs (control vs LPS), one pig per individual pen under freely moving conditions for 48 h, starting at 0800 h of day 1. The animals were continuously monitored by the personnel involved in the study. Control group pigs received continuous i.v. normal saline infusion. LPS-treated pigs received endotoxin (*Escherichia coli* 0111:B4, Sigma–Aldrich) infusion as follows: a 5-min bolus of 3.5  $\mu$ g/kg per min followed by a continuous infusion of 3.5  $\mu$ g/kg per h during the first 12 h. At 2000 h, if the body temperature increased more than 2 °C over pre-infusion temperature, the infusion rate was not changed; however, if the body temperature was lower than 2 °C over pre-infusion temperature, LPS infusion was

increased with a 5-min bolus of 4 µg/kg per min followed by a continuous infusion of 4 µg/kg per h during the next 12 h. At day 2, 0800 h, changes in infusion rate followed the same requirements, but if needed, LPS was increased with a 5-min bolus of 5 µg/kg per min followed by a continuous infusion of 5 µg/kg per h until day 3, 0800 h. Heart rate and skin temperature were continuously monitored with a Jacketed External Telemetry system (Data Sciences International, St Paul, MN, USA) attached to skin and protected by an in-house made jacket. Food and water were available *ad libitum*. Food intake was estimated by weighing the food in the animal pen at the beginning of the infusion period and at necropsy; if an animal died before 48 h, food in the pen was weighed and compared with its pair at the time of death. Presence of food in the animal's stomach was recorded at necropsy.

At the end of the infusion period, animals were killed by i.v. pentobarbital sodium (Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI, USA). Tissue samples were taken immediately after death from cerebral frontal lobe, cerebellum, hypothalamus, pituitary gland, thyroid gland, lung, heart left ventricle, liver, spleen, duodenum, ascendant colon, adrenal gland, and skeletal muscle (quadriceps femoris). Tissue samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further analysis.

### Hormonal studies

Serum hormonal levels were measured in blood samples obtained immediately before infusion began and at the end of the infusion period. Chemiluminescence was used to measure serum-free  $T_4$  (FT<sub>4</sub>), free  $T_3$  (FT<sub>3</sub>) (ADVIA Centaur, Bayer Diagnostics), and cortisol (Immulite 2000, DPC, Gwynedd, UK). Serum rT<sub>3</sub> was measured by RIA (Alpco Diagnosis, Salem, NH, USA).

$T_4$  and  $T_3$  levels were measured in frontal lobe, lung, heart left ventricle, liver, kidney cortex, and skeletal muscle by in-house RIAs after extraction and purification of tissue samples (Morreale de Escobar *et al.* 1985, Calvo *et al.* 1997). Frozen tissue samples (100–150 mg) were homogenized in methanol with tracer amounts of [<sup>131</sup>I]- $T_4$  and [<sup>125</sup>I]- $T_3$  added to each homogenate. The iodothyronines were extracted using chloroform–methanol (2:1), back-extracted into an aqueous phase, and purified through Bio-Rad AG 1X2 resin columns (Bio-Rad Laboratories S.A.) using a pH gradient. The acetic extracts were then evaporated to dryness and dissolved in RIA buffer. Each extract was counted to determine the recovery of [<sup>131</sup>I]- $T_4$  and [<sup>125</sup>I]- $T_3$  in each sample. The samples were

submitted to highly sensitive RIA for the determination of  $T_4$  and  $T_3$ , the limits of sensitivity being 2.5 pg  $T_4$  and 1.5 pg  $T_3$ /tube. Each sample was processed in duplicate. Concentrations are calculated using the amounts of  $T_4$  and  $T_3$  found in the RIAs, the individual recovery of the [<sup>131</sup>I]- $T_4$  and [<sup>125</sup>I]- $T_3$  added to each sample, and the weight of the tissue sample extracted. Cross-reactivity of both RIAs has been previously reported (Ruiz de Oña *et al.* 1991).

High specific activity [<sup>131</sup>I]- $T_4$ , [<sup>125</sup>I]- $T_3$ , and [<sup>125</sup>I]- $T_4$  ( $\geq 3000$  µCi/µg) were synthesized in our laboratory as described previously (Morreale de Escobar *et al.* 1985) and used for the in-house RIAs and as recovery tracers for tissue extractions.

### Gene expression analysis by quantitative real-time PCR

Total RNA was extracted from tissue and reverse transcribed as described previously (Rodriguez-Perez *et al.* 2008). Expression of *THRB*, TH receptor  $\alpha 1$  (*THRA1*), TH receptor  $\alpha 2$  (*THRA2*), retinoid X receptor  $\alpha$  (*RXRA*), retinoid X receptor  $\beta$  (*RXR $\beta$* ), nuclear receptor co-repressor 1 (*NCOR1*), monocarboxylate transporter 8 or *SLC16A2* (*MCT8*), type I iodothyronine DIO (*DIO1*), type II iodothyronine DIO (*DIO2*), v-rel reticuloendotheliosis viral oncogene homolog A (*avian*) nuclear factor NF-kappa-B p65 subunit (*RELA*), and of the internal control ribosomal protein L4 gene (*RPL4*) genes were quantified by real-time PCR in an Applied Biosystems 7500 instrument (Applied Biosystems) using RT<sup>2</sup>SYBR Green ROX FAST master mix (Qiagen Sciences) and specific oligonucleotide primers (sequences are available upon request). The reaction mixtures contained 5 µl RT<sup>2</sup>SYBR Green ROX FAST master mix, 4.2 µl ddH<sub>2</sub>O, 1.0 µl template cDNA (up to 250 ng), and 0.4 µl gene-specific 10 µM PCR oligonucleotides primers. The reaction conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 20 s and 60 °C for 30 s. Relative gene expression was calculated as  $\Delta\text{Ct}$  (the difference between the cycle threshold values, Ct, of the internal control, *RPL4*, and Ct of gene of interest) and confirmed by  $2^{-\Delta\Delta\text{Ct}}$  method (Livak & Schmittgen 2011). Interassay variability was corrected by measuring RNA polymerase II Ct in the same cDNA batch obtained from HepG2 cells.

### DIOs activity

DIO1 and DIO2 activities were assayed in tissue sample homogenates prepared in 0.1 M potassium phosphate, 2 mM EDTA, and 2 mM dithiothreitol (DTT), pH 7.0.

The same conditions were used for DIO3 activity except DTT was changed to 10 mM. Protein concentration was determined by Bradford's method using BSA as a standard. Tissue samples were assayed in duplicates.

DIO1 activity was measured using 25–50 µg protein in 100 µl reaction mixture, [<sup>125</sup>I]-rT<sub>3</sub> (100 000 c.p.m./tube), 400 nM rT<sub>3</sub>, and 2 mM DTT. Incubation was carried at 37 °C for 1 h, with or without 1 mM propylthiouracil (PTU). Results are expressed in pmol/min per mg protein.

DIO2 activity was measured using 50 µg protein in 100 µl reaction mixture consisting of [<sup>125</sup>I]-T<sub>4</sub> (200 000 c.p.m./tube), 2 nM T<sub>4</sub> or 500 nM T<sub>4</sub> (to inhibit DIO2 activity), 1 µM T<sub>3</sub> (to inhibit DIO3 activity), 20 mM DTT, and 1 mM PTU for 2 h at 37 °C. Results are expressed in fmol/min per mg protein. DIO3 activity was assayed using 50–100 µg protein in 100 µl reaction mixture, [<sup>125</sup>I]-T<sub>3</sub> (200 000 c.p.m./tube), 1 nM T<sub>3</sub>, or 1 µM T<sub>3</sub> (to inhibit DIO3 activity), at 37 °C for 2 h. Results are expressed in fmol/min per mg protein.

Before each assay, [<sup>125</sup>I]-T<sub>4</sub>, [<sup>125</sup>I]-T<sub>3</sub>, and [<sup>125</sup>I]-rT<sub>3</sub> (Perkin Elmer, Billerica, MA, USA) were purified by dialysis as described previously (Dumitrescu *et al.* 2005).

For DIO1 and DIO2 activity assays, reactions were stopped by adding 100 µl of cold 2% BSA and 800 µl of 10% trichloroacetic acid. After centrifugation at 2053 g for 10 min, 800 µl supernatant was applied to AG 50W-X2 columns (bed volume=1 ml) (Bio-Rad Laboratories) and eluted with 10% glacial acetic acid. The [<sup>125</sup>I] generated in the assay was counted in a gamma scintillation counter (Packard Cobra Gamma Counter, Perkin Elmer, Waltham, MA, USA). For DIO3 activity assay, reactions were stopped by adding 100 µl cold methanol. After centrifugation, 150 µl supernatant was applied to a 250×4.6 mm ID, 5 µm Viva C18 analytical column (Restek Corporation, Bellefonte, PA, USA) connected to a HPLC system (Rainin Instruments, Woburn, MA, USA) and eluted isocratically with a mixture of methanol and H<sub>2</sub>O (55:45), pH 3.0, at a flow of 1 ml/min. Fractions of 0.3 min were collected and counted for radioactivity.

Blanks were performed in quadruplicates by substituting tissue homogenates for buffer homogenates. Assay detection limit was calculated as the average of [<sup>125</sup>I<sup>-</sup>] (DIO1 and DIO2 assay) or [<sup>125</sup>I]-T<sub>2</sub> (DIO3 assay) produced by blanks multiplied by 3.3 times SD.

### NF-κB nuclear binding activity

EMSA was used to investigate NF-κB nuclear binding activity in samples obtained from frontal lobe, cerebellum,

heart left ventricle, lung, liver, kidney cortex, spleen, adrenal gland, and skeletal muscle of six control and six LPS-treated pigs. NF-κB binding activity was correlated with *THRB* gene expression in these tissues.

Nuclear protein was extracted using a nuclear extraction kit (Signosis, Inc., Sunnyvale, CA, USA) and protein concentration was determined by Bradford's method. Oligonucleotides containing a consensus binding site for NF-κB (5'-AGTTGAGGGGACTTTCCAGGC-3') or mutated binding site (5'-AGTTGA**ATTC**ACTTTCCAGGC-3') were annealed, end-labeled using T4 polynucleotide kinase (New England BioLabs, Ipswich, MA, USA) and γ<sup>32</sup>P-ATP (3000 Ci/mmol at 10 mCi/ml, Perkin Elmer), and separated from unincorporated nucleotides over a Sephadex G-50 column (Illustra ProbeQuant, GE Healthcare, Piscataway, NJ, USA). Nuclear extracts (10 µg) were incubated with labeled consensus or mutated probes in a binding reaction buffer (5 mM MgCl<sub>2</sub>, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 50 mM Tris-HCl, pH 7.5, 20% glycerol, 0.25 mg/ml poly(dI-dC)) for 20 min at room temperature. In competition studies, 100-fold molar excess of unlabeled mutant oligonucleotide was added to the binding reaction in addition to labeled probe. The protein-DNA complexes were resolved on a non-denaturing 6% polyacrylamide gel in 0.5X TBE buffer. The gel was run at 250 V for 2 h, dried, and exposed to autoradiography film at -80 °C. For supershift assays, 0.4 µg antibody against p50 (sc-114) or p65 (sc-109; Santa Cruz Biotechnology) NF-κB subunits was added to the reaction buffer for 20 min at room temperature before addition of poly(dI-dC) and γ<sup>32</sup>P-labeled probe.

Activated NF-κB-positive control was obtained from TNFα-stimulated HepG2 cells. Briefly, HepG2 cells were incubated as described previously (Lado-Abeal *et al.* 2005); at 70% confluence, medium was removed and cells were grown in serum-free Eagle's minimum essential medium for 24 h before treatment with TNFα (50 ng/ml) (Sigma) for 30 min. HepG2 cells were harvested to obtain nuclear extracts used for EMSA as described earlier.

### Statistical analysis

Data were analyzed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Gaussian distribution of variables was checked by Kolmogorov-Smirnov test. Results are expressed as mean ± s.d. or as Tukey box-and-whisker plot. Student's *t*-test or Mann-Whitney *U* test were used for same variable comparison between groups when appropriate.

## Results

LPS administration caused high mortality. Of the nine pigs treated with LPS, three pigs survived the 48-h LPS infusion period, one pig died at 12 h, and three pigs died between 6 and 8 h from the beginning of LPS infusion. Two pigs died in the first 4–5 h and were excluded from the statistical analysis. All control pigs survived the 48-h period of saline infusion.

LPS infusion increased skin temperature by more than 0.5 °C in the first 1–3 h. Elevated temperature was sustained in four animals. Hypothermia occurred in five pigs after the initial raise in body temperature, and all these animals died within 48 h. Food was always present in animal stomach at necropsy. Control pigs consumed  $4.42 \pm 0.52$  kg of food, while LPS-treated pigs that survived the 48 h consumed  $0.24 \pm 0.11$  kg.

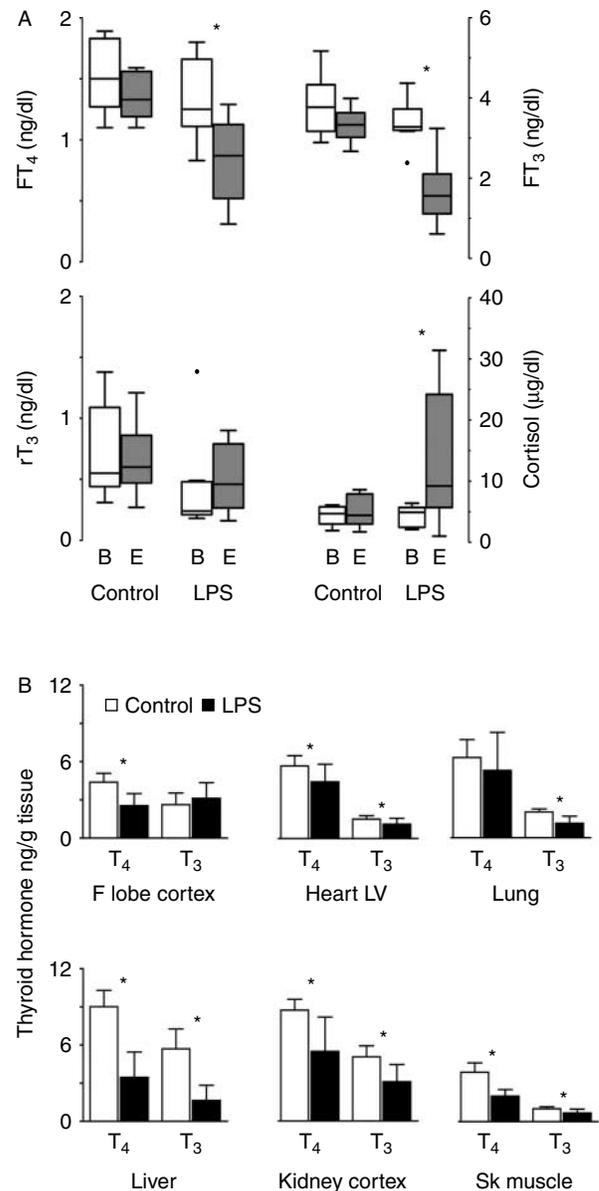
### Effect of LPS on serum and tissue hormonal levels

LPS infusion ( $n=7$ ) caused a decrease in serum FT<sub>4</sub> and FT<sub>3</sub> and an increase in serum cortisol compared with control ( $n=8$ ) (Fig. 1). No differences were observed in serum rT<sub>3</sub> levels. However, in LPS-treated pigs, the rT<sub>3</sub>/FT<sub>4</sub> ratio was threefold higher at the end of the infusion compared with pre-infusion values ( $0.30 \pm 0.06$  pre-LPS vs  $0.92 \pm 0.30$  post-LPS,  $P < 0.05$ ). LPS-treated pigs had significantly lower T<sub>4</sub> and T<sub>3</sub> levels in most analyzed tissues, except for T<sub>4</sub> in lung and T<sub>3</sub> in brain (Fig. 1).

### DIOs gene expression and activity

*DIO1* mRNA expression was detected in all tissues from the control group, while *DIO1* activity was detected only in liver and kidney cortex and medulla, the two organs with the highest *DIO1* mRNA expression levels (Fig. 2). LPS decreased *DIO1* mRNA expression in liver and kidney as well as *DIO1* activity in kidney cortex. Although *DIO1* activity tended to decrease in liver and kidney medulla of LPS-treated pigs, the decrease did not achieve statistical significance (Fig. 2).

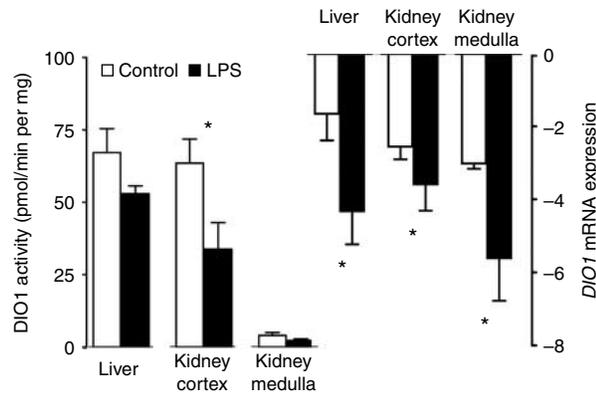
*DIO2* mRNA expression was detected in all control tissues. The highest expression was detected in frontal lobe, hypothalamus, pituitary gland, liver, and skeletal muscle. LPS infusion was associated with increased *DIO2* mRNA expression in kidney cortex and decreased expression in liver. *DIO2* activity was detected in control hypothalamus, pituitary gland, and thyroid gland, and LPS did not change enzyme activity in the hypothalamus (median: 0.22 (range: 0–0.56) fmol/mg per min control vs



**Figure 1**

(A) Serum levels of thyroid hormones and cortisol in pigs. Basal (B) levels of serum-free T<sub>4</sub> (FT<sub>4</sub>), FT<sub>3</sub>, rT<sub>3</sub>, and cortisol were not significantly different between control ( $n=8$ ) and LPS-treated ( $n=9$ ) pigs. In LPS-treated pigs, serum FT<sub>4</sub> and FT<sub>3</sub> decreased and cortisol increased at the end (E) of the infusion period. Hormonal values are represented with Tukey box-and-whisker plot. (B) T<sub>4</sub> and T<sub>3</sub> levels in tissues from control ( $n=8$ ) and LPS-treated ( $n=9$ ) pigs. Values are represented as mean  $\pm$  s.d. (\* $P < 0.05$ ; closed circle, outlier value).

0.23 (0–0.66) fmol/mg per min LPS, NS), pituitary gland (median: 0.58 (0–2.85) fmol/mg per min control vs 0.51 (0–3.19) fmol/mg per min LPS, NS), or thyroid gland (median: 0.08 (0–0.34) fmol/mg per min control vs 0.09 (0–0.23) fmol/mg per min LPS, NS).



**Figure 2**

DIO1 activity (left) and relative gene expression (right) in liver and kidney cortex and medulla. *DIO1* gene relative expression is represented as  $\Delta Ct$ . A  $\Delta Ct$  difference of 1 indicates a twofold difference in gene expression. LPS induced a significant decrease in *DIO1* gene expression in liver and kidney and in DIO1 activity in kidney cortex. The decrease in DIO1 activity observed in liver did not achieve statistical significance. Values are represented as mean  $\pm$  s.d. (\* $P < 0.05$ ).

DIO3 activity was detected in all analyzed tissues except in heart left ventricle and spleen, although important differences between tissues were noticed (Fig. 3). LPS-treated pigs showed a remarkable increase in liver DIO3 activity, and a significant increase was also observed in the hypothalamus and thyroid gland (Fig. 3).

#### ***MCT8*, *THR*s, and *THR* cofactor gene expression**

*MCT8*, *THR*, and *THR* cofactor mRNA expression showed remarkable differences between tissues. *THRB*, *THRA1*, and *THRA2* mRNA expression in control pig tissues are shown in Fig. 4.

LPS effects on *MCT8*, *THRB*, *THRA1*, *THRA2*, *RXRA*, and *RXRB* mRNA expression are shown in Fig. 5. In cerebellum, hypothalamus, thyroid gland, duodenum, colon, spleen, and skeletal muscle, expression of these genes was not significantly different between control and LPS-treated pigs. *NCOR1* mRNA expression did not differ between groups in any of the studied tissues.

#### **NF- $\kappa$ B nuclear binding activity**

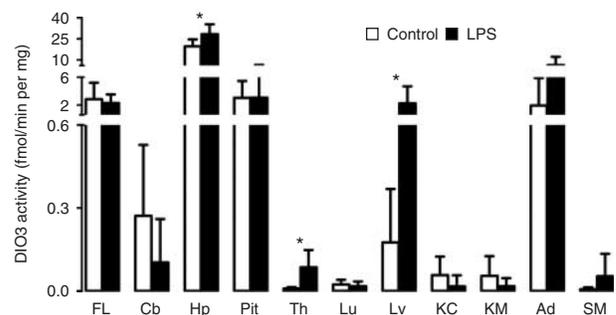
NF- $\kappa$ B p50–p65 nuclear binding was detected in all studied tissues except in cerebellum and skeletal muscle in both control and LPS-treated animals. LPS induced an increase in NF- $\kappa$ B nuclear binding activity in frontal lobe, lung, kidney cortex, and adrenal gland (Fig. 6A). No differences between groups were found in heart left ventricle, liver, or

spleen (Fig. 6A). Tissues *RELA* mRNA expression did not change significantly after LPS. NF- $\kappa$ B nuclear binding activity and *THRB* mRNA expression in corresponding animals are represented in Fig. 6B.

#### **Discussion**

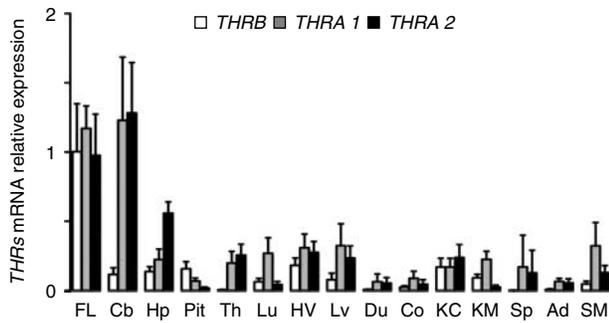
The domestic pig is a large animal that closely resembles human TH metabolism (Wassen *et al.* 2004). In this study, we have shown that pigs have serum levels of FT<sub>4</sub> and FT<sub>3</sub> similar to that of humans. Characterization of DIOs revealed high DIO1 activity in liver and kidney; DIO2 activity in hypothalamus, pituitary gland, and thyroid gland; and high DIO3 activity in hypothalamus, frontal lobe, pituitary, and adrenal gland. A remarkable difference between pig and human DIO activity was a lack of significant DIO1 enzyme activity in pig thyroid gland; however, our results agree with a previous report in pig (Wassen *et al.* 2004). Finally, relative gene expression of TH transporter *MCT8*, *THR*s, and cofactors has been characterized in all analyzed tissues.

LPS administration induced a profound decrease in serum TH levels resulting in tissue hypothyroidism. The observed decrease in *MCT8* expression may also reduce TH supply to those tissues depending on *MCT8* for TH transport. Furthermore, the LPS-induced decrease in *DIO1* gene expression and enzyme activity in liver and kidney, a common finding in models of NTIS (Bianco *et al.* 2002, Boelen *et al.* 2011), contributed to decreased T<sub>3</sub> production.



**Figure 3**

DIO3 activity in tissues from control (n:8) and LPS (n:9)-treated pigs. DIO3 activity was detected in all studied tissues, although large differences between tissues were observed. LPS administration caused a statistically significant increase in DIO3 activity in liver, hypothalamus, and thyroid gland. Values are represented as mean  $\pm$  s.d. (\* $P < 0.05$ ; FL, frontal lobe; Cb, cerebellum; Hp, hypothalamus; Pit, pituitary; Th, thyroid gland; Lu, lung; Lv, liver; KM, kidney medulla; KC, kidney cortex; Ad, adrenal gland; SM, skeletal muscle).

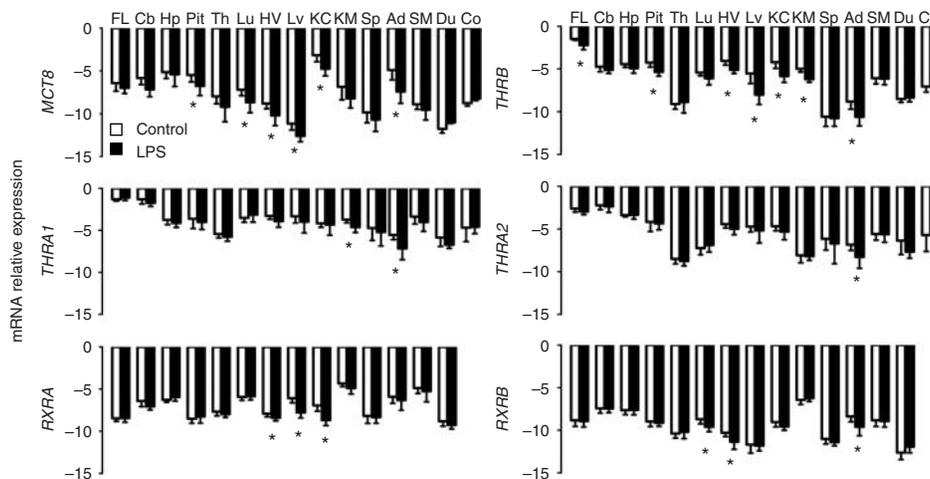
**Figure 4**

Relative gene expression of *THR*s in tissues from control pigs (n:8). Relative expression of *THR*B, *THRA*1, and *THRA*2 genes calculated using the  $2^{-\Delta\Delta CT}$  method are normalized against *THR*B expression in frontal lobe. *RPL4* mRNA expression was used as a reference gene. Values are represented as mean  $\pm$  s.d. (FL, frontal lobe; Cb, cerebellum; Hp, hypothalamus; Pit, pituitary; Th, thyroid gland; Lu, lung; HV, heart left ventricle; Lv, liver; Du, duodenum; Co, colon; KC, kidney cortex; KM, kidney medulla; Ad, adrenal gland; SM, skeletal muscle).

LPS infusion stimulated DIO3 activity in several tissues and such an increase promotes conversion of  $T_4$  into  $rT_3$  and accelerates  $T_3$  clearance, contributing to NTIS hypothyroidism (Gereben *et al.* 2008). NTIS is often associated with elevated serum levels of  $rT_3$  as a consequence of an increase in DIO3 activity (Wartofsky & Burman 1982, DeGroot 1999). However,  $rT_3$  levels did not increase in LPS-treated pigs, likely a consequence of the

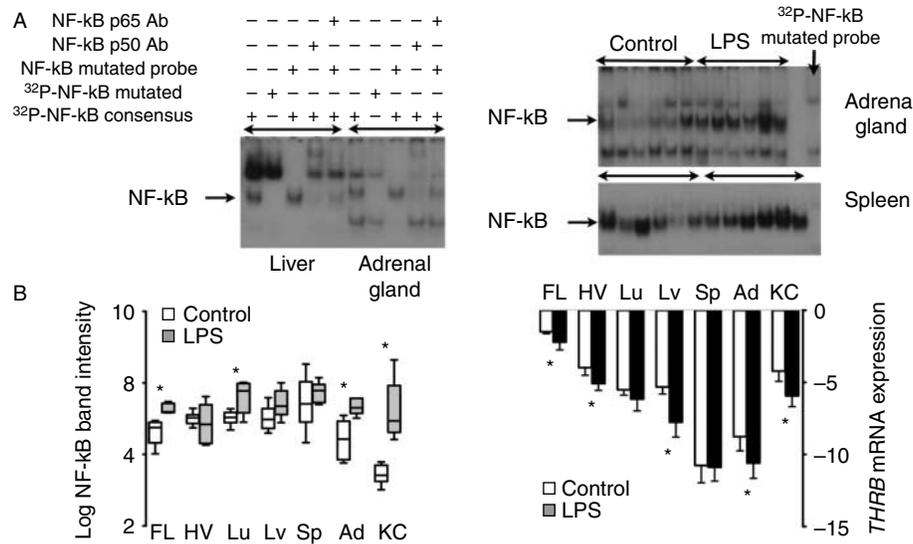
low tissue levels of  $T_4$ . However, the serum  $rT_3/T_4$  ratio did increase after LPS treatment, indicating that increased DIO3 activity was effective, especially when  $rT_3/T_4$  ratio is not expected to increase in hypothyroidism due to low levels of  $rT_3$  (Burman *et al.* 1997). The observation that pig liver DIO3 activity increased after LPS agrees with previous studies in critically ill patients (Peeters *et al.* 2003). However, in mouse models of NTIS, liver DIO3 activity and expression decreased (Boelen *et al.* 2005, 2008). Also, our finding that DIO3 activity increased in pig hypothalamus after LPS does not agree with studies on rodents that showed no changes in DIO3 activity during prolonged NTIS (Mebis *et al.* 2009) and a decrease in *DIO3* mRNA expression during acute (Boelen *et al.* 2009) and chronic (Boelen *et al.* 2006) NTIS. Differences between animal models, study time frame, and severity of inflammatory response may account for differences observed between the pig and rodent models. However, an increase in DIO3 activity in liver and hypothalamus agrees with the reduced levels of TH observed in these two tissues from humans with NTIS (Arem *et al.* 1993, Peeters *et al.* 2003). Interestingly, thyroid gland DIO3 activity increased in LPS-treated pigs and it is tempting to speculate that this might contribute to decreased  $T_4$  secretion from the thyroid gland during NTIS.

Hypoxia-inducible factor 1  $\alpha$  subunit (HIF-1 $\alpha$  (HIF1A)), a transcription factor that regulates cellular response to hypoxia, has been proposed as an activator

**Figure 5**

Monocarboxylate transporter 8 (*MCT8*); thyroid hormone receptors  $\beta$  (*THR*B),  $\alpha$ 1 (*THRA*1), and  $\alpha$ 2 (*THRA*2); and retinoid X receptors  $\alpha$  (*RXRA*) and  $\beta$  (*RXRB*) gene expression changes between control (n:8) and LPS-treated (n:9) pigs. Relative gene expression is represented  $\Delta Ct$ . A  $\Delta Ct$  difference of 1 indicates a twofold difference in gene expression. Values are represented

as mean  $\pm$  s.d. (\* $P < 0.05$ ; FL, frontal lobe; Cb, cerebellum; Hp, hypothalamus; Pit, pituitary; Th, thyroid gland; Lu, lung; HV, heart left ventricle; Lv, liver; KC, kidney cortex; KM, kidney medulla; Sp, spleen; Ad, adrenal gland; SM, skeletal muscle; Du, duodenum; Co, colon (*RXRA* and *RXRB* mRNA expression was not measured in colon)).

**Figure 6**

(A) NF- $\kappa$ B nuclear binding activity in pig tissues evaluated by EMSA. (Left) NF- $\kappa$ B p50–p65 nuclear binding was identified in pig tissues using radioactively labeled oligonucleotides containing a consensus and/or a mutated NF- $\kappa$ B binding site. Non-specific bands were identified using a probe containing a mutated NF- $\kappa$ B binding site in both radiolabeled and non-labeled competition assays (lanes 2, 3, 7, and 8). Specific bands were identified by supershift assays using antibodies against p50 and p65 NF- $\kappa$ B subunits (lanes 4, 5, 9, and 10). (Right upper) Adrenal gland from LPS-treated (n:6) pigs showed higher NF- $\kappa$ B nuclear binding activity than controls (n:6). (Right lower) Spleen from control (n:6) pigs had a high basal NF- $\kappa$ B nuclear binding activity that did not increase significantly after LPS (n:6). (B) Relationship between NF- $\kappa$ B nuclear binding (left) and *THRB*

mRNA expression (right) in pig tissues. In frontal lobe (FL), adrenal gland (Ad), and kidney cortex (KC), LPS induced a significant increase in NF- $\kappa$ B nuclear binding activity and a decrease in *THRB* mRNA expression. In spleen (Sp), no differences in NF- $\kappa$ B nuclear binding or *THRB* gene expression were observed between groups. In lungs (Lu), LPS caused an increase in NF- $\kappa$ B nuclear binding activity but no change in *THRB* gene expression was observed between groups. Finally, in heart left ventricle (HV) and liver (Lv), LPS induced a decrease in *THRB* mRNA expression that did not correspond with a significant increase in NF- $\kappa$ B nuclear binding activity. NF- $\kappa$ B nuclear binding activity is represented with Tukey box-and-whisker plot. *THRB* mRNA relative expression is represented as  $\Delta$ Ct and values as mean  $\pm$  s.d. (\* $P < 0.05$ ).

of *DIO3* gene expression and activity (Simonides *et al.* 2008). It has been hypothesized that under circumstances of cardiac hypoxia, HIF-1 $\alpha$  increases *DIO3* activity creating a situation of local hypothyroidism that could reduce oxygen consumption and energy expenditure (Gereben *et al.* 2008). Although *DIO3* activity was not detected in pig heart, in those tissues where LPS induced an increase in *DIO3* activity, *HIF-1 $\alpha$*  gene expression did not increase, and even decreased in the hypothalamus (unpublished data). These findings suggest that the tissue-specific increase in *DIO3* activity induced by LPS is not related to increased *HIF-1 $\alpha$*  gene expression levels.

*DIO2* activity was detected in the hypothalamus, pituitary gland, and thyroid gland, and LPS administration did not induce significant changes in *DIO2* activity. Previous studies in rats have shown that LPS administration induces *DIO2* activity in mediobasal hypothalamus tanycytes leading to the hypothesis that an increase in hypothalamic *DIO2* activity causes a local hyperthyroidism responsible for low *TRH* mRNA expression in NTIS (Fekete *et al.* 2004). However, in a

rabbit model of prolonged critical illness, an increase in *DIO2* activity or in TH levels was found (Mebis *et al.* 2009). Moreover, in autopsy samples from humans with severe NTIS,  $T_3$  concentration decreased in the hypothalamus and pituitary gland (Arem *et al.* 1993). All together, these results suggest that changes in hypothalamic and pituitary *DIO2* activity are not necessary for central hypothyroidism in NTIS.

LPS-treated pigs showed remarkable tissue-specific changes in expression of THRs. Endotoxemic pigs showed decreased levels of TH and *THRB* mRNA expression in heart left ventricle, liver, and kidney cortex, while in lung and skeletal muscle TH levels decreased, but no changes in *THRB* mRNA expression were observed. These findings suggest that heart, liver, and kidneys of LPS-treated animals are not only hypothyroid but may also have a decreased availability of THR $\beta$ . Although quantification of THR $\beta$  protein expression by western blot was attempted, our results were not reliable due to the poor specificity of the commercially available THR $\beta$  antibodies against pig protein; however, in a previous study using tissues from NTIS

patients, we showed that decreased *THRB* mRNA expression levels were associated with decreased protein level (Lado-Abeal *et al.* 2010).

MCT8 is so far the best-characterized TH transporter. In the brain, MCT8 regulates T<sub>3</sub> uptake, and MCT8-null mice show resistance to TH action in the hypothalamus and pituitary gland (Heuer & Visser 2009). However, in liver and kidney, MCT8 is not required for TH uptake but plays a role in TH efflux (Liao *et al.* 2011, Trajkovic-Arsic *et al.* 2010). *MCT8* gene expression decreased in several tissues after LPS administration, although the consequences of such an effect are unknown. Based on the present knowledge, a decrease in pituitary *MCT8* expression may aggravate central TH resistance, especially in the presence of decreased *THRB* gene expression as observed in LPS-treated pigs. By contrast, LPS-induced decrease in liver and kidney *MCT8* mRNA expression should not affect TH uptake but efflux resulting in an increase in T<sub>3</sub> retention. The consequences of LPS-induced inhibition of *MCT8* gene expression needs further study.

In frontal lobe cortex, LPS-treated animals had lower T<sub>4</sub> than controls, but no differences were observed in T<sub>3</sub>. These findings are similar to those in pregnant rats near term, where decreased levels of T<sub>4</sub> and T<sub>3</sub> were observed in all the studied tissues with the exception of the cerebral cortex, which maintained a normal concentration of T<sub>3</sub> (Calvo *et al.* 1990). Normal levels of T<sub>3</sub> in the frontal lobe of LPS-treated pigs are difficult to explain because T<sub>4</sub> levels were lower than controls, and no differences in DIO3 activity or *MCT8* mRNA expression were observed. Also, frontal lobe was among the tissues with the highest level of *DIO2* expression, but no enzyme activity was detected. As our experimental design does not allow differentiation of *DIO2* activity in a specific subset of brain cells, the mechanisms responsible for sustaining normal T<sub>3</sub> levels in frontal lobe after LPS exposure remain unexplained.

Overall, the changes in expression of *MCT8* and *THRB* genes after LPS observed in our study agrees with previous studies on rodent models of NTIS where no changes in hypothalamic expression of *MCT8* or *THRs* have been noticed (Boelen *et al.* 2006, Mebis *et al.* 2009), and a decrease in *THRB* mRNA expression has been reported in the pituitary gland (Boelen *et al.* 2006), liver (Beigneux *et al.* 2003), heart (Feingold *et al.* 2004), and kidney (Feingold *et al.* 2008). LPS induced a decrease in *RXRA* in rodent liver (Beigneux *et al.* 2000), which was also observed in our pig model. The mechanism behind the tissue-specific inhibition of *THRs* and transporters remains unknown.

Based on our results, it seems reasonable to hypothesize that TH replacement in animals with LPS-induced

NTIS will normalize thyroid homeostasis in some but not all tissues. Specifically, those organs with decreased expression of *MCT8*, *THRs*, and/or increased DIO3 activity may have a decreased sensitivity to TH. The described situation would be more severe in heart left ventricle, liver, adrenal gland, and kidney, where gene expression of *RXRs* is also decreased.

LPS activates the innate immune response and induces the release of pro-inflammatory cytokines in pigs (Williams *et al.* 2009). LPS and inflammatory cytokines activate NF- $\kappa$ B, a family of transcription factors central to activation of the immune response (Oeckinghaus *et al.* 2011). NF- $\kappa$ B activation inhibits the induction of DIO1 and other positively regulated T<sub>3</sub>-responsive genes by T<sub>3</sub> *in vitro*, an observation that leads to the hypothesis that activation of NF- $\kappa$ B plays a key role in NTIS pathogenesis (Nagaya *et al.* 2000). In this study, we observed that LPS-induced septic shock was associated with an increase in NF- $\kappa$ B nuclear binding activity in some but not all analyzed tissues. In spleen, liver, and heart left ventricle, no increase in NF- $\kappa$ B nuclear binding activity was observed. In spleen, control pigs had a high basal NF- $\kappa$ B nuclear binding activity that could be caused by the elevated number of resident immune cells continuously exposed to antigens; under such circumstances, LPS-induced NF- $\kappa$ B activation could be more difficult to observe. The findings in liver and heart left ventricle are more difficult to explain. The liver results could be related to lack of statistical power as there was a clear trend toward an increase in NF- $\kappa$ B nuclear binding activity in the LPS-treated group. The absence of significant NF- $\kappa$ B activation in heart left ventricle after LPS administration is intricate. Septic shock causes high mortality in part due to myocardial depression (Parrillo *et al.* 1990) through mechanisms triggered by pro-inflammatory cytokine-induced high levels of nitric oxide and reactive oxygen species (Finkel *et al.* 1992, Yokoyama *et al.* 1993, Goldhaber *et al.* 1996). Although pro-inflammatory cytokines are mostly released by stimulated circulating immune cells, it has been shown *in vitro* that LPS induces the release of inflammatory cytokines by myocardial cells through the activation of NF- $\kappa$ B, causing a decrease in cardiomyocyte contractility (Boyd *et al.* 2006). However, a similar conclusion in pigs cannot be made from our study because NF- $\kappa$ B nuclear binding activity in control pigs' myocardium was not different from activity in myocardium from septic shock pigs, which was remarkably low in both experimental groups. Although the observed discrepancies could be due to different animal models (rodents vs pigs) and experimental design (*in vivo* vs

*in vitro*), our study was not designed to investigate the role of NF- $\kappa$ B activation in myocardial depression during sepsis.

Our results showed a relationship between increased NF- $\kappa$ B nuclear binding activity and decreased *THRB* mRNA expression after LPS in some but not all studied tissues. *THRB* mRNA in the heart and liver decreases despite there being no activation of NF- $\kappa$ B, indicating that NF- $\kappa$ B pathway activation is not solely responsible for the decreased expression of *THRB*. There are several possible mechanisms that could be responsible for the observed decrease in *THRB* gene expression, including other inflammatory pathways, epigenetic changes, or increased mRNA degradation. Additionally, the *THRB* gene has multiple transcription start sites (Franton *et al.* 2004), which raises the possibility that multiple promoter regions regulate *THRB* gene expression. Under these circumstances, tissue-specific expression of *THRB* could be regulated by the use of different promoters resulting in a tissue-specific gene expression profile. As *THRB* gene expression was not affected by NF- $\kappa$ B activation in the lung, it is possible that *THRB* gene transcription in the lung is regulated differently from *THRB* gene transcription in the frontal lobe, adrenal, and kidney cortex, which showed a decrease in *THRB* gene expression and an increase in NF- $\kappa$ B nuclear binding activity. Participation of the NF- $\kappa$ B pathway in regulating *THRB* mRNA in frontal lobe, adrenal gland, and kidney cortex cannot be excluded based on these results; however, an alternate mechanism must be responsible for the decrease in *THRB* expression in heart, liver, and lung. Further studies are needed to identify the different pig *THRB* promoters and the potential regulation by NF- $\kappa$ B.

This study has several limitations. Food intake decreased in LPS-treated pigs, indicating that most food present in these animals' gastrointestinal system resulted mostly from intake before LPS administration. As no nutritional support was provided, the effect of low energy intake on observed changes cannot be quantified. Our model describes changes occurring early in a severe form of NTIS associated with high mortality during the first 24 h of LPS infusion. In previous studies, we treated pigs with lower doses of LPS and, in spite of the fact that all animals presented fever, NTIS phenotype was not observed, indicating that in pigs treated with LPS there is a narrow margin between absence of NTIS phenotype and presence of severe septic shock. Nowadays, fulminant cases of septic shock are uncommon in clinical settings and consequences of TH replacement in such situations would probably be difficult to assess. A pig model of LPS-induced

septic shock NTIS with airway, hemodynamic, and nutritional support as in hospital intensive care units will provide more practical information for human NTIS pathophysiology in clinical settings.

In conclusion, LPS-induced septic shock NTIS in pigs caused a severe hypothyroidism with decreased levels of THs in tissues. Conditions for reduced sensitivity to TH action are also observed in some tissues. LPS-induced NF- $\kappa$ B nuclear binding activity was present in most but not all the analyzed tissues and, overall, a relationship between NF- $\kappa$ B activation and decrease in *THRB* gene expression was not observed.

#### 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

I C and L Q have contributed equally to the manuscript and should be given equal consideration. They participated in the animals study and performed most of the molecular studies. R-M C and M-J O measured the tissue hormonal levels. M-J O participated in drafting the manuscript and provided research funds. J L-A designed the study, performed the animals' surgery, collected the tissues, draft the manuscript, and provided most of the research funds. He is the research Principal investigator. All the authors participated in the manuscript discussion.

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