Thyrostimulin deficiency does not alter peripheral responses to acute inflammation-induced nonthyroidal illness

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van Zeijl CJ, Surovtseva OV, Kwakkel J, van Beeren HC, Bassett JH, Williams GR, Wiersinga WM, Fliers E, Boelen A. Thyrostimulin deficiency does not alter peripheral responses to acute inflammation-induced nonthyroidal illness. Am J Physiol Endocrinol Metab 307:E527–E537, 2014. First published August 12, 2014; doi:10.1152/ajpendo.00266.2014.—Thyrostimulin, a putative glycoprotein hormone, comprises the subunits GPA2 and GPB5 and activates the TSH receptor (TSHR). The observation that proinflammatory cytokines stimulate GPB5 transcription suggested a role for thyrostimulin in the pathogenesis of nonthyroidal illness syndrome (NTIS). In the present study, we induced acute inflammation by LPS administration to GPB5−/− and WT mice to evaluate the role of thyrostimulin in peripheral thyrotronic hormone metabolism during NTIS. In addition to serum thyroid hormone concentrations, we studied mRNA expression and activity of deiodinase types I, II, and III (D1, D2, and D3) in peripheral T3 target tissues, including liver, muscle, and white and brown adipose tissue (WAT and BAT), of which the latter three express the TSHR. LPS decreased serum free (f)T4 and fT3 indexes to a similar extent in GPB5−/− and WT mice. Serum reverse (r)T3 did not change following LPS administration. LPS also induced significant alterations in tissue D1, D2, and D3 mRNA and activity levels, but only the LPS-increased in WAT D2 mRNA expression differed between GPB5−/− and WT mice. In conclusion, lacking GPB5 during acute illness does not affect the LPS-induced decrease of serum thyroid hormones while resulting in subtle changes in tissue D2 expression that are unlikely to be mediated via the TSHR.

thyrostimulin; type II deiodinase; inflammation; thyroid hormone

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In addition to central alterations of the HPT axis, NTIS results in altered peripheral thyroid hormone metabolism and plasma thyroid hormone binding capacity (26). As the TSHR is expressed in several peripheral extrathyroidal tissues (10), and activation of the TSHR is known to regulate the expression and activity of deiodinating enzymes, which are involved in thyroid hormone metabolism (6, 11), we hypothesized that altered thyrostimulin expression in peripheral tissues could also account for changes in thyroid hormone metabolism seen in NTIS. To this end, we investigated a possible role for GPB5 in peripheral thyroid hormone metabolism during acute inflammation, induced by LPS administration using GPB5−/− and WT mice. We measured serum free T3 (fT3) and free T3 (fT3) indexes and serum reverse T3 (rT3) concentrations. In addition, we determined deiodinase types I, II, and III (D1, D2, and D3) mRNA expression and activities in key peripheral thyroid hormone target tissues, including muscle and white and brown adipose tissue (WAT and BAT), which also express the TSHR, and liver. Furthermore, to investigate underlying molecular mechanisms, we stimulated a murine skeletal muscle cell line and adipocyte cell line with GPB5, GPA2, thyrostimulin, and TSH and measured cAMP activation together with D2 and D3 mRNA expression. The present study aimed to clarify whether thyrostimulin plays a significant role in illness-induced alterations in thyroid hormone metabolism.

MATERIALS AND METHODS

Animals. Female GPB5−/− and WT mice (129SvEv background) were studied between 7 and 11 wk of age (6 mice per group). GPB5−/− mice were generated by Lexicon Genetics (The Woodlands, TX), bred, and genotyped as described previously (24). We observed no differences between the GPB5−/− and WT lineages in breeding behavior. Litter sizes and sex ratios were similar in both strains. The mice were kept in 12:12-h light-dark cycles (light on from 0700 to 1900) in a temperature-controlled room (22°C). One week before the experiment, the mice were housed in groups according to the experimental set-up. Acute illness was induced by intraperitoneal (ip) injection of 200 μg LPS (endotoxin; E. coli 127: B8; Sigma Chemical, St. Louis, MO) diluted in 0.5 ml of sterile 0.9% NaCl. Control mice received 0.5 ml of sterile 0.9% NaCl. At different time points after LPS injection (t = 0, 4, 8, and 24 h), mice were anesthetized with

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isoflurane and euthanized by cervical dislocation. To correct for diurnal variations, all experiments started at 0900, and each time point had its own control. Blood was taken by cardiac puncture, and serum was stored at –20°C until analyzed. Liver, hindlimb muscle (including quadriceps femoris), gonadal WAT, and scapular BAT were obtained and immediately stored in nitrogen. The Local Animal Welfare Committee approved the study, and animals were treated according to ETS123 (Council of Europe) guidelines for use of laboratory animals.

**In vitro experiments.** To investigate the effect of thyrostimulin in vitro, a mouse myoblast cell line, C6C12 (Sigma) and differentiated mouse adipocytes (3T3-L1 cells, kindly provided by Dr. Marc Tol, Medical Biochemistry, Academic Medical Center, Amsterdam) were stimulated with thyrostimulin (A2/B5), GPB5, or GPA2. CAMP production and D2 mRNA expression were evaluated in response to these treatments. To obtain biologically active thyrostimulin, bicistronic pBuCE4 plasmid vectors containing either GPA2 or GPB5 cDNA alone or both GPA2 and GPB5 were transfected into COS-7 cells, and conditioned medium containing the subunits was collected (generation of GPA2- and GPB5-containing expression vectors is described by Bassett et al., personal communication). Briefly, COS-7 cells were cultured in growth medium [DMEM (GIBCO, Breda, The Netherlands) supplemented with l-glutamine, 0.1 mM MEM Non-Essential Amino Acids Solution, 100 U/ml penicillin G (P), 0.1 mg/ml streptomycin (S), and 10% fetal calf serum (FCS; Invitrogen, The Netherlands)]. One day before transfection, medium was replaced by growth medium without P-S. Plasmid DNA (0.5 μg) was diluted in 50 μl of Opti-MEM (Opti-MEM I Reduced Serum Medium, GIBCO) and mixed gently. Lipofectamine (1 μl, Lipofectamine 2000, Invitrogen) was also diluted in 50 μl of Opti-MEM and incubated for 5 min at room temperature (RT). Subsequently, the DNA was combined with the Lipofectamine and incubated for 20 min at RT. The complex was added to the COS-7 cells and incubated for 48 h at 37°C, 5% CO2, and medium was replaced by growth medium containing P-S but lacking FCS. Cells were incubated for another 48 h at 37°C, 5% CO2, and the supernatant was harvested and stored at –80°C until use.

A Chinese hamster ovary cell line (CHO-JP26) stably transfected with the human TSHR (CHO.hTSHR cells, kindly provided by Dr. S. Costagliola, Université Libre de Bruxelles, Brussels, Belgium) was used to measure the ability of thyrostimulin and its subunits to activate the TSHR and induce cAMP production. CHO-JP02 (empty vector) and CHO-JP26 cells were cultured in DMEM-F12 modified medium (GIBCO) containing 5% FCS, P-S, and 400 μg/ml G418 (Sigma). One day before the assay, the culture medium was replaced by serum-free DMEM-F12 supplemented with 1 μg/ml Aeaptac insulin (Novo Nordisk, The Netherlands), 5 μg/ml human apo-transferrin (Sigma), and P-S. The next day, medium was changed in assay medium [DMEM-F12, 1 μg/ml Aeaptac insulin, 5 μg/ml human apo-transferrin, P-S, and 20 μM rolipram (Sigma)]. After 1 h, the cells (1.106 cells/well) were stimulated with control supernatant, GPA2, GPB5, A2/B5 (100 μl), bovine (b)TSH (100 μU/ml), and forskolin (50 μM) for 60 min at 37°C, 5% CO2. The supernatant was removed, and the cells were treated with 0.1 M HCl for 10 min, and samples were subsequently centrifuged. Supernatant was collected and stored at –80°C until use.

C6C12 cells ([1.106 cells/well] cultured in DMEM with 4.5 g/l glucose and l-glutamine, supplemented with 5% FCS and P-S) and 3T3-L1 cells ([2.106 cells/well] cultured in DMEM with 4.5 g/l glucose and l-glutamine, supplemented with 5% FCS and P-S) were also stimulated with control supernatant, GPA2, GPB5, A2/B5 (100 μl/1.106 cells), bTSH (100 μU/ml), and forskolin (50 μM) for 60 min at 37°C, 5% CO2 to measure cAMP production.

C6C12 cells (5.105 cells/well) and 3T3-L1 cells (1.106 cells/well) were also stimulated with control supernatant, GPA2, GPB5, A2/B5 (25 μl/well), bTSH (10 μU/ml) and forskolin (50 μM) for 6h at 37°C, 5% CO2 to measure D2 and D3 mRNA expression. Cells were washed with PBS and lysed in 250 μl of lysis buffer from the MagnaPure LC RNA isolation kit-High Performance (Roche Molecular Biochemicals, Mannheim, Germany). RNA was isolated on the Magna Pure apparatus (Roche Molecular Biochemicals), and RNA concentrations were measured using Nanodrop (Nanodrop, Wilmington, DE) to ensure cDNA synthesis with equal RNA input as described below.

**cAMP production.** cAMP produced by the cells after stimulation was measured using cAMP ELISA (Sigma CA200 cAMP Enzyme Immunoassay Kit) according to the manufacturer’s protocol.

**Charcoal thyroid hormone uptake assay.** Serum T4 and T3 had previously been measured with in-house RIAs (24). We measured serum rT3, using an in-house RIA (27). Serum thyroid hormone (TH) uptake was measured using a charcoal TH uptake assay to estimate the free fraction of serum T3 and T4, as previously described by Zavacki et al. (29). Briefly, 10 μl of mouse serum was incubated with 0.5 ml of PBS containing [125I]T3 (~28,000 cpm) with a specific activity of 3,390 μCi (125 MBq/μg; NEN Life Science Products, Boston, MA) for 45 min at RT to allow equilibration. Samples were transferred to an ice bath for 15 min, cold 0.008% activated charcoal (0.5 ml) solution in PBS (Sigma) was added, and samples were incubated on ice for an additional 15 min. Samples were subsequently centrifuged for 15 min at 2,500 rpm at 4°C, and the charcoal-containing pellets were counted. The assay was optimized to bind ~30% of the tracer to charcoal in sera from control mice, and samples were assayed in triplicate for each mouse. All samples of one experiment were analyzed in the same assay to avoid interassay variation; the intraassay variation was 5%. An estimate of the rT3 index and rT4 index was calculated by multiplying respectively the total T3 and total T4 serum concentration by the normalized TH charcoal uptake.

**mRNA isolation and reverse transcription PCR.** Liver, hindlimb muscle, WAT, and BAT mRNAs were isolated on the Magna Pure apparatus (Roche Molecular Biochemicals) using the MagNA Pure LC mRNA HS Kit and ~25 mg of tissue. We used the protocol and buffers supplied with the corresponding kit. cDNA synthesis was performed using the Transcripter First-Strand cDNA Synthesis kit for real-time PCR with oligo(dT) primers (Roche Molecular Biochemicals). We performed real-time PCR for quantitation of hypoxanthine phosphoribosyltransferase (HPRT), TSHR, GPB5, GPA2, IL-1β, D1, D2, and D3 using the Lightcycler480 (Roche Molecular Biochemicals) and the Lightcycler 480 Sybr Green I Master kit (Roche Molecular Biochemicals) as previously described (1, 24). Primers were either intron spanning or genomic DNA contamination was tested using a cDNA synthesis reaction without the addition of reverse transcriptase (1, 4, 22, 24). Samples were corrected for their mRNA content using HPRT as a housekeeping gene, as LPS administration tested using a cDNA synthesis reaction without the addition of reverse transcriptase (1, 4, 22, 24). Samples were checked for their PCR efficiency (18). The median of the efficiency was calculated for each assay, and samples that differed by more than 0.05 of the efficiency median value were not taken into account. Aberrant PCR efficiencies occurred randomly and therefore did not influence the results in a systematic way.

**Deiodinase activities.** Deiodinase activity was measured as described previously (7, 8, 17). Tissue (~150 mg) was homogenized on ice in 600 μl of PE buffer (0.1 M sodium phosphate, 2 mM EDTA, pH 7.2) containing 50 mM dithiothreitol (DTT; PES50), using a Polytron (Kinematica, Lucerne, Switzerland). For D1 and D3 activity measurements, samples were either immediately frozen in liquid nitrogen and stored at –80°C until use; for D2 activity measurements, homogenates were used immediately. Samples were measured in duplicate. We measured protein concentration with the Bio-Rad protein assay (Bio-Rad Laboratories, Veenendaal, The Netherlands), according to the manufacturer’s instructions.

For D1 activity measurements, we diluted the homogenates 100–200 times in PED20 (20 mM DTT) buffer with equal protein input for all samples. D1 activity was measured using a 50-μl sample incubated for 30 min in a final volume of 100 μl with 0.1 μM rT3, with the addition of –1 × 105 cpm [5-125I]T3 in PED20. Reactions were stopped by adding 0.1 ml of 5% BSA on ice. We precipitated the
protein-bound iodothyronines by adding 10% (wt/vol) trichloroacetic acid. After centrifugation, 125I— was separated from the supernatant by chromatography on Sephadex LH-20 columns with a bed volume of 0.25 ml, equilibrated, and eluted with 0.1 M HCl. Released 125I— was counted with the Packard Cobra Auto-Gamma Counting System (Canberra Packard, Zurich, Switzerland) in the eluate. D1 activity was expressed as 125I picomole release per minute per milligram of liver protein.

D2 activity was measured in BAT and muscle in duplicate using 75 μl (BAT ~210 μg protein and muscle ~1,200 μg protein) of homogenate in a final volume of 0.15 ml with 1 nM T4 with the addition of ~1.5 × 10^6 cpm [3^-125I]T4 in PE (NEX110X; PerkinElmer, Groningen, The Netherlands; purified with Sephadex LH-20). Samples were incubated for 2 h (BAT) or 4 h (muscle) at 37°C. D1 activity was blocked by 0.5 mM PTU.

D3 activity was measured in liver, BAT, WAT, and muscle in duplicate using 75 μl (liver ~100 μg, BAT ~210 μg, WAT ~175 μg and muscle ~1,500 μg protein) of homogenate in a final volume of 0.15 ml in the presence of 1 nM T3 or 500 nM T3 with the addition of ~1 × 10^6 cpm [3^-125I]T3 (NEX110X, PerkinElmer) in PE. Samples were incubated for 2 h (liver, WAT, and muscle) or 4 h (BAT) at 37°C. D1 activity was blocked by 0.5 mM PTU.

D2 and D3 activity reactions were stopped by adding 0.15 ml ice-cold ethanol. After centrifugation, 0.125 ml of the supernatant was added to 0.125 ml of 0.02 M ammonium acetate (pH 4), and 0.1 ml of the mixture was applied to 4.6 × 250 mm Symmetry C18 column connected to a Waters HPLC system (Model 600E pump, model 717 WISP autosampler, Waters, Ettten-Leur, The Netherlands). Mobile phase A: 0.02 M ammonium acetate (pH 4.0), mobile phase B: acetonitril. The column was eluted with a linear gradient (28–42% B in 15 min) at a flow of 1.2 ml/min. The activities of T4, T3, and T2 in the eluate were measured online using a Radiomatic Flow-One/Beta scintillation detector (Packard, Meriden, CT).

For D2 activity, for each group we included one sample with 500 nM T4 incubation, as this concentration T4 saturates D2. D2 activity when incubated with 1 nM T4, minus the activity measured when incubated with 500 nM T4 represents true D2 activity. D2 activity was expressed as femtomoles of generated T3 per minute per milligram of protein. If the %conversion was below the cut-off value (mean ± 3 SD of the %generated T3 of the 500 nM T4 incubations), we labeled the sample as undetectable.

For D3 activity, for each group we included one sample with 500 nM T3 incubation, as this concentration T3 saturates D3; D3 activity when incubated with 1 nM T3 minus the activity measured when incubated with 500 nM T3 represents true D3 activity. D3 activity was expressed as femtomoles of generated 3,3'-T2 per minute per milligram of tissue. If the %conversion was below the cut-off value (mean ± 3 SD of the %generated T2 of the 500 nM T3 incubations), we labeled the sample as undetectable.

Statistics. Normal distribution and equality of variances of the data were tested using the Shapiro-Wilk and Levene's tests, respectively. Differences between saline-treated and LPS-treated GPB5−/− or WT mice and between LPS-treated GPB5−/− and WT mice were evaluated using nonparametric proportional odds regression. To correct for testing multiple outcomes, (Bonferroni) corrected P values of ≤0.0085 were considered statistically significant. In case of an overall significant

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**Fig. 1.** Effects of LPS on serum free thyroxine (T4) and free triiodothyronine (T3) indexes in the glycoprotein thyrostimulin subunit GPB5 knockout (GPB5−/−) and WT mice. A, D: saline-treated (●) and LPS-treated (○) WT mice. B, E: saline-treated (●) and LPS-treated (○) GPB5−/− mice. C, F: LPS-treated WT (●) and GPB5−/− (○) mice. These are expressed as percentage of their saline controls (set at 100% and given as a dotted line). Mean values ± SE are shown (n = 6). P values indicate differences between groups by nonparametric proportional odds regression; P ≤ 0.0085 was considered statistically significant because of Bonferroni correction. Differences at individual time points were tested using Mann-Whitney U-tests with Bonferroni-corrected significance threshold (P ≤ 0.017) and indicated by *P ≤ 0.017 and **P ≤ 0.010.
treatment or strain effect, differences at the individual time points were tested using Mann-Whitney U-tests with a Bonferroni-corrected significance threshold for these post hoc analyses ($P \leq 0.017$). Variations between groups in in vitro experiments were evaluated by one-way ANOVA or Kruskal-Wallis tests with one grouping factor (treatment). In case of a significant overall test, differences between groups were analyzed by Student’s $t$-test or by the Mann-Whitney U-test where appropriate, with a Bonferroni-corrected significance threshold ($P \leq 0.01$). All analyses were carried out in SPSS v. 16.0.2 (SPSS, Chicago, IL) or in R (v. 2.14.2; R Foundation for Statistical Computing, Vienna, Austria).

**RESULTS**

**Effect of LPS on the inflammatory response.** The inflammatory response to LPS treatment was determined by measuring IL-1β mRNA expression in liver, muscle, WAT, and BAT. In all tissues, IL-1β mRNA expression was increased 4 h after LPS administration and returned to basal levels after 24 h. We observed no differences in IL-1β mRNA expression between GPB5−/− and WT mice in liver, muscle, WAT, or BAT (data not shown).

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**Fig. 2.** Thyroid-stimulating hormone receptor (TSHR) mRNA expression [normalized to hypoxanthine phosphoribosyltransferase (HPRT)] in muscle and white and brown adipose tissues (WAT and BAT) of GPB5−/− and WT mice after LPS administration. A: saline-treated (■) and LPS-treated (●) WT mice. B: saline-treated (■) and LPS-treated (●) GPB5−/− mice. C: LPS-treated WT (●) and GPB5−/− (○) mice. These are expressed as percentage of their saline controls (set at 100% and given as a dotted line). Mean values ± SE are shown ($n = 6$). $P$ values indicate differences between groups by nonparametric proportional odds regression; $P \leq 0.0085$ was considered statistically significant because of Bonferroni correction. Differences at individual time points were tested using Mann-Whitney U-tests with Bonferroni-corrected significance threshold ($P \leq 0.017$) and indicated by *$P \leq 0.017$ and **$P \leq 0.010$.**
Fig. 3. Effects of LPS on deiodinases D1 and D3 mRNA expression (normalized to HPRT) and D1 and D3 activity in liver of GPB5−/− and WT mice. A, D, G, J: saline-treated (●) and LPS-treated (○) WT mice. B, E, H, K: saline-treated (▼) and LPS-treated (▲) GPB5−/− mice. C, F, I, L: LPS-treated WT (●) and GPB5−/− (○) mice. These are expressed as percentage of their saline controls (set at 100% and given as a dotted line). Mean values ± SE are shown (n = 6). P values indicate differences between groups by nonparametric proportional odds regression; P ≤ 0.0085 was considered statistically significant because of Bonferroni correction. Differences at individual time points were tested using Mann-Whitney U-tests with Bonferroni-corrected significance threshold (P ≤ 0.017) and indicated by *P ≤ 0.017 and **P ≤ 0.010.
Effect of LPS on systemic TH concentrations. Basal serum rT₃, the fT₄ index, and fT₃ index were similar in GPB5⁻/⁻ and WT mice. Serum rT₃ did not change following LPS administration (data not shown), and the LPS-induced alterations in serum fT₄ and fT₃ indexes were similar in both genotypes (Fig. 1).

Effect of LPS on peripheral GPB5 and TSHR mRNA expression. GPB5 mRNA was expressed in liver, WAT, and BAT of WT mice, albeit at very low levels near the limit of detection. LPS did not increase GPB5 mRNA expression in liver, WAT, and BAT (data not shown). GPA2 was expressed in liver, muscle, WAT, and BAT of both WT and GPB5⁻/⁻ mice (data not shown). TSHR mRNA expression showed a strong diurnal variation in muscle, WAT and BAT (Fig. 2; ANOVA, \( P_{\text{time}} \leq 0.001 \)) that was similar in saline-treated GPB5⁻/⁻ and saline-treated WT mice. LPS treatment suppressed TSHR mRNA expression in WAT of WT (\( P_{\text{treat}} = 0.005 \)) and GPB5⁻/⁻ mice (\( P_{\text{treat}} \leq 0.001 \)). In muscle and BAT, LPS treatment did not significantly alter TSHR mRNA levels in WT or GPB5⁻/⁻ mice. The relative TSHR mRNA expression in muscle, WAT, and BAT did not differ in LPS-treated WT mice compared with LPS-treated GPB5⁻/⁻ mice.

**Fig. 4.** Effects of LPS on D2 and D3 mRNA expression (normalized to HPRT) and activity in muscle of GPB5⁻/⁻ and WT mice (D2 activity was not detectable). A, D, G: saline-treated (■) and LPS-treated (●) WT mice. B, E, H: saline-treated (○) and LPS-treated (●) GPB5⁻/⁻ mice. C, F, I: LPS-treated WT (●) and GPB5⁻/⁻ (○) mice. These are expressed as percentage of their saline controls (set at 100% and given as a dotted line). Mean values ± SE are shown (\( n = 6 \)).

\( P \) values indicate differences between groups by nonparametric proportional odds regression; \( P \leq 0.0085 \) was considered statistically significant because of Bonferroni correction. Differences at individual time points were tested using Mann-Whitney U-tests with Bonferroni-corrected significance threshold (\( P \leq 0.017 \)) and indicated by \(* P \leq 0.017\) and \(** P \leq 0.010\).
In summary, thyrostimulin deficiency did not alter TSHR mRNA responses to LPS administration.

**Effect of LPS on D1 and D3 expression in liver.** LPS decreased hepatic D1 mRNA expression in both GPB5\(^{-/-}\) and WT mice (ANOVA, \(P_{\text{treat}} \leq 0.001\)). The decrease in D1 mRNA was similar in GPB5\(^{-/-}\) and WT mice. Similarly, hepatic D1 activity decreased following LPS administration in WT and GPB5\(^{-/-}\) mice. The decrease in D1 activity was similar in GPB5\(^{-/-}\) and WT mice. Liver D3 mRNA expression also decreased at all time points after LPS administration in both GPB5\(^{-/-}\) and WT mice (both ANOVA, \(P_{\text{treat}} \leq 0.001\)), and the relative LPS-induced D3 mRNA suppression was similar in both strains. Hepatic D3 activity also decreased following LPS administration in WT mice (ANOVA, \(P_{\text{treat}} = 0.001\)), but no difference in the response to LPS was present when effects in WT were compared with effects in GPB5\(^{-/-}\) mice (Fig. 3).

Thus, thyrostimulin deficiency did not alter LPS-induced changes in hepatic D1 and D3 expression.

**Effect of LPS on D2 and D3 expression in skeletal muscle.** Basal D2 mRNA expression was higher in saline-treated WT mice compared with saline-treated GPB5\(^{-/-}\) mice (data not shown, ANOVA, \(P_{\text{strain}} \leq 0.001\)). D2 mRNA expression did not increase after LPS administration in WT mice, whereas a marked increase in D2 mRNA expression was observed in GPB5\(^{-/-}\) mice after LPS treatment (ANOVA, \(P_{\text{treat}} \leq 0.001\)). The relative increase in D2 mRNA expression, however, did not differ between GPB5\(^{-/-}\) and WT mice. Despite increases

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**Fig. 5.** Effects of LPS on D2 and D3 mRNA expression (normalized to HPRT) and D3 activity in WAT of GPB5\(^{-/-}\) and WT mice (D2 expression was not detectable). A, D, G: saline-treated (●) and LPS-treated (○) WT mice. B, E, H: saline-treated (□) and LPS-treated (○) GPB5\(^{-/-}\) mice. C, F, I: LPS-treated WT (●) and GPB5\(^{-/-}\) (○) mice. These are expressed as percentage of their saline controls (set at 100% and given as a dotted line). Data are presented as median with the interquartile range (IQR) (D2 mRNA, \(n = 6\)) or as means ± SE (D3 mRNA and D3 activity, \(n = 6\)). \(P\) values indicate differences between groups by nonparametric proportional odds regression; \(P \leq 0.0085\) was considered statistically significant because of Bonferroni correction. Differences at individual time points were tested using Mann-Whitney \(U\)-tests with Bonferroni-corrected significance threshold (\(P \leq 0.017\)) and indicated by *\(P \leq 0.017\) and **\(P \leq 0.010\).
in D2 mRNA expression, muscle D2 enzyme activity was not detectable in either GPB5⁻/⁻ or WT mice (Fig. 4).

LPS administration did not alter D3 mRNA expression or D3 enzyme activity in GPB5⁻/⁻ and WT.

In summary, muscle D2 mRNA expression increased following LPS administration in GPB5⁻/⁻ but not in WT mice. The observed increase in D2 mRNA expression, however, was not accompanied by detectable D2 enzyme activity in skeletal muscle.

Effect of LPS on D2 and D3 expression in WAT. Basal D2 mRNA expression was lower in saline-treated WT mice than in saline-treated GPB5⁻/⁻ mice (data not shown, ANOVA, Pstrain < 0.002). LPS treatment increased WAT D2 mRNA expression in WT (ANOVA Pstrain = 0.006) but not in GPB5⁻/⁻ mice. Thus, the relative D2 mRNA expression after LPS was lower in GPB5⁻/⁻ than in WT mice (ANOVA Pstrain = 0.003). D2 activity was not detectable in WAT from GPB5⁻/⁻ or WT mice (Fig. 5).

D3 mRNA expression was decreased to a similar extent in GPB5⁻/⁻ and WT mice following LPS treatment (ANOVA, Pstrain = 0.001). LPS decreased WAT D3 activity in GPB5⁻/⁻ (ANOVA Pstrain = 0.001) but not in WT mice, but no difference in the response to LPS was present when effects in WT were compared with effects in GPB5⁻/⁻ mice.

Thus, D2 mRNA expression in WAT increased following LPS administration in WT mice but not in GPB5⁻/⁻ mice. However, a role for GPB5 in the regulation of D2 mRNA expression in WT mice is unlikely, as GPB5 expression did not increase in WAT following LPS administration. Furthermore, the observed increase in D2 mRNA expression was not accompanied by an increase in D2 enzyme activity, suggesting that the response is of minor significance.

**Fig. 6.** Effects of LPS on D2 and D3 mRNA expression (normalized to HPRT) and activity in BAT of GPB5⁻/⁻ and WT mice (D3 activity was not detectable). A, D, G: saline-treated (○) and LPS-treated (●) WT mice. B, E, H: saline-treated (□) and LPS-treated (●) GPB5⁻/⁻ mice. C, F, I: LPS-treated WT (●) and GPB5⁻/⁻ (○) mice. These are expressed as percentage of their saline controls (set at 100% and given as a dotted line). Data are presented as median with IQR (D2 and D3 mRNA, n = 6) or as means ± SE (D2 activity, n = 6). P values indicate differences between groups by nonparametric proportional odds regression; P < 0.0085 was considered statistically significant because of Bonferroni correction.
Effect of LPS on D2 and D3 expression in BAT. D2 and D3 mRNA expression did not differ following LPS treatment in either GPB5<sup>−/−</sup> or WT mice. LPS did not significantly affect BAT D2 activity in GPB5<sup>−/−</sup> and WT mice, and the relative D2 activity after LPS treatment was also similar in both genotypes. D3 activity in BAT was not detectable in GPB5<sup>−/−</sup> or WT mice (Fig. 6).

Effect of thyrostimulin and GPB5 and GPA2 subunits on cAMP production and D2 mRNA in muscle cells and adipocytes. Conditioned medium produced by COS-7 cells expressing thyrostimulin induced a robust cAMP response in CHO cells expressing the TSHR, but no response was evident in CHO-TSHR cells after stimulation with GPA2 or GPB5. Both C<sub>2</sub>C<sub>12</sub> and differentiated 3T3-L1 cells express TSHR mRNA (data not shown). Stimulation of C<sub>2</sub>C<sub>12</sub> and 3T3-L1 cells with GPA2, GPB5, or A2/B5 failed to induce cAMP production, whereas TSH induced cAMP production in differentiated 3T3-L1 cells and to a lesser extent in C<sub>2</sub>C<sub>12</sub> cells (Fig. 7).

Stimulation of C<sub>2</sub>C<sub>12</sub> cells with A2/B5 for 6 h did not result in increased D2 mRNA expression, whereas TSH administration resulted in a small increase in D2 mRNA expression. D2 mRNA expression was not increased in differentiated 3T3-L1 cells following treatment with GPA2, GPB5, A2/B5, or TSH. Forskolin increased D2 mRNA expression in both C<sub>2</sub>C<sub>12</sub> and differentiated 3T3-L1 cells (Fig. 7). D3 mRNA expression was at the limit of detection in C<sub>2</sub>C<sub>12</sub> cells but was undetectable in 3T3-L1 cells.

**DISCUSSION**

Thyrostimulin, comprising GPA2 and GPB5 subunits, is a putative glycoprotein hormone that activates the TSHR. In addition to the thyroid gland, TSHR expression has been reported in a number of peripheral tissues including WAT, BAT, muscle, kidney, skin, and bone (10). Activation of the TSHR is known to regulate deiodinase expression and activity in several tissues (6, 11–13, 23, 28). As acute illness results in a marked increase in pituitary GPB5 transcription (21, 25), we hypothesized that altered thyrostimulin expression in peripheral tissues could also account for changes in thyroid hormone metabolism seen in the NTIS.

Thus, we investigated the role of peripheral thyrostimulin expression in the regulation of thyroid hormone metabolism during LPS-induced acute illness in GPB5<sup>−/−</sup> and WT mice. We determined 1) serum fT<sub>4</sub> and fT<sub>3</sub> indexes and rT<sub>3</sub> in GPB5<sup>−/−</sup> and WT mice, 2) TSHR mRNA expression in key T<sub>3</sub> target tissues, 3) mRNA expression and activities of the deiodinases in T<sub>3</sub> target tissues, and 4) D2 and D3 mRNA expression in response to treatment of skeletal muscle and adipocyte cell cultures with thyrostimulin.

LPS administration is a well-established mouse model of acute NTIS associated with dysregulation of the HPT axis at
several levels. In the present study, LPS administration resulted in decreased serum \( fT_4 \) and \( fT_3 \) indexes in WT mice, as observed before (9). Of note, the degree of inflammation was comparable in GPB5\(^{-/-}\) and WT mice, as the LPS-induced IL-1\(\beta\) mRNA increase was similar between WT and mutant strains in liver, muscle, WAT, and BAT. Thus, the study design was suitable to investigate the role of GPB5 in peripheral thyroid hormone metabolism during acute illness.

**Thyrostimulin deficiency does not alter hepatic responses to LPS.** LPS decreased liver D1 and D3 mRNA expression as well as enzyme activities, in accord with earlier reports in WT mice (1, 2). LPS-induced suppression of hepatic D1 mRNA expression and D1 activity was similar in GPB5\(^{-/-}\) and WT mice, which is in agreement with the observation that TSHR mRNA expression was not detected in liver.

**Thyrostimulin deficiency does not alter muscle responses to LPS.** We studied D2, D3, and TSHR expression in muscle tissue and observed no prominent differences between GPB5\(^{-/-}\) and WT mice. Previous studies had suggested that muscle TSHR mRNA expression resulted from expression in fibroblasts rather than myocytes (10). We stimulated a skeletal muscle cell line with TSH, thyrostimulin, or GPB5. TSH treatment resulted in a small cAMP response accompanied by a small increase in D2 mRNA, whereas treatment with thyrostimulin had no effect. In contrast, activation of the cAMP pathway in C\(_2\)C\(_1\)2 cells by forskolin resulted in a marked increase in D2 mRNA expression, suggesting that the D2 increase in muscle in response to LPS might be a consequence of inflammation-dependent cAMP activation (8).

In summary, these findings suggest that thyrostimulin does not have a significant role in regulation of muscle D2 and D3 in acute illness.

**Thyrostimulin deficiency does not alter the LPS responses in WAT and BAT.** LPS administration increased D2 mRNA expression in WAT of WT but not of GPB5\(^{-/-}\) mice. However, thyrostimulin is unlikely to have an important role in this response for several reasons: 1) TSHR expression decreased after LPS administration to a similar extent in WAT of GPB5\(^{-/-}\) and WT mice, as demonstrated previously in pituitary, hypothalamus and thyroid (1, 25); 2) thyrostimulin failed to induce a cAMP or D2 mRNA response in 3T3-L1 adipocytes; 3) TSH stimulated a robust cAMP response, as expected (13), indicating a functional TSHR present, but there was no accompanying increase in D2 mRNA expression; and 4) stimulation of cAMP by forskolin treatment of 3T3-L1 adipocytes resulted in a robust increase in D2 mRNA expression. Thus, similar to muscle, circulating inflammatory mediators may contribute to the LPS-induced increase in D2 in WAT.

In summary, LPS-induced changes in D3 mRNA and activity in WAT, and D2, and D3 mRNA expression and D2 activity in BAT are similar in GPB5\(^{-/-}\) and WT mice, whereas LPS-induced alterations in D2 mRNA expression in WAT differed in GPB5\(^{-/-}\) mice compared with WT.

TSHR activation has been shown to increase expression of D2 in several tissues, including human thyroid, rat BAT, human osteosarcoma cells, and rat astrocytes (11–13, 28). It was also demonstrated that pituitary GPB5 transcription was increased markedly during acute illness (25). Thus, we hypothesized that thyrostimulin deficiency in GPB5\(^{-/-}\) mice might regulate D2 expression in TSHR-expressing tissues. However, the current studies demonstrate that thyrostimulin has no major role in the regulation of thyroid hormone metabolism in peripheral tissues during acute inflammation.

Currently, it is uncertain whether thyrostimulin acts as a functional glycoprotein hormone in vivo, as the GPA2 and GPB5 subunits are unlikely to form a stable heterodimer in the circulation (5, 14). However, local coexpression of GPA2 and GPB5 suggests that thyrostimulin may have a paracrine function in peripheral tissues (14–16, 19). In the present study, however, LPS administration resulted in similar changes in thyroid status and had no effect on GPB5 mRNA expression in thyrostimulin-deficient and WT mice. Furthermore, thyrostimulin failed to induce cAMP or D2 mRNA expression in skeletal muscle cells or 3T3-L1 adipocytes. Taken together, the current studies exclude a major role for thyrostimulin during acute illness.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


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