Increased FGF21 in brown adipose tissue of tyrosine hydroxylase heterozygous mice: implications for cold adaptation

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Abstract  Tyrosine hydroxylase (TH) catalyzes the first step in catecholamines synthesis. We studied the impact of reduced TH in brown adipose tissue (BAT) activation. In adult heterozygous (TH+/−) mice, dopamine and noradrenaline (NA) content in BAT decreased after cold exposure. This reduced catecholaminergic response did not impair cold adaptation, because these mice induced uncoupling protein 1 (UCP-1) and maintained BAT temperature to a similar extent than controls (TH+/+). Possible compensatory mechanisms implicated were studied. Prdm16 and Fgf21 expression, key genes in BAT activation, were elevated in TH+/− mice at thermoneutrality from day 18.5 of embryonic life. Likewise, plasma FGF21 and liver Fgf21 mRNA were increased. Analysis of endoplasmic reticulum (ER) stress, a process that triggers elevations in FGF21, showed higher phospho-IRE1, phospho-JNK, and CHOP in BAT of TH+/− mice at thermoneutrality. Also, increased lipolysis in BAT of cold-exposure TH+/− mice was demonstrated by increased phosphorylation of hormone-sensitive lipase (HSL), as well as diacylglycerol (DAG) and FFA content. Overall, these results indicate that the mild effects of TH haplinsufficiency on BAT function are likely due to compensatory mechanisms involving elevations in Fgf21 and Prdm16 and through adaptive changes in the lipid profile.—Vázquez, P., C. Hernández-Sánchez, C. Escalona-Garrido, L. Pereira, C. Contreras, M. López, J. Balsinde, F. de Pablo, and A. M. Valverde. Increased FGF21 in brown adipose tissue of tyrosine hydroxylase heterozygous mice: implications for cold adaptation. J. Lipid Res. 2018. 59: 2308–2320.

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Obesity is an important global health problem that results from an imbalance between energy intake and expenditure (1). Three types of adipose cells (white, brown, and beige) have been described with different developmental origins, cell-specific gene expression, and distinct functions (2). Whereas white adipocytes accumulate energy in the form of triglyceride deposits that are distributed in a large fat droplet and contain few mitochondria, brown
adipocytes present multilocular fat droplets and contain a higher number of mitochondria (3). Brown adipocytes are responsible for nonshivering thermogenesis by uncoupling the proton gradient generated by oxidative phosphorylation and ATP synthesis, due to the specific expression of the uncoupling protein 1 (UCP-1) in the inner mitochondrial membrane. This brings out energy dissipation as heat (4). Recently, a third class of adipocytes has been identified and termed “beige” or “brite” adipose cells. They reside within white adipose tissue (WAT), but can acquire characteristics of brown adipocytes under different environmental or pharmacological stimuli (5, 6).

It is known that brown adipose tissue (BAT), which is maintained in adult humans (7), uses glucose and FAs as fuel (8), and its activity is reduced in obese and diabetic individuals (1). For this reason, understanding the mechanisms involved in the control of BAT activity could lead to improving the treatment of these patients. BAT is a highly vascularized and innervated tissue (9). Classical activation of thermogenesis occurs via noradrenaline (NA) released from the sympathetic nervous system, which binds to β3 adrenergic receptors (Adrb3) present in the brown adipocyte. Receptor activation increases intracellular cAMP, which induces lipolysis, leading to the release of FFAs that, in turn, activate UCP-1. In fact, the surgical denervation of interscapular BAT reduced the activation of brown adipocytes after cold exposure (10).

Tyrosine hydroxylase (TH) catalyzes the first and rate-limiting step in the synthesis of catecholamines. A null mutation in the mouse Th gene causes a depletion of catecholamines and lethality at prenatal stages because of cardiovascular failure (11–13). Additionally, this mutation has an impact on pancreatic β-cell development (14). Recently, it was reported that haploinsufficiency of Th increases mouse half-life by protecting against age-associated hypertension (15). In contrast, patients with pheochromocytomas that produce and secrete large amounts of catecholamines (NA or adrenaline) present body weight loss, among other abnormalities (16). The importance of the sympathetic stimulation in BAT for cold adaptation has also been shown because adrenal medullar TH is involved in the response to stress induced by cold (17). More recently, a role of TH on the antiinflammatory responses triggered by cold exposure has been reported (18).

BAT is also a secretory tissue that releases different molecules (batokines) with autocrine and paracrine roles (19). One of these batokines is the fibroblast growth factor 21 (FGF21), which was initially identified as a hepatokine and is considered, rather than a mitogenic factor, an atypical GFG due to its role in endocrine function over carbohydrates and FA metabolism (20). Fgf21-null mice have reduced hepatic FA oxidation and a concomitant increase in liver fat accumulation (21). More recently, it has been demonstrated that FGF21 is highly produced and released by BAT after cold exposure (19). FGF21 also impacts “browning” of WAT, and, as a consequence, it can improve lipid metabolic disorders and insulin resistance (22).

In this study, we examined how Th-haploinsufficiency in mice affects the response to the stress induced by cold exposure in BAT. Our data show that, despite reduced catecholamine levels, Th-haploinsufficiency does not impair cold adaptation in mice. We also demonstrate that this unaltered adaptive response is associated with increased FGF21 and increased lipolytic capacity in BAT. This evidence suggests a new mechanism for cold adaptation in a context of low adrenergic signaling.

**MATERIALS AND METHODS**

**Animal procedures**

All procedures involving animals were approved by the Ethics Committee of Consejo Superior de Investigaciones Científicas and Comunidad de Madrid in accordance with the European Union guidelines. The C57BL6/J Th heterozygote mouse strain was kindly provided by R. D. Palmer (University of Washington, Seattle, WA) (12). All the studies were performed using littersmates. We used three groups named adult mice (2–3 months), embryos at embryonic day (e) 18.5, and aged mice (15 months). The animals were maintained on 12 h:12 h light-dark cycles and at 22°C and allowed free access to standard rodent chow and water ad libitum. The adult mice were randomly divided into two experimental groups: One group was maintained at thermoneutral conditions (29°C), and the other one was subjected to cold challenge (4°C) for 6 h. Prior to the cold exposure, the two groups of mice were housed at 29°C for at least 1 week. Body weight and rectal temperature were measured in all mice before and after cold exposure.

**Preparation of tissue extracts and Western blot**

BAT samples were homogenized at 4°C in lysis buffer (in mmol/l: 50 Tris HCl, 2 EGTA, 10 EDTA, 2 orthovanadate, 50 NaF, and 1 sodium pyrophosphate) containing 1% (w/v) Triton X-100 and a mini EDTA-free protease inhibitor tablet. Samples were homogenized in lysis buffer using The Brinkmann Pt 10/35 Polytron. Tissue lysates were centrifuged at 20,000 g for 30 min at 4°C, and the supernatants were collected and stored at −80°C until further analysis. Total protein extracts (15–30 μg) were loaded into Any kD Criterion TGX Precast Gels (catalog no. 567-1124, Bio-Rad, CA) and transferred onto PVDF membranes (catalog no. 170-4157, Bio-Rad) for 14 min at 25 V using a Trans-Blot Turbo Transfer System (Bio-Rad). Membranes were activated with 100% methanol for 2 min and blocked for 1 h at room temperature with 5% (w/v) BSA or nonfat milk in TBS-Tween (Santa Cruz, 73614) Abs as loading controls. Additionally, we used Ponceau Red as a loading control (Sigma). Abs were detected with the corresponding HRP-labeled secondary Abs (Pierce, Rockford, IL) and visualized with the Super Signal West Pico chemiluminescent substrate (Pierce). Different experiments were quantified by scanning densitometry using ImageJ software.

**RNA isolation and quantitative real-time PCR**

Total RNA from BAT, inguinal WAT (iWAT), or liver was extracted using TRIzol reagent, and reverse transcription was...
performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA) using random primers and Superscript III enzyme according to the manufacturer’s instructions. Quantitative real-time PCR was performed in a 7900 HT-Fast real-time PCR (Life Technologies) with SYBR or Taqman Universal PCR Master Mix. The following probes and primers are listed in Table 1. Results are expressed using the 2−ΔΔCt (cycle threshold) quantification method. Results were normalized according to expression levels of Tbp or Rhph0 RNA.

**Immunohistochemistry**

For immunohistochemical analysis, a piece of BAT was fixed overnight at 4°C in 4% (w/v) parafomaldehyde, then rinsed with PBS and dehydrated through an ethanol gradient before embedding in paraffin for microtome sectioning. Paraffin sections (7–8 µm) were dewaxed in Histo-Clear-II (CONDA, Madrid, Spain) and rehydrated in paraffin for microtome sectioning. Paraffin sections (7–8 µm) were dewaxed in Histo-Clear-II (CONDA, Madrid, Spain) and rehydrated through a descending series of ethanol dilutions. Anti-Agr1 (Santa Cruz, catalog no. 73614) as a loading control. The amount of glycerol released by the cells to the culture medium after the different treatments was analyzed using a commercial kit (F628, Sigma).

**Analysis of FFAs and triacylglycerol levels**

Plasma glycerol levels were quantified using a commercial kit (catalog no. F6428, Sigma) and plasma nonesterified (“free” or unsaturated) FAs (FFAs) were quantified using commercial kits (catalog nos. 919898, 91696, and 91096, WAKo Chemicals, Richmond, VA), according to the manufacturer’s instructions. Samples were normalized by protein concentration.

**Lipidomic analysis**

Total lipids from BAT were extracted according to Folch et al. (24). Lipid classes were separated by TLC. For neutral lipid separation, a solvent mixture consisting of n-hexane/diethyl ether/acetonic acid (70:30:1, v/v/v) was used as the mobile phase (25). The various neutral lipid classes cholesteryl esters (CEs), triacylglycerol

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**Table 1.** Primer sequences and Taqman probes used in real-time PCR

| Gene   | Forward | Reverse | Taqman Probe     |
|--------|---------|---------|-----------------|
| Atp2   | 5′-CGGTTGCTATTCCTGCATCAA-3′ | 5′-TTGCTTCATCGAAGCCTGTT-3′ | Mn02901891_g1  |
| Prkaz  | 5′-AGGCCGCCATCTCTGCTGCT-3′ | 5′-ACTGCTATCGAAGACACAA-3′ | Mn00456425_m1  |
| Rhph0(364B) | 5′-ACTGGTTCATGACGACCAGGAG-3′ | 5′-TGGGACCTCTGACGTCTCAGT-3′ | Mn00475988_m1  |
| Adipq  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Agl    | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| β klotho | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Bmp8b  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| CebpA  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Cidea  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Csnb  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Cptia  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Dna  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Fgf21  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Fgf1  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Nos2  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Pparg  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Pparg1a | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Prdm16 | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Tbp   | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |
| Ucp1  | Mn00475988_m1  | Mn00475988_m1  | Mn00475988_m1  |

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In vitro experiments

The immortalized brown adipocyte cell line was generated from mouse interscapular BAT and fully differentiated to brown adipocytes following the protocol previously described (23). Cells (day 7 of differentiation) were incubated with or without 1 µg/ml recombinant FG21 (catalog no. RD272108100-B, BioVendor) for 18 h. Then, cells were treated with 0.1 µM NA for a further 4 h. After washing with PBS, cells were lysed in lysis buffer (10 mM Tris-HCl, 5 mM EDTA, 50 mM NaCl, 1% Triton X-100, 30 mM sodium pyrophosphate, 5 mM fluoride, 1 mM phenylmethylsulfonfyl fluoride, and 10 µg/ml protease inhibitors, pH 7.4–7.6). After centrifugation at 12,000 g for 7 min at 4°C, the total protein in supernatants was quantified with the BCA Protein Assay Kit. FG21 plasma levels were quantified by ELISA (catalog no. RD21108200R, BioVendor) following the manufacturer’s instructions.
(TAG), diacylglycerol (DAG), and FFAs were scraped and extracted from the silica with 1 ml of chloroform/methanol (1:2, v/v) followed by 1 ml of chloroform/methanol (2:1, v/v). The CE, TAG, and DAG fractions were treated with 500 µl of 0.5 M KOH in methanol for 60 min at 37°C to obtain FA methyl esters. To neutralize, 500 µl of 0.5 M HCl was added. The FFA fraction was treated with 450 µl of MeOH in 0.5% HCl for 20 min at 45°C. Neutralization of the FA methyl esters was conducted with one vol of 0.14 M KOH and 500 µl of deionized water was added. Extraction of the FA methyl esters was carried out with 2 ml of n-hexane, and 1 µl was subjected to GC/MS analysis as previously described (26, 27), using an Agilent DB23 column (60 m length × 0.25 mm internal diameter × 0.15 µm film thickness). Data analysis was carried out with the Agilent G1701EA MSD Productivity Chemstation software, revision E.02.00.

Measurement of skin temperature surrounding BAT
The skin temperature surrounding BAT was recorded with an infrared camera (B335:Compact-Infrared-Thermal-Imaging-Camera; FLIR; West Malling, Kent, UK) and analyzed with a specific software package (FLIR-Tools-Software; FLIR), as previously shown (28, 29). Pictures were taken at both thermoneutral conditions or immediately after cold exposure (6 h).

Indirect calorimetry

Th+/+ and Th+/− mice at 2–3 months of age were individually housed and maintained at thermoneutrality (28–29°C) under a 12:12 h light-dark cycle in a Phenomaster system (TSE Systems, Bad Homburg, Germany). Food and water were provided ad libitum in the appropriated devices. Mice were acclimated to the chambers for at least 24 h and then monitored for an additional 48 h period. After that, mice were placed into the cold room (4°C) for 6 h and then immediately moved to their original cages of the TSE system to continue the measurement for additional 24 h.

Statistical analysis
Statistical analyses were performed using a Student’s t-test, a nonparametric test (Mann-Whitney U test), or two-way ANOVA with Bonferroni’s post hoc test. All statistical analyses were performed with GraphPad Prism software version 5 (GraphPad Software, Inc., San Diego, CA). Statistical significance was set at *P < 0.05, **P < 0.01, and ***P < 0.001, Th+/− versus Th+/+ groups; and a P < 0.05, b P < 0.01, and c P < 0.001, 4°C versus 29°C groups.

RESULTS
Characterization of BAT in Th+/− and Th+/− mice at thermoneutrality
We first characterized adult (3 month old) Th+/− and Th+/− mice at thermoneutral conditions (29°C). As shown in Fig. 1A, no differences between the two genotypes of mice were found in body weight and rectal temperature. Likewise, temperature of skin surrounding BAT, energy expenditure, and respiratory exchange ratio were similar in the two genotypes (Fig. 1B and supplemental Fig. S1). As expected, Western blot analysis revealed a 50% decrease of TH protein content in the BAT of Th+/− mice compared with levels of their Th+/+ littermates (Fig. 1C). The analysis of paraffin sections showed reduced TH-positive immunostaining.

Fig. 1. Characteristics of Th+/− mice in thermoneutrality. Body weight and rectal temperature (A), skin temperature surrounding BAT, and energy expenditure (B) were measured in Th+/− and Th+/− mice housed 1 week at 29°C. C: Western blot analysis and quantification of lysates from BAT collected from Th+/− and Th+/− adult mice showing TH levels using GAPDH as a loading control. D: Representative immunohistochemistry of TH at paraffin sections. Nuclei were stained with DAPI (blue) and TH+ fibers were detected by immunostaining (cyan). E: Representative sections of BAT from Th+/− and Th+/− mice stained with H&E and quantification of the percentage of white area versus total area. F: Quantitative RT-PCR of thermogenic gene expression in BAT. The graph represents the relative gene expression compared with Th+/+ mice. The data were normalized by Tbp RNA expression. Data are mean ± SEM. n = 5–10/group in A–C, n = 5–7/group in E, and n = 4–10/group in F. Mann-Whitney U test was used. *P < 0.05; **P < 0.01 (between Th+/− and Th+/+ mice); ###P < 0.001 (between each genotypes in dark cycle versus light cycle). Scale bars: 75 µm.
in the BAT of $Th^{+/−}$ mice (Fig. 1D), although no major differences were observed in the amount of innervating fibers, as shown by Western blot and immunohistochemistry for β-III-tubulin (supplemental Fig. S2A, B). H&E staining revealed similar BAT histology in $Th^{+/−}$ and $Th^{+/+}$ mice (Fig. 1E), although a slight increase in white area (Fig. 1E) and decreased cell density (supplemental Fig. S2C) was found in $Th^{+/−}$ mice. We next analyzed the expression of some key genes for BAT identity. Whereas the expression of thermogenic-related genes such as $Ucp1$, $Ppargc1a$ (which encodes PGC1α), and $Cidea$ was similar between the two genotypes, the levels of $Prdm16$, a relevant gene in brown fat adipogenesis, were significantly higher in the $Th^{+/−}$ mice than in their WT littermates (Fig. 1F). Further characterization of BAT-related gene expression showed no differences between the two genotypes of mice at thermoneutral conditions (supplemental Fig. S2D).

**$Th^{+/−}$ mice responded normally to cold exposure**

Next, we addressed a possible impact of reduced TH expression in cold adaptation in young mice at 3 months of age. After 6 h of cold exposure (4°C), the BAT of $Th^{+/−}$ mice maintained lower TH protein levels than their WT littermates (supplemental Fig. S3A). Concordantly, dopamine and NA content, determined by ELISA, were diminished in BAT of $Th^{+/−}$ mice after 6 h of cold exposure (Fig. 2A). Similar to thermoneutrality, H&E-stained sections revealed more white area in $Th^{+/−}$ compared with $Th^{+/+}$ mice after cold exposure (supplemental Fig. S3B). Surprisingly, body weight (Fig. 2B), rectal temperature, and temperature of skin surrounding BAT (Fig. 2C, D), all indicators of cold adaptation, were similar in both genotypes of mice. Moreover, energy expenditure was elevated in $Th^{+/−}$ mice immediately after cold exposure and also during the following night period (Fig. 2E). Notably, despite the impaired catecholamine production, the expression of $Ucp1$ and $Ppargc1a$ was similarly increased in $Th^{+/−}$ and $Th^{+/+}$ mice upon cold challenge (Fig. 2F, G). It has been previously reported that cold exposure does not modify RNA expression levels of $Prdm16$ and $Cidea$ (30). In agreement with these results, no differences were found in either $Th^{+/−}$ or $Th^{+/+}$ mice (Fig. 2H, I), although $Prdm16$ levels, which were elevated at 29°C in the $Th^{+/−}$ mice, decreased to levels of the $Th^{+/+}$ mice after cold exposure (Fig. 2H). Body weight, rectal temperature, and the expression of $Ucp1$ and $Ppargc1a$ remained similar in both genotypes after more prolonged (16 h) cold exposure (supplemental Fig. S4). These results indicate that reduction of catecholamine levels does not alter the BAT acute response to cold. Notably, this effect was unrelated to compensatory changes in adrenergic signaling, because no differences between both genotypes were found in $Adrb3$ RNA at basal conditions. Indeed, $Adrb3$ RNA levels decreased similarly in both groups of mice after cold challenge (supplemental Fig. S5A), as reported (31–33).

Besides UCP-1, BAT thermogenesis relies on deiodinase 2 (DiO2) enzymatic activity (34). The conversion of T4 into
Increased FGF21 expression in BAT of TH heterozygous mice

T3 by Dio2 amplifies the cAMP response after adrenergic stimulation, thereby enhancing lipolysis and mitochondrial thermogenesis (35). In this context, we analyzed Dio2 RNA levels and found similar basal levels at thermoneutrality in both genotypes, which increased to the same extent (about 100-fold) after cold exposure (supplemental Fig. S5B). Similarly, additional BAT-related gene expression analysis indicated no differences between the two genotypes after 6 h of cold exposure (supplemental Fig. S5C).

**Th** haploinsufficiency does not impact browning of iWAT

Ptdm16 has been described to be essential in activating the thermogenic phenotype in inguinal subcutaneous depots (36). During cold acclimation, iWAT has been also reported to play a role in thermogenesis (37). The analysis of RNA levels of *Ucp1, Ppargc1a, Cidea, and Ptdm16* in iWAT at 3 months old mice showed a similar expression pattern to that found in BAT at 29°C in the two genotypes (Fig. 3A; compare with Fig. 1F), a significant increase being detected exclusively in *Ptdm16* in **Th**- mice (Fig. 3A). Moreover, upon cold exposure, the iWAT of **Th** heterozygous animals responded similarly to that of the controls, displaying comparable RNA levels of *Ucp1, Ppargc1a, Ptdm16, and Cidea* genes (Fig. 3B–E), as well as levels of *Adrb3* and *Fgf21* (supplemental Fig. S6A, B).

**Th** haploinsufficiency increases FGF21 in BAT

After finding unaltered the key players in the thermogenic response in BAT and iWAT in **Th**+/− mice, we investigated possible factors that could compensate the catecholamine deficit. We hypothesized that unbalanced catecholamine levels may represent a stress with an impact on the expression of the batokine *Fgf21*, as occurred in the liver under lipotoxic conditions (38). As shown in Fig. 4A, significantly higher *Fgf21* RNA levels were found in BAT of **Th**−/− animals at 3 months of age at thermoneutral conditions. As occurred with other thermogenic genes, cold exposure increased BAT *Fgf21* expression to a similar extent in both genotypes of mice (Fig. 4A). Furthermore, plasma FGF21 levels were increased about 5-fold in **Th**−/− mice at thermoneutrality (Fig. 4B) in parallel to an elevated expression of *Fgf21* in the liver (Fig. 4C), the main source of circulating FGF21 (39). These results showed that, at thermoneutrality, **Th**−/− mice displayed an increase in both local BAT production and circulating levels of FGF21, suggesting a close relationship between the mild phenotype of **Th** haploinsufficiency and the elevated *Fgf21* expression. Conversely, upon cold exposure plasma FGF21 and hepatic *Fgf21* RNA levels did not change in WT mice as described (40, 41), whereas both parameters decreased in **Th**−/− mice up to the levels of the WT. In BAT, FGF21 signals through heterocomplexes of the receptors klotho (Klb) and FGFR1 (42). The analysis of the expression of both coreceptors at thermoneutrality and after cold exposure showed no differences in their RNA levels (Fig. 4D, E).

We next checked the timing of the onset of *Fgf21* up-regulation. The analysis of BAT in e18.5 embryos showed
higher levels of Fgf21 RNA in the Th+/− embryos than in their WT littermates (Fig. 4F). Similarly, Prdm16 RNA was increased at e18.5 in the Th+/− BAT, as described in adult mice. However, we did not observe differences in the RNA levels of the thermogenic-related genes Ucp1 and Ppargc1a. Of note, a trend toward an increase in embryonic plasma and liver FGF21 content was detected in Th+/− embryos (supplemental Fig. S7A, B). In addition, the BAT of Th+/− old animals (15 months of age) showed a nearly significant increase in Fgf21 RNA levels (supplemental Fig. S7C), reinforcing a possible role of this batokine in Th+/− mice.

**Th+/− mice show increased BAT ER stress levels at thermoneutrality**

To further explore a possible mechanism by which Fgf21 expression was elevated in the BAT of Th+/− mice, we focused on endoplasmic reticulum (ER) stress, one of the well-known triggers of FGF21 expression (39, 43). The analysis of CHOP protein levels in BAT extracts showed a significant increase of this ER stress long-term marker in Th+/− mice at 3 months in thermoneutral conditions (Fig. 5A). However, in agreement with the absence of differences between both genotypes in Fgf21 RNA levels in BAT after cold exposure, no differences in CHOP levels were found after cold challenge. Likewise, the relative phosphorylation of IRE1 and JNK, important signaling mediators in the unfolded protein response, was also increased in the BAT of Th+/− mice respect to the Th+/− control mice only at thermoneutrality (Fig. 5B, C).

**Increased lipolytic capacity and TAG availability in the BAT of Th+/− mice**

To get additional insight into the compensatory response of mice with reduced catecholamines during cold adaptation, we examined BAT lipolytic activity by the analysis of phosphorylation of the hormone-sensitive lipase (HSL) at the regulatory residue Ser-660. Figure 6A shows that although no differences were detected at thermoneutral, HSL Ser-660 phosphorylation was significantly higher in the BAT of Th+/− mice at 3 months compared with that of the Th+/+ controls after 6 h of cold exposure. This result indicated that a sustained lipolytic capacity of BAT in Th+/− mice was probably contributing to the activation of UCP-1. However, plasma glycerol and FFA levels were similar in both genotypes of mice (data not shown). To explain these results, we performed in vitro experiments using differentiated brown adipocytes previously characterized (23). As an experimental approach to mimic in vivo Th haploinsufficiency, brown adipocytes were stimulated with submaximal (0.1 μM) concentrations of NA for 4 h with or without a pretreatment with recombinant FGF21 (1 μg/ml) for 18 h. As shown in Fig. 6B, there was an increase in phospho-HSL in brown adipocytes pretreated with FGF21 prior to NA stimulation. Importantly, under these experimental conditions, a significant elevation was found in glycerol released to the culture medium (Fig. 6C) and, more importantly, in UCP-1 protein levels (Fig. 6D).
To further investigate in detail the profile of different lipidic species in the two groups of mice, we performed a mass-spectrometry-based lipidomic study of total neutral lipid classes in BAT samples from mice at thermoneutral and cold conditions (Tables 2 and 3 and Fig. 7A–D). Interestingly, at thermoneutrality, several FAs in TAG and DAG fractions were significantly elevated in Th+/−mice, suggesting that increased overall fat content, and likely lipotoxicity, which would lead to elevated ER stress in the BAT of Th+/−mice. Conversely, the levels of FFAs C16:0 (palmitic acid) and C18:0 (stearic acid) were reduced. The choleseryl ester (CE) content showed no differences between the two genotypes (Fig. 7D). After cold exposure, the Th+/−animals maintained higher levels of TAG or DAG species compared with those of their WT littermates. In addition, the total amount of FFAs was higher in Th+/−BAT (Fig. 7C) compared with levels of the Th+/+BAT with significant increases in palmitic acid (Table 3).

DISCUSSION

Catecholamines are essential neurotransmitters for the maintenance of physiological homeostasis under basal and stress conditions. In BAT, catecholamines are responsible for the activation of nonshivering thermogenesis (5, 9). Also, dopamine has been shown to directly impact mitochondrial mass and thermogenesis in brown adipocytes (44). Whereas human pathologies with elevations of circulating catecholamines, such as pheochromocytoma, concur with excessive BAT activation (16, 45, 46), much less is known about the impact of reduced catecholamine biosynthesis in BAT functionality. The present work, using a murine preclinical model of global Th haploinsufficiency, has revealed an unexpectedly normal cold adaptation under reduced catecholamine availability. The early and life-long increase in FGF21 levels of Th+/−mice represents a potential new insight on the compensatory mechanisms that may operate in Th+/−BAT to ensure its full activation upon cold challenge.

The unique role of the sympathetic-mediated NA stimulation of BAT was highlighted by Lee et al. (10) showing that ablation of TH protein in BAT by denervation abolished UCP-1 induction during cold stress. More recently, a role of adipose tissue M2 macrophages or eosinophils as an additional catecholamine source in BAT or iWAT has been postulated (47–50), although controversies have also been raised regarding the contribution of these immune cells in secreting enough catecholamines to promote BAT activation or browning of iWAT (48). In our model, we found unexpected mild consequences of the global reduction of TH expression in BAT thermogenesis since Th+/−mice adapted normally to cold stress as manifested by the maintenance of rectal temperature, body weight, temperature of the skin surrounding BAT, and Ucp1 induction. In particular, the significant increase in the expression of the thermogenic-related gene Prdm16 that was detected in BAT of Th+/−mice suggests an early relationship between this master regulator of BAT development and TH. Prdm16 has been previously described as a critical transcriptional
regulator of thermogenesis that is essential for the differentiation of brown adipocytes, as well as for the browning process in beige fat cells (9, 51). A recent study has demonstrated that PRDM16 stabilizes the response to β3-adrenergic signaling to increase thermogenic gene expression and mitochondrial biogenesis in subcutaneous WAT by interacting with the transcription factor HLX (52). In agreement with these data, the iWAT of $Th^{+/−}$ mice showed an increase in Prdm16 RNA levels, supporting the relevance of TH in the process of browning. These results were reinforced by the upregulation of Prdm16 even in the embryonic BAT of $Th^{+/−}$ mice, indicating that during embryonic development, these mice have already activated compensatory mechanisms to overcome Th haploinsufficiency; Prdm16 seems to be a relevant contributor to this phenotype. In fact, catecholamines are required to respond to stress during gestation. In this regard, it has been reported that Th-null mice die at midgestation due to cardiovascular failure (11, 12). This effect is likely a consequence of their inability to adapt to the gestational hypoxia (53). However, additional posttranslational modifications in PRDM16 such as sumoylation that primes its stabilization, as recently reported (54), cannot be excluded.

Beside changes in Prdm16 gene expression, the largest changes found in BAT of $Th^{+/−}$ mice were detected in FGF21, which was significantly elevated under thermoneutral conditions compared with the $Th^{+/+}$ controls; this effect was maintained during aging. This batokine has been previously found to be increased under various situations including starvation, ketogenic diets, and overfeeding, as well as by deficiency or excess in dietary proteins and carbohydrates, respectively (43, 55). Although FGF21 is mainly synthetized and released by the liver (56), the BAT is another source of FGF21, where it has autocrine/paracrine functions related to upregulation of UCP-1 and thermogenesis (19, 57). We also found elevated FGF21 in the plasma from $Th^{+/−}$ mice, an effect that could be explained by the increased liver expression and secretion. Altogether, these results suggest that less TH availability induces regulatory mechanisms in BAT, likely mediated by Prdm16 and/or Fgf21, which overall appear to compensate thermoregulatory changes and lead to normal adaptation to cold.

We next explored the possible molecular mechanisms that could explain the elevation of FGF21 in $Th^{+/−}$ mice. Several recent reports have demonstrated that the expression of Fgf21 in the liver is induced by ER stress (58, 59). However, Fgfr1 deficiency in WAT led to a sustained lipid droplet expansion and ER stress (42). In BAT, a direct relationship between ER stress and Fgf21 levels has been well described under cold exposure or after birth during suckling (60). In agreement with the involvement of ER stress in the $Th^{+/−}$ phenotype, elevation of several ER stress markers was detected in the BAT of $Th^{+/−}$ mice at thermoneutrality, allowing us to speculate that ER stress in BAT,
TABLE 2. FA distribution profile of TAG, DAG, FFA, or CE in BAT from Th+/− and Th+/+ mice

| Percent of Th+/− Content vs. Th+/+ (29°C), % | Statistical Significance |
|--------------------------------------------|--------------------------|
| TAG | 14:00 | 189.71 | ns |
|    | 16:0 | 175.58 | ns |
|    | 16:1n-9 | 151.91 | *** |
|    | 16:1n-7 | 201.60 | ** |
|    | 18:0 | 141.10 | ns |
|    | 18:1n-9 | 195.98 | *** |
|    | 18:1n-7 | 22.24 | ns |
|    | 18:2n-6 | 164.42 | ns |
|    | 18:3n-3 | 155.56 | ns |
|    | 20:0 | 187.69 | ns |
|    | 20:1n-9 | 230.35 | ns |
|    | 20:1n-7 | 70.53 | ns |
|    | 20:2n-6 | 319.92 | ns |
|    | 20:3n-6 | 281.95 | ns |
|    | 20:4n-6 | 155.93 | ns |
|    | 22:0 | 191.58 | ns |
|    | 22:1n-9 | 284.10 | ns |
| CE | 16:0 | 99.99 | ns |
|    | 16:1n-9 | 87.56 | ns |
|    | 18:0 | 105.60 | ns |
|    | 18:1n-9 | 194.13 | ns |
| DAG | 14:00 | 97.02 | ns |
|    | 16:0 | 144.27 | *** |
|    | 16:1n-9 | 110.07 | ns |
|    | 16:1n-7 | 156.77 | ns |
|    | 18:0 | 132.05 | ns |
|    | 18:1n-9 | 137.05 | * |
|    | 18:2n-6 | 122.32 | ns |
| FFA | 14:0 | 44.79 | ns |
|    | 16:0 | 56.52 | *** |
|    | 16:1n-9 | 48.05 | ns |
|    | 16:1n-7 | 49.12 | ns |
|    | 18:0 | 64.87 | *** |
|    | 18:1 | 73.91 | ns |
|    | 18:2 | 78.66 | ns |
|    | 20:0 | 36.71 | ns |
|    | 20:1n-9 | 66.85 | ns |
|    | 20:4n-6 | 57.76 | ns |
|    | 22:0 | 4.86 | ns |

Percentage of FAs in BAT of Th+/− versus Th+/+ mice at 29°C. Data are mean ± SEM. n = 10 or 11/group. Two-way ANOVA was used. ns, not significant. *P < 0.05; **P < 0.01; ***P < 0.001.

probably due to reduced Th expression, may upregulate Fgf21.

Catecholamines are the inducers of lipolysis in adipose tissues. Zeng et al. (61) described that sympathetic denervation (or glucocorticoid administration) increased lipid accumulation in adipocytes due to defective lipolysis. In addition, lipolysis requires the activation of lipases such as adipose triglyceride lipase and HSL, as well as their stable association with the lipid droplets (62, 63). The analysis of phosphorylation of HSL at Ser-660, which is a direct indicator of its enzymatic activity, revealed elevations in the BAT of Th+/− mice after cold exposure, regardless of lower catecholamine levels. This response contrasted with decreased Ser-660 HSL phosphorylation in cold-exposed Th+/+ mice, indicating that at least 6 h after cold exposure, the time at which the experiments were performed, the lipolytic activity due to HSL-mediated phosphorylation might be more sustained in the BAT of Th+/− mice, which would explain their maintained thermogenic capacity within a decreased catecholamine milieu. Interestingly, the stimulation of differentiated brown adipocytes with a submaximal dose of NA in the presence of FGF21 increased lipolysis and UCP-1 protein levels, reinforcing the in vivo data and indicating that the relationship between FGF21 and HSL might be important in this setting.

To find a possible explanation for such an effect, we analyzed TAG, DAG, and FFA content in BAT from the two genotypes of mice. Again, substantial differences were found between genotypes. Decreased Th expression was accompanied by higher DAG and TAG content in BAT under basal conditions, and, importantly, TAG, DAG, and FFA content was increased after cold exposure compared with the levels of the Th+/+ mice. This suggests an increased lipolysis rate, in accordance with the higher HSL phosphorylation. Interestingly, the differences observed in FFA levels

TABLE 3. FA distribution profile of TAG, DAG, FFA, or CE in BAT from Th+/− and Th+/+ mice

| Percent of Th+/− Content vs. Th+/+ (4°C), % | Statistical Significance |
|--------------------------------------------|--------------------------|
| TAG | 14:00 | 181.11 | ns |
|    | 16:0 | 173.28 | *** |
|    | 16:1n-9 | 140.46 | ns |
|    | 16:1n-7 | 159.13 | ns |
|    | 18:0 | 188.45 | ns |
|    | 18:1n-9 | 175.79 | *** |
|    | 18:1n-7 | 154.75 | ns |
|    | 18:2n-6 | 151.93 | *** |
|    | 18:3n-3 | 136.76 | ns |
|    | 20:0 | 135.04 | ns |
|    | 20:1n-9 | 156.04 | ns |
|    | 20:1n-7 | 153.91 | ns |
|    | 20:3n-6 | 509.70 | ns |
|    | 20:4n-6 | 163.72 | ns |
|    | 22:0 | 71.27 | ns |
|    | 22:1n-9 | 618.31 | ns |
| CE | 16:0 | 115.01 | ns |
|    | 16:1n-9 | 78.46 | ns |
|    | 18:0 | 103.95 | ns |
|    | 18:1n-9 | 100.63 | ns |
| DAG | 14:0 | 105.54 | ns |
|    | 16:0 | 149.88 | *** |
|    | 16:1n-9 | 45.13 | ns |
|    | 16:1n-7 | 143.62 | ns |
|    | 18:0 | 142.08 | ** |
|    | 18:1n-9 | 141.47 | *** |
|    | 18:2n-6 | 112.74 | ns |
|    | 20:0 | 110.29 | ns |
| FFA | 14:0 | 121.51 | ns |
|    | 16:0 | 129.40 | *** |
|    | 16:1n-9 | 142.10 | ns |
|    | 16:1n-7 | 148.56 | ns |
|    | 18:0 | 121.47 | ns |
|    | 18:1 | 166.77 | ns |
|    | 18:2 | 98.75 | ns |
|    | 20:0 | 73.85 | ns |
|    | 20:1n-9 | 99.28 | ns |
|    | 20:4n-6 | 59.88 | ns |
|    | 22:0 | 63.89 | ns |

Percentage of FAs in BAT of Th+/− versus Th+/+ mice after 6 h exposure to cold at 4°C. Data are mean ± SEM. n = 10 or 11 per group. Two-way ANOVA was used. ns, not significant. *P < 0.05; **P < 0.01; ***P < 0.001.
upon cold exposure (i.e., significant increase in BAT from Th+/− mice was observed only for the saturated palmitic acid) suggest that BAT may adapt to temperature changes by modifying the ratio saturated versus unsaturated/polyunsaturated FFAs within neutral lipids via selective lipolysis. Thus, the loosening of acyl chain packing within TAG and DAG may provide BAT with a mechanism to finely adjust to temperature changes, as needed. These results suggest a more rapid replenishment and remodeling of fat stores in the lipid droplets in the BAT of Th+/− mice. In addition, the increased levels of highly lipotoxic species, such as DAG, might explain the existence of lipotoxicity and, subsequently, ER stress in Th haploinsufficiency. These complex mechanisms are likely to be necessary to respond to the stress of cold exposure in conditions of decreased Th expression because, as stated above, Th+/− mice adapted to cold with a similar pattern of thermogenic-related gene expression, body weight, and rectal and skin surrounding BAT temperature as the Th+/+ mice. This adaptation may also be facilitated by elevated lipolytic substrates DAG and TAG under basal conditions, thereby yielding more availability of FFAs that ultimately are the fuels for UCP-1 activation and thermogenesis. This evidence indicates a new mechanism for cold adaptation in a context of low adrenergic signaling, that may be therapeutically relevant. However, further research is needed to unravel whether additional intermediate factors are involved.

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