INTRODUCTION

Fibroblast growth factor-21 (FGF21) is a pleiotropic protein involved in glucose, lipid metabolism and energy homeostasis, with main tissues of expression being the liver and adipose tissue. Brown adipose tissue (BAT) is responsible for cold-induced thermogenesis in rodents. The role of FGF21 in BAT biology has not been investigated. In the present study, wild-type C57BL/6J mice as well as a brown adipocyte cell line were used to explore the potential role of cold exposure and β3-adrenergic stimulation in the expression of FGF21 in BAT. Our results demonstrate that short-term exposure to cold, as well as β3-adrenergic stimulation, causes a significant induction of FGF21 mRNA levels in BAT, without a concomitant increase in FGF21 plasma levels. This finding opens new routes for the potential use of pharmaceuticals that could induce FGF21 and, hence, activate BAT thermogenesis.

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Brown Adipose Tissue Responds to Cold and Adrenergic Stimulation by Induction of FGF21

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the liver, but also overexpresses FGF21 after cold exposure or selective β3-adrenergic stimulation. This FGF21 might act as an autocrine factor.

**MATERIALS AND METHODS**

**Mice**

Mice were bred and housed in the animal facility of the University of Patras Medical School at 22°C with *ad libitum* access to standard laboratory chow diet. We used male age-matched (24 weeks) C57BL/6J wild-type mice (The Jackson Laboratory, Bar Harbor, ME, USA). For the cold experiments, mice were individually housed and fasted for 12 h, and during the last 4 h of fasting, they were exposed to either control (22°C) or low temperature (4°C). At the end of the cold exposure, blood was collected and interscapular BAT, epididymal WAT and liver were harvested in RNA later solution. Similarly, the selective β3-adrenergic receptor agonist CL316243 (Sigma, Germany; 2 mg/kg body weight) was given by intraperitoneal injection 4 h before the end of the experiment.

All animal procedures were approved by the institutional review board of the University of Patras Medical School and were in accordance with EC (European Commission) Directive 86/609/EEC.

**Measurements of Hormones and Metabolites**

Plasma was collected by using heparin as an anticoagulant and was centrifuged at 2,000 g for 20 min at 4°C. Plasma measurements were conducted following the manufacturer’s instruction for each kit. Enzyme-linked immunosorbent assay (ELISA) kits were used for plasma leptin (ALPCO, Salem, NH, USA) and FGF21 (R&D, Minneapolis, MN, USA). Cholesterol and triglycerides were measured by using an Olympus AU640 analyzer (Hamburg, Germany).

**Quantification of Gene Expression Levels**

Liver, BAT and WAT were submerged immediately after collection in RNA later solution (Ambion, Foster City, CA, USA). Total RNA was isolated by using Trizol reagent (Invitrogen) and further purified by using the RNasy mini kit (Qiagen, Hilden, Germany). A DNAse (Turbo-DNAse; Ambion) digestion step was included to prevent genomic DNA contamination. cDNA was synthesized by using the Superscript first-strand synthesis system (Invitrogen) and real-time (RT)-polymerase chain reactions (PCRs) were performed in triplicate on a Step One Plus instrument (Applied Biosystems, Foster City, CA) using Taqman Gene Expression assays on demand (Applied Biosystems): FGF21, Mm00840165_g1; PGC-1α, Mm00447183_m1; PPARα, Mm00440939_m1; GAPDH (glyceraldehyde-3-phosphate dehydrogenase), 4352339E. Relative mRNA levels were calculated by the comparative threshold cycle method using GAPDH as the housekeeping gene.

**Cell Culture and Treatments**

SV40T-immortalized brown adipocytes from the C57BL/6J strain of mice were provided by Prof. Johannes Klein (Lübeck, Germany) (25). Preadipocytes were grown to confluence in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies, Paisley, Strathclyde, UK) supplemented with 20% fetal bovine serum, 4.5 g/L glucose, 20 nmol/L insulin, 1 nmol/L triiodothyronine (“differentiation medium”) and penicillin/streptomycin.

**Table 1.** Plasma metabolic parameters of C57BL/6J mice maintained at 22°C or after exposure to 4°C for 4 h.

|                | 22°C     | 4°C     |
|----------------|----------|---------|
| Triglycerides (mg/dL) | 92 ± 8   | 74 ± 6b |
| Cholesterol (mg/dL)   | 94 ± 6   | 96 ± 6  |
| Leptin (pg/mL)        | 5200 ± 78| 3296 ± 70b|
| FGF21 (pg/mL)         | 3620 ± 451.1| 2755 ± 173.4|

Data are means ± SEM; n = 10.

bP < 0.05.
bP < 0.001.
Adipocyte differentiation was induced by complementing the medium further with 250 μmol/L indomethacin, 500 μmol/L isobutylmethylxanthine and 2 μg/mL dexamethasone for 24 h when confluence was reached. After this induction period, cells were changed back to differentiation medium. Cell culture was continued for 5 more days before cells were starved for 24 h with serum-free medium prior to carrying out the experiments. Maximally differentiated cells were treated, when indicated, with 50 μmol/L of the β3-adrenergic receptor agonist CL316243 for 6 h or with 10 μmol/L of the PPARα antagonist GW6471 (Sigma, Germany) for 16 h before harvesting the cells.

Statistical Analyses
Experiments were performed three times by using at least triplicate samples per group. Data were expressed as the mean ± SEM. Student t test or one-way analysis of variance (ANOVA) followed by Tukey test was performed by using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). P < 0.05 was considered significant.

RESULTS
Cold Exposure Induces FGF21 Gene Expression in BAT
Exposure of wild-type C57BL/6J mice to 4°C for 4 h caused a significant fold increase in FGF21 mRNA levels in BAT over the baseline (25.6 ± 1.3, P < 0.01, Figure 1A). It is known that after cold exposure, PGC-1α mRNA levels of BAT are induced as part of the cold-induced thermogenesis program. In the same experiment, PGC-1α mRNA levels indeed increased by 15-fold, as expected (20). In contrast, cold did not change FGF21 mRNA levels in the liver or the WAT tissues, where FGF21 is known to be mainly expressed (see Figure 1A).

Bearing in mind that the induction of FGF21 with fasting is PPARα dependent (10), PPARα mRNA levels were tested in BAT. No difference however was found between control and cold-exposed animals (Figure 1B).

Plasma Chemistries after Exposure to Cold
Taking into account that FGF21 might be secreted and act as a hormone, plasma FGF21 levels were measured after exposure of mice to cold for 4 h. No differences were found between the two states. By contrast, plasma leptin and triglyceride levels dropped in accordance with other studies (Table 1) (26,27).

β3-Adrenergic Receptor Stimulation Induces FGF21 Gene Expression in BAT But Not in the Liver
Because it is known that cold activates BAT via the sympathetic nervous system, a selective β3-agonist (CL316243) was tested for its ability to reproduce the above findings (28). The intraperitoneal administration of CL316243 led to a significant fold increase in FGF21 mRNA levels in BAT over the baseline (19 ± 0.8, P < 0.01, Figure 2A). As a control,
PGC-1α mRNA levels were measured and found to be induced by both cold and CL316243, as expected (Figure 2B). No differences in the expression of PPARα were found (Figure 2C).

**Treatment of Differentiated Mouse Brown Adipocytes with the β3-Agonist Increases FGF21 mRNA Levels**

To discriminate between cell autonomous or nonautonomous effect of β3-adrenergic stimulation on FGF21 expression, a brown fat cell line able to differentiate into mature brown adipocytes and respond to the β3-agonist was used (25). Differentiated brown adipocytes were treated with CL316243 (50 μmol/L) for 6 h. The levels of FGF21 mRNA were increased more than five-fold (5.1 ± 0.8, P < 0.001, Figure 3). No change in FGF21 mRNA was observed in undifferentiated brown preadipocytes (data not shown), since they do not express β3-adrenergic receptors (18). Treatment of the adipocytes with the PPARα antagonist GW6471 did not affect the response of these cells to the β3 agonist.

**DISCUSSION**

In the present study, we have shown that short-term exposure of mice to 4°C induced FGF21 mRNA levels in brown fat. This induction is restricted to BAT, whereas no change is observed in liver and WAT. The baseline expression level of FGF21 in BAT is lower than that in WAT, but after cold exposure, it is induced well above that in the latter. At the same time, plasma FGF21 was not found to increase accordingly by this short-term exposure to cold.

BAT is heavily innervated by sympathetic nerves and is responsible for thermogenesis during cold exposure. β3-adrenergic receptor agonists cause an increase in energy expenditure that is comparable to that induced by cold in both rodents and humans (19). In our experiments, stimulation of either mice or a brown adipocyte cell line with the β3 agonist CL316243 had the same affect on FGF21 expression as exposure to cold. This points to a novel mechanism of action of β3 agonists in brown fat through induction of FGF21 and suggests that some of the favorable effects of β3 agonists might be mediated via induction of FGF21 (11–13, 19). PGC-1α, a known target of β3-adrenergic receptor stimulation in brown fat (19), was also induced in our experimental system. It is noteworthy that FGF21 induces PGC-1α and regulates carbohydrate and fatty acid metabolism during the adaptive starvation response in liver (29). It remains to be seen if this applies to BAT as well.

Leptin levels fall after cold exposure, and this might be a signal for initiating a broad program of adaptation to starvation (30). FGF21 is considered a starvation factor as well (31). In this vein, the reduction of leptin levels observed after exposure to cold could be a permissive factor for the observed induction of FGF21 in BAT, although this does not explain the selectivity of the induction only in BAT.

In liver, FGF21 was shown to be induced after fasting in a PPARα-dependent manner. In our in vivo or in vitro experiments, no differences were found in the expression of PPARα that could account for the aforementioned increase of FGF21. Nevertheless, activation of PPARα without any change in its expression levels could lead to elevation of FGF21. This result is not supported by our findings, since the PPARα antagonist GW6471 did not change the response of brown adipocytes to β3 agonist regarding FGF21 induction.

The fact that FGF21 plasma levels did not change could be attributed to the small contribution of brown fat to the overall production of FGF21. In this case, it is conceivable that FGF21 could be induced in BAT after cold exposure and act in an autocrine fashion. A general model for this kind of action of FGF21 is favored by a recent review (31). Alternatively, the duration of cold exposure might have not been sufficient enough to reveal any differences. Direct action of the sympathetic nervous system on BAT and induction of FGF21 remains as the most plausible explanation. Hepatic FGF21 expression is induced at birth via PPARα in response to milk intake and contributes to thermogenic activation of neonatal brown fat (23). It can be envisioned that medications that augment the expression of FGF21 in brown fat could activate BAT thermogenesis and be used in the treatment of obesity. Experiments are under way in our laboratory to delineate the mechanisms of FGF21 induction by cold/β-adrenergic stimulation, as well as the significance of this induction in the thermogenic program of the brown fat adipocyte. We note that during finalizing this report, a paper addressing the same issue was published online, corroborating our main finding of FGF21 induction by cold in BAT (32).

**DISCLOSURE**

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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