Cell Metabolism
Short Article

Irisin and FGF21 Are Cold-Induced Endocrine Activators of Brown Fat Function in Humans

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SUMMARY

Rediscovery of cold-activated brown adipose tissue (BAT) in humans has boosted research interest in identifying BAT activators for metabolic benefits. Of particular interest are cytokines capable of fat browning. Irisin, derived from FNDC5, is an exercise-induced myokine that drives brown-fat-like thermogenesis in murine white fat. Here we explored whether cold exposure is an afferent signal for irisin secretion in humans and compared it with FGF21, a brown adipokine in rodents. Cold exposure increased circulating irisin and FGF21. We found an induction of irisin secretion proportional to shivering intensity, in magnitude similar to exercise-stimulated secretion. FNDC5 and/or FGF21 treatment upregulated human adipocyte brown fat gene/protein expression and thermogenesis in a depot-specific manner. These results suggest exercise-induced irisin secretion could have evolved from shivering-related muscle contraction, serving to augment brown fat thermogenesis in concert with FGF21. Irisin-mediated muscle-adipose crosstalk may represent a thermogenic, cold-activated endocrine axis that is exploitable in obesity therapeutics development.

INTRODUCTION

Cold-induced thermogenesis (CIT) is the increase in heat production in response to acute ambient temperature reduction. It comprises nonshivering thermogenesis (NST) and shivering thermogenesis (ST). In rodents, the chief tissue mediating NST is brown adipose tissue (BAT), which releases heat through the action of uncoupling protein 1 (UCP1) (Cannon and Nedergaard, 2004). Heat demand not met by NST recruits ST, thereby generating heat from muscle contractions. Long-term cold exposure reduces shivering, conceivably a result of NST enhancement from cold acclimatization (Davis, 1961). In humans, the rediscovery of cold-activated BAT suggests a possible regulatory role of BAT in NST (Cypess et al., 2009; Saito et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009). However, the physiologic cues orchestrating NST and ST recruitment are unclear.

While adequate shelter and clothing in modern society have minimized the hazards of cold temperatures, the obesity epidemic has reignited interest into exploring whether harnessing BAT may benefit weight control (Yoneshiro et al., 2013). Activated BAT may contribute up to 20% of CIT following mild cold exposure (Chen et al., 2013), representing a proportion of total energy expenditure (EE) sufficient to impact long-term energy balance. Identification of BAT endocrine activators may open new directions in obesity therapeutics development (Lee et al., 2013b).

Irisin is an exercise-induced myokine that is secreted into the circulation following proteolytic cleavage from its cellular form, fibronectin-type III domain-containing 5 (FNDC5) (Boström et al., 2012). It reverses diet-induced obesity and diabetes by stimulating thermogenesis in rodents through increasing brown adipocyte-like cell abundance (brite [Petrovic et al., 2010]/beige [Wu et al., 2012] adipocytes) within white fat. As it appears paradoxical that exercise should increase secretion of a thermogenic hormone, it has been hypothesized that the mechanism evolved from shivering-related muscle contraction to augment NST through BAT expansion (Boström et al., 2012).

In this study, we tested this hypothesis by investigating the impact of cold exposure in healthy adults on irisin secretion and compared its excursion with the sympatho-thyroid-adrenal axes, principal regulators of CIT (Celi et al., 2010), as well as fibroblast growth factor 21 (FGF21), a recently identified brown adipokine that predicts NST response in humans (Lee et al., 2013a, 2013c). Finally, we examined in vitro the bioenergetic profiles of FNDC5- and FGF21-treated human adipocytes to determine their thermogenic significance.

RESULTS AND DISCUSSION

Irisin Detection in Human Serum

Circulating irisin, cleaved from FNDC5, is heavily glycosylated, and multiple bands are visible on serum immunoblot against anti-FNDC5 antibody (Boström et al., 2012). Because of the recent controversy over the circulating form of irisin (Erickson,

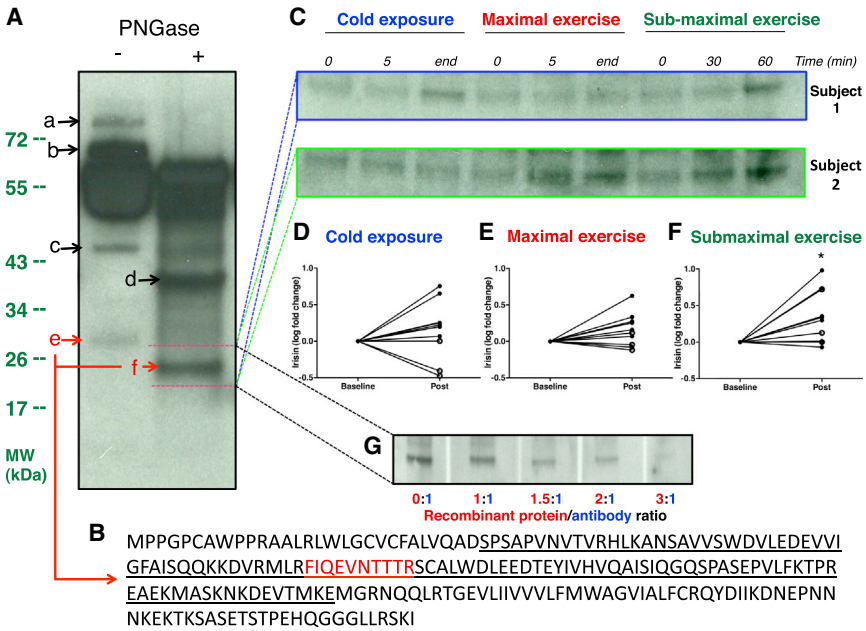

Figure 1. Validation of Immunoblot-Detected Irisin by Mass Spectrometry

(A) Immunoblot of paired serum samples following albumin/immunoglobulin depletion against anti-FNDC5 antibody revealed multiple distinct bands (a–f). PNGase treatment reduced size of band e (~32 kDa) to band f (~24 kDa).

(B) Shown is the amino acid sequence of full-length FNDC5 with the secreted irisin segment underlined. Mass spectrometry analysis of all bands (a–f) identified a specific peptide (in red), unique to irisin, only in band e and band f, with molecular weights matching those of glycosylated and deglycosylated irisin, respectively.

(C) Shown are representative immunoblots of serum irisin for fold change quantification from two subjects during cold exposure, maximal exercise, and submaximal exercise. Subject 1 shivered during cold exposure, while subject 2 did not. Accordingly, deglycosylated irisin band (~24 kDa) was stronger at the end of cold exposure only in subject 1. In contrast, irisin band was stronger after submaximal exercise in both subjects. Full-sized blots are shown in Figure S1.

(D–F) Graphical representation of serum irisin fold changes during cold exposure, maximal, and submaximal exercise tests, respectively, of all ten subjects. "Post" indicates the average band intensity of irisin extracted from the mid and final blood samples of each clinical test. Similar results were obtained when analysis was conducted comparing irisin band intensity between baseline and final sample alone. Irisin level rose significantly following sub-maximal exercise (F) and trended higher ($p = 0.07$) after maximal exercise (E). Irisin levels increased only in the seven subjects who shivered (closed circles, D), but not those who did not (open circles, D).

(G) Neutralization of anti-FNDC5 antibody by FNDC5 recombinant protein. FNDC5 antibody mixture in increasing ratio resulted in quenching of western signal in a dose-dependent manner by excess FNDC5 recombinant protein. *$p < 0.05$. Data are presented as mean ± SD.

2013), we first determined the identity of FNDC5-immunoreactive bands detectable in human serum by mass spectrometry (MS).

Consistent with previous reports, immunoblot of albumin/immunoglobulin-depleted serum revealed multiple bands reactive to anti-FNDC5 antibody. Deglycosylation reduced the size of a 32 kDa band to 24 kDa, corresponding to reported molecular weights (MWs) of glycosylated and deglycosylated irisin, respectively (Figure 1A) (Boström et al., 2012; Schumacher et al., 2013). MS analysis identified a unique peptide, mapped to the known sequence of irisin, only within the 32 kDa and 24 kDa bands (Figure 1B). These results thus validated immunoblot identification of circulating irisin in humans. To further ascertain specificity of antibody used, we also demonstrated successful quenching of irisin signal by excess recombinant protein (Figure 1G).

Exercise Increases Serum Irisin Levels in Humans

To understand the interrelationships between exercise, cold exposure, and irisin, we compared irisin secretion in ten healthy adults (four females, 27 ± 5 years old, body mass index [BMI] 22 ± 2 kg/m^2, body fat (BF) 24% ± 9%) following graded, stepwise cold exposure (27°C–12°C, see Figure S1 available online) with two forms of standard exercise tests: exercise on cycloergometer to maximal capacity (VO_{2max}) and submaximal exercise test at 40% VO_{2max} for 1 hr.

Serum irisin levels trended higher ($p = 0.07$) after maximal exercise (Figures 1C and 1E). After 60 min of submaximal exercise, irisin levels rose by 3.1- ± 2.8-fold ($p < 0.05$) (Figures 1C and 1F). These results thus replicated the known stimulatory effect of exercise on irisin secretion (Boström et al., 2012; Huh et al., 2012). The greater irisin increase during submaximal exercise compared to maximal exercise suggests endurance exercise maybe a more potent stimulus of irisin secretion, consistent with the finding of higher FNDC5 expression in oxidative versus glycolytic muscle fibers (Wrann et al., 2013).

Shivering Is an Afferent Signal of Irisin Secretion

We next determined the impact of cold temperature on irisin changes. Upon cold exposure, skin temperature decreased in all subjects ($p < 0.0001$) while core temperature was preserved (Table 1; Figure 2A). Arm-to-hand, skin-to-core, and supraclavicular-to-chest temperature gradients increased by 13% ± 18% ($p = 0.01$), 45% ± 15% ($p < 0.0001$), and 4% ± 3% ($p < 0.0001$), signifying vasoconstrictive, insulative, and thermogenic responses, respectively. EE rose by 48% ± 37% ($p < 0.01$), representing CIT response (Figure 2A). Seven subjects reported shivering, and shivering activity, quantified by surface electromyography (EMG), increased during cold exposure ($p < 0.01$) (Table 1). The increase in EMG activity was 88% ± 80% in individuals who shivered and 13% ± 9%, among those who did not ($p < 0.05$). Our cooling protocol thus elicited the full spectrum of CIT response, allowing the interrogation of irisin-CIT interrelationships. Irisin changes correlated the strongest with shivering among all CIT components (Table S1). Circulating irisin rose in the seven subjects who shivered (Figures 1C and 1D), and changes in irisin levels correlated positively with shivering activity ($r = 0.91$, $p < 0.001$) (Figure 2B). To ensure accuracy of our quantification, we also demonstrated concordant irisin changes measured with a commercially available irisin enzyme-linked immunosorbant assay (Figures 2E and

Table 1. Physiologic Changes during Cold Exposure and Hormonal Profile during Cold Exposure and Exercise Tests in Ten Subjects

Physiologic Variables						
Body Temperature (°C)	27°C	18°C	16°C	14°C	12°C	Trend p Value
Core	36.9 ± 0.2	36.9 ± 0.2	36.9 ± 0.2	36.9 ± 0.2	36.9 ± 0.2	0.65
Skin	32.1 ± 0.5	31.7 ± 0.7	31.1 ± 0.8	30.7 ± 0.9	30.0 ± 1.0	<0.0001
Gradient, core-to-skin	4.5 ± 0.8	4.8 ± 0.9	5.4 ± 1.1	5.9 ± 1.2	6.5 ± 1.2	<0.0001
Gradient, arm-to-hand	−6.7 ± 1.7	−7.1 ± 1.8	−7.4 ± 1.8	−7.2 ± 1.8	−7.3 ± 1.5	0.01
Gradient, supraclavicular-to-chest	1.04 ± 0.03	1.05 ± 0.03	1.06 ± 0.04	1.07 ± 0.05	1.09 ± 0.06	<0.0001
Resting EE (kcal/day)	1,488 ± 196	1,731 ± 337	1,926 ± 673	2,100 ± 785	2,235 ± 738	0.007
Respiratory quotient	0.78 ± 0.03	0.85 ± 0.07	0.79 ± 0.07	0.77 ± 0.07	0.79 ± 0.08	0.001
Surface electromyography ($\times 10^{-6}$ RMS)	2.5 ± 5.7	3.1 ± 2.1	4.0 ± 4.0	4.7 ± 3.6	4.9 ± 3.3	0.044

Hormonal Variables						
	Cold Exposure		Maximal Exercise		Submaximal Exercise	
	Baseline	End	Baseline	End	Baseline	End
Epinephrine (pg/ml)	38 ± 25	110 ± 73[a]	237 ± 418	300 ± 316	180 ± 406	311 ± 558 [a]
Norepinephrine (pg/ml)	658 ± 306	1,101 ± 449[a]	727 ± 264	1,568 ± 541[a]	714 ± 173	974 ± 209[a]
Glucose (mg/dL)	84 ± 3	86 ± 2	91 ± 6	117 ± 22[a]	87 ± 4	90 ± 10
Insulin (U/L)	4.2 ± 1.1	5.3 ± 1.1	6.8 ± 3.6	21.4 ± 22.6[a]	6.6 ± 3.5	7.9 ± 5.2
HOMA IR	1.0 ± 0.8	1.3 ± 0.8	1.5 ± 0.9	6.5 ± 6.7[a]	1.4 ± 0.8	1.8 ± 1.2
NEFA (μEq/L)	0.55 ± 0.07	0.57 ± 0.07	0.49 ± 0.19	0.45 ± 0.19	0.47 ± 0.23	0.65 ± 0.22[a]
TSH (mIU/L)	1.9 ± 0.3	2.1 ± 0.3	2.1 ± 0.7	2.5 ± 0.9[a]	1.9 ± 0.6	1.9 ± 0.7
Free T4 (ng/dL)	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Free T3 (pg/dL)	320 ± 17	346 ± 13	299 ± 43	296 ± 47	312 ± 45	311 ± 41
Total T3 (ng/dL)	134 ± 22	139 ± 17	115 ± 29	114 ± 16	121 ± 23	117 ± 17
ACTH (pg/ml)	17 ± 9	19 ± 6	27 ± 16	156 ± 133[a]	19 ± 10	37 ± 31[a]
Cortisol (μg/dL)	12 ± 3	15 ± 5	16 ± 5	18 ± 6	14 ± 4	16 ± 5
FGF21 (pg/ml)	129 ± 76	98 ± 90	135 ± 80	102 ± 97	119 ± 79	97 ± 85

Data are presented as mean ± SD.
[a]$p < 0.05$ compared to baseline.

S1D–S1F), which has been validated against immunoblotting (Wen et al., 2013).

As exercise is the only known activator of irisin secretion, we compared cold- and exercise-induced irisin changes. The increment in irisin was similar during the two tests (Figure 2C). However, the increase in EE was significantly greater during maximal exercise, compared to cold exposure (9.8- ± 2.4-fold versus 1.5- ± 0.4-fold, $p < 0.0001$). The dissociation between irisin and EE responses suggests additional cold-specific signals, unrelated to muscle contraction, potentiating irisin secretion during shivering. As NST, mediated by BAT (Cannon and Nedergaard, 2004) and muscle (Bal et al., 2012), is activated with ST, we explored whether FGF21, a brown adipokine in rodents (Chartoumpekis et al., 2011; Hondares et al., 2011) and humans (Lee et al., 2013c), relates to shivering-induced irisin secretion.

Distinct Involvement of Irisin and FGF21 during Shivering and Nonshivering Thermogenesis

In agreement with known diurnal reduction in circulating FGF21 levels in humans (Yu et al., 2011), FGF21 concentration trended lower during all three tests (cold exposure and exercise tests) undertaken between 8:00 and 10:00 a.m. (Table 1). Greater FGF21 reduction was associated with more intense shivering, although it did not reach significance ($p = 0.08$) (Figure 2D). We interpret a greater reduction in FGF21 levels as lesser FGF21 secretion, indicating a lower NST response (Lee et al., 2013a) and leading to ST recruitment for additional heat generation. This is corroborated by a positive correlation observed between supraclavicular skin temperature (i.e., an index of BAT activity [Lee et al., 2011]) and FGF21 changes (Table S1; Figure 2F). To further substantiate our interpretation of cold-induced FGF21 secretion, we undertook two additional experiments in separate groups of subjects to characterize (1) relationships between BAT and FGF21 and (2) temperature dependency of FGF21 diurnal rhythm.

First, to elucidate whether BAT is a significant source of cold-augmented FGF21 secretion, we profiled FGF21 excursions in five men (21 ± 2 years old; BMI, 22 ± 1 kg/m^2; BF, 21% ± 2%) stratified to BAT status during 5 hr of either mildly cold, nonshivering condition (19°C) versus thermoneutrality (24°C) (Figures 2G and 2H). FGF21 diurnal reduction was blunted at 19°C in the group as a whole by 23% ± 17% ($p < 0.05$). However, the blunting effect was markedly greater in BAT-positive, compared to BAT-negative, subjects (Figures 2K and 2L), translating to a total FGF21 output more than 6-fold higher in BAT-positive individuals. Since the subjects were of similar age and leanness and differed only by BAT status, these associative

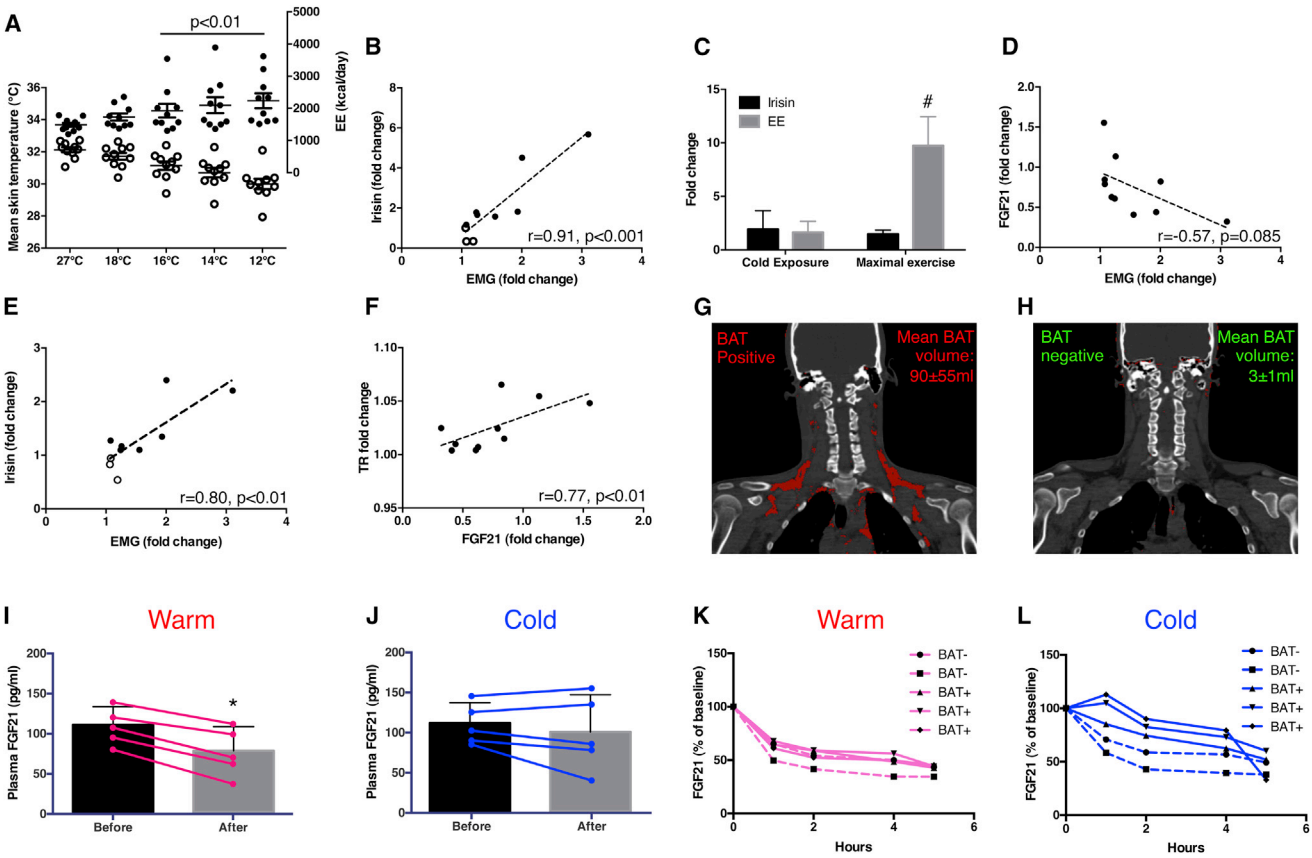

Figure 2. Relationship between Irisin, FGF21, BAT, and Temperature

Cold exposure resulted in reduction in skin temperature (open circles, A), accompanied by a rise in energy expenditure (EE) (closed circles), both reaching significance from 16°C to 12°C. Panels (B) (by immunoblot) and (E) (by ELISA) show positive associations between irisin and EMG fold changes during cold exposure. Panel (C) compares irisin and EE fold changes during cold exposure with maximal exercise test. Changes in FGF21 levels correlated negatively with shivering (D) but positively with thermogenic response (TR; difference between supraclavicular skin and chest T°C) (F). In (G) and (H), representative PET-CT images of BAT-positive (n = 3) and -negative (n = 2) individuals are shown, respectively (BAT in red). FGF21 diurnal reduction was more markedly blunted in BAT-positive (solid lines) compared to BAT-negative (dashed lines) individuals at 19°C (L) versus 24°C (K). Panels (I) and (J) compared FGF21 changes (n = 5) measured between 8 and 10 a.m. at either warm (27°C) or shivering (12°C) conditions. FGF21 reduction was significantly blunted in the cold. *p < 0.05 compared to warm condition; #p < 0.001 compared to cold exposure. Data are presented as mean ± SD.

results support BAT as a source of FGF21 during cold exposure in humans.

Second, to verify that FGF21 diurnal rhythm is indeed temperature sensitive, we measured FGF21 changes in another five men (26 ± 6 years old; BMI, 23 ± 2 kg/m^2; BF, 19% ± 3%) under the same shivering-inducing cold exposure employed in the main study but on a separate day exposed the same subjects to a warm temperature (27°), during which FGF21 was measured at matching time points. Cold exposure blunted FGF21 diurnal reduction by 28% ± 23% (p = 0.02) (Figures 2I and 2J). Pooling the results in these five subjects with the ten subjects originally studied, we observed a significant positive association between FGF21 diurnal reduction and shivering intensity (r = 0.53, p < 0.05).

Collectively, our findings indicate concerted stimulated secretion of FGF21 and irisin during NST and ST, respectively. In other words, shivering stimulates irisin secretion in a FGF21-primed milieu, through a mechanism mimicked by muscle contraction during exercise, which offers a plausible reconciliation for the paradox of why exercise, an energy-dissipating process, should stimulate the release of a thermogenic hormone.

FNDC5/FGF21 Induce Expression of Beige Gene Transcriptome in Human Neck Adipocytes

These results led us to probe the biological significance underlying enhanced irisin and FGF21 secretion during cold exposure. We hypothesize the two hormones are cold activated to boost whole-body thermogenic capacity by switching on brown-fat-like program in white fat. We thus examined in vitro the bioenergetic profiles of FNDC5- and FGF21-treated primary human adipocytes established from neck fat biopsies, a location known to be enriched with beige adipocytes.

While stimulated beige adipocytes manifest thermogenic capacity similar to that of classic brown adipocytes, they are characterized by a distinct gene signature (Cypess et al., 2013; Jespersen et al., 2013; Sharp et al., 2012; Wu et al., 2012). We therefore first determined the impact of FNDC5 and/or FGF21 treatment on classic brown and beige gene expression in human

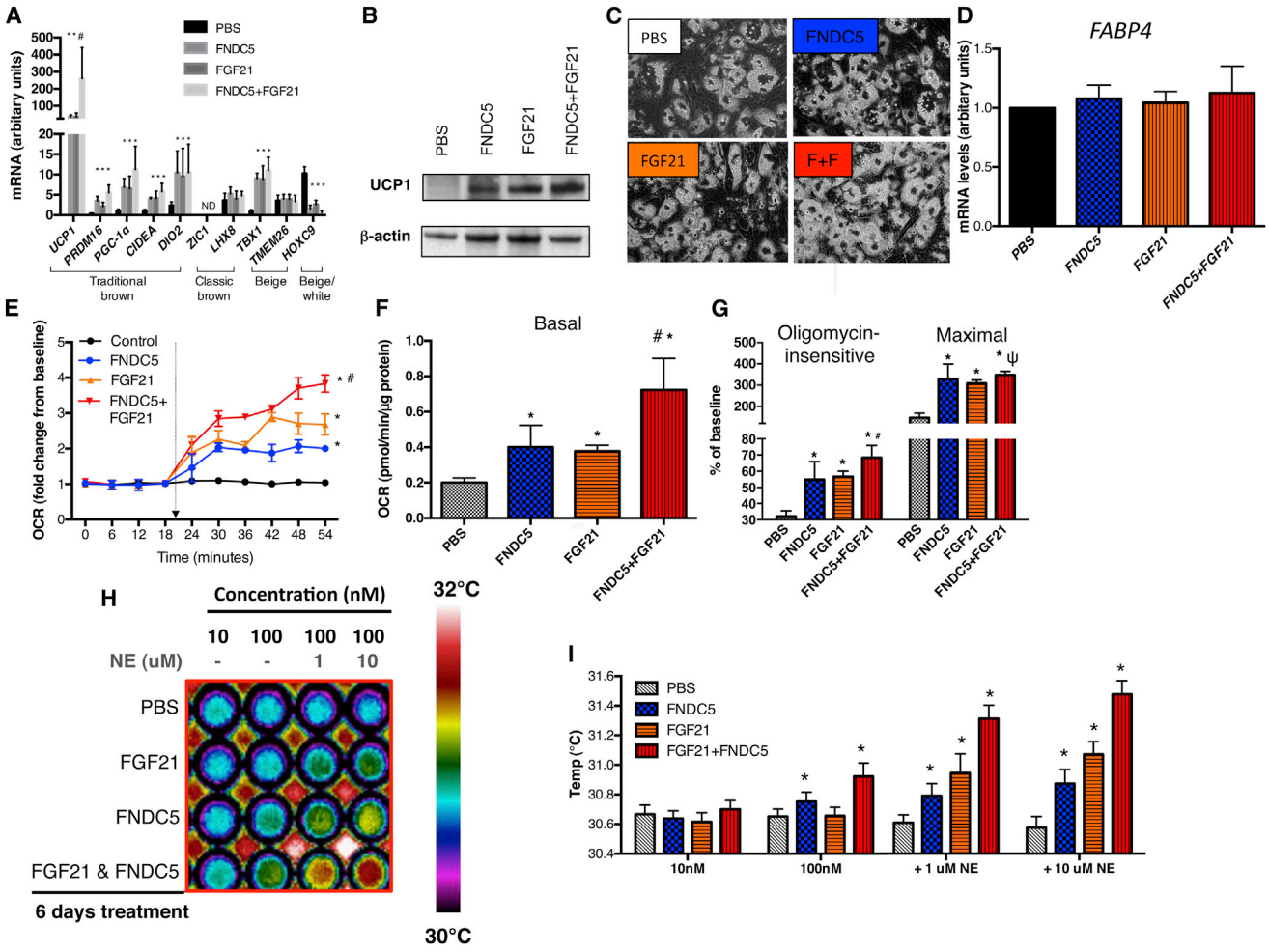

Figure 3. Effects of FNDC5 and/or FGF21 Treatment on Gene/Protein Expression and Bioenergenetics of Neck Adipocytes

(A) Effects of FGF21 and/or FNDC5 treatment on BAT/beige/white gene markers in neck adipocytes (n = 6). UCP1 protein was absent in PBS-treated adipocytes but was detected following FGF21 and/or FNDC5 treatment (B). UCP1 protein was highest in adipocytes treated with dual FGF21/FNDC5. Neck adipocytes displayed multilobulated lipid droplets (40×), similar before and after treatment (C, F+F = FNDC5+FGF21 treatment). Expression of *FABP4*, a general adipogenic gene, was not different following treatment (D). Induction of UCP1 was accompanied by upregulation of basal (F), oligomycin-insensitive, maximal uncoupled (G), and norepinephrine-induced (E) oxygen consumption, most robust in dual FGF21/FNDC5-treated adipocytes (n = 4). (H) Infrared thermographic images of adipocytes in microplates treated with PBS, FGF21, and/or FNDC5 (n = 4). The temperature scale showed color representation of temperature variation. Heat production was increased in the basal state by FNDC5 but not FGF21. Addition of norepinephrine (NE) increased heat production in increasing magnitude in FGF21-, FNDC5-, and dual FGF21/FNDC5-treated adipocytes. These results are displayed in graphical format in (I). *$p < 0.05$ compared to PBS; #$p < 0.05$ compared to FNDC5- or FGF21-treated adipocytes; $^{\psi}p < 0.05$ compared to FGF21-treated adipocytes. Data are presented as mean ± SD.

neck adipocytes. FNDC5 and/or FGF21 treatment increased general BAT and beige gene expression without altering those belonging to the classic brown fat lineage (Figure 3A). UCP1 protein, absent in untreated adipocytes, became strongly expressed following FNDC5 and/or FGF21 treatment (Figure 3B). Pre- and posttreatment adipocytes displayed similar morphology, lipid accumulation, and general adipogenic gene expression (Figures 3C and 3D), indicating thermogenic gene upregulation was not a result of more efficient differentiation.

Bioenergetic Activation of Human Neck Adipocytes by FNDC5/FGF21

We next investigated the functional impact of FNDC5 and FGF21 on adipocyte thermogenic function. We chose a treatment duration of 6 days, as guided by previous studies reporting a reduction of shivering in cold acclimatized humans after 1 week (Davis, 1961). FNDC5 and/or FGF21 enhanced adipocyte basal oxygen consumption rate (OCR) (Figure 3F), as measured using an extracelluar fluid bioanalyzer. Pharmacological interrogation of mitochondrial respiration revealed augmentation of both forms of respiratory uncoupling (oligomycin-insensitive and maximal) by FNDC5 and FGF21 treatment (Figure 3G). To mimic cold exposure in vitro, we measured norepinephrine-induced thermogenesis. While untreated adipocytes did not respond to norepinephrine, FNDC5 and FGF21 both induced a robust increase in OCR upon norepinephrine exposure (Figure 3E) and thus recapitulated the observed cold-induced hormonal response in vivo. Combined FNDC5/FGF21 treatment produced greater

responses than either hormone alone (Figure 3). Treating adipocytes with irisin instead of FNDC5 resulted in a similar extent of fat browning on gene, protein, and functional levels (Figures S2A and S2C–S2E), consistent with previous findings in murine fat cells (Wu et al., 2012), and suggests both FNDC5 and irisin in the circulation could be biologically active at adipose tissue.

FNDC5/FGF21 Increases Human Neck Adipocyte Heat Production

As the primary function of BAT is to generate heat, we next quantified heat production from adipocytes directly by infrared thermography (IRT) (Figure S2G). FNDC5 treatment enhanced adipocyte heat production dose dependently (Figures 3H and 3I), which was further augmented by norepinephrine. In contrast, FGF21 increased heat production only after norepinephrine exposure. Additive effects were again observed following combined FNDC5/FGF21 treatment.

Taken together, these in vitro experiments provide mechanistic insight into our in vivo observations. It is conceivable that shivering-stimulated irisin, in concert with FGF21, phenotypically transforms white adipocytes to BAT-like cells to expand overall thermogenic capacity. This heat-generating hormonal response may confer an evolutionary advantage in the defense against environmental hypothermic challenges by boosting the more energy-efficient NST response over shivering.

Fat Depot-Specific Effects of FNDC5/FGF21

From a clinical perspective, a 2- to 3-fold increase in adipocyte OCR following FNDC5/FGF21 treatment, if extrapolated to the whole-body EE level, could be substantial. However, the in vivo relevance is dependent on the generalizability of our findings to other fat depots. We therefore repeated FNDC5/FGF21 experiments in primary human subcutaneous and visceral adipocytes. FNDC5/FGF21 enhanced BAT-like thermogenic program in subcutaneous but not omental adipocytes, and the magnitude of thermogenic activation was less compared to those observed in neck adipocytes (Figures S3 and S2F). Beige gene expression was either low (subcutaneous adipocytes) or absent (omental adipocytes). In rodents, irisin treatment only increased UCP1 in beige fat gene-expressing adipocytes (Wu et al., 2012). Lower or absent beige gene expression in subcutaneous and omental adipocytes may account for the modest or lack of response of these adipocytes to FNDC5/FGF21.

Metabolic Significance and Clinical Implications

Although our sample size is relatively small, our results are physiologically and clinically relevant. First, they uncover an intriguing evolutionary interconnection between exercise and shivering, juxtaposing at the muscle-fat interface through cold-induced endocrine BAT activators. As irisin levels were higher in shivering subjects, we hypothesize muscle to be the main contributor to the observed irisin rise, although adipose-derived irisin cannot be excluded (Roca-Rivada et al., 2013). Second, while the sympathetic nervous system (SNS) is the best-known mediator of CIT, recent evidence points to the existence of specific cold-induced neuroendocrine signals in animals, whose actions are highly fat specific, without undesirable global SNS activation (Villarroya and Vidal-Puig, 2013). Our study provides evidence supporting similar fat browning capacity of two of these cytokines, irisin and FGF21. Third, our irisin detection validation clarifies recent concerns over specificity of irisin immunoblotting (Erickson, 2013). Although the antibody used recognizes a peptide present in FNDC5, which is theoretically lost during irisin-specific proteolytic cleavage, it is possible that shivering induces release of FNDC5 fragments that harbor the antibody-reactive peptide. While the dynamics of irisin secretion remain to be clarified in future studies, identification of the same peptide sequence shared by circulating irisin (shown in our clinical study) and recombinant FNDC5/irisin proteins used in our in vitro experiments offers renewed perspective over controversy on the relevance of irisin in human biology (Atherton and Phillips, 2013). Fourth, our finding of fat depot-specific browning by FNDC5 suggests adipocyte browning potential may impact response to FNDC5/irisin and may account for negative results utilizing solely subcutaneous adipocytes (Raschke et al., 2013). Finally, long-term maintenance of regular exercise is challenging, and natural human tendency for thermal comfort limits cold exposure in contemporary society. Irisin and FGF21 may represent endocrine mimics of these thermogenic stimuli and are therefore potential therapeutic targets to attain weight control and to improve overall metabolic profile.

EXPERIMENTAL PROCEDURES

Clinical Studies

Healthy volunteers provided written informed consent. The NIDDK-NIAMS institutional review board approved the studies (http://clinicaltrials.gov, NCT00521729 and NCT01730105). We conducted three sets of studies. (1) Main Study consisted of three experiments: cold exposure, maximal, and submaximal exercise tests; (2) PET-CT Study involved FGF21 profiling in volunteers stratified to BAT status; and (3) Temperature Study determined temperature dependence of FGF21 diurnal rhythm.

Main Study

These tests allowed comparison of hormone/substrate profiles during active muscle contractions with cold-induced shivering. Volunteers were admitted after an overnight fast (September 2012–March 2013) for each test, performed at least 3 days apart.

Cold Exposure Test

Ten volunteers wearing hospital scrubs rested in beds in a room at 24°C. Two water-infused thermoblankets (Gaymar Medi Therm) were used to adjust rapidly temperature exposure (Figure S1A). Thirty minutes of resting EE measurement was obtained by indirect calorimetry when water temperature was at 27°C, after which it was cooled to 18°C, then further lowered by 2°C every 3 min until 12°C was reached. EE measurement continued throughout this period, and the test concluded after 5 min at 12°C. Shivering intensity was measured by surface electromyography (EMG) (Trigno, DelSys Inc.).

Exercise Tests

Maximal exercise test was performed on a mechanically braked cycle ergometer (Ergomedic 839E, Monark Exercise). A stepwise incremental exercise test was performed to assess maximal aerobic capacity (VO_{2max}) using breath-by-breath analysis. For the submaximal exercise test, volunteers cycled for 1 hr at an intensity of 40% of VO_{2max}.

Laboratory Measurements

Three blood samples were obtained during each test for hormone/substrate measurements: baseline, 5 min after the start of cooling or maximal exercise, and at the conclusion of the test. For the submaximal exercise test, samples were obtained at baseline, 30 min into, and at the end of the test. Serum irisin was measured by western blotting (Boström et al., 2012) and plasma FGF21 by enzyme immunoabsorbant assays.

PET-CT Study

Five subjects were studied after an overnight fast. They were exposed to either 24 hr of mild cold (19°C) or thermoneutral temperature (24°C). Blood samples were obtained at 08:00, 09:00, 10:00, 12:00, and 13:00, corresponding to 0, 1, 2, 4, and 5 hr after exposure to testing temperature. FGF21 levels were measured by same ELISA as in Main Study. At the end of 24 hr exposure to 19°C, each subject received a 5 mCi dose of ^{18}flurodeoxyglucose (FDG) at 08:00 and underwent PET-CT scanning.

Temperature Study

Five subjects underwent the same cold exposure study as those in Main Study. Blood samples were obtained at baseline and at the end of the cold exposure study for FGF21 measurements and correlation with shivering activity. On a separate day, subjects returned and were exposed to a constant warm temperature at 27°C for the same duration as the cold exposure testing period. Blood samples were obtained at matching time points for FGF21 measurements to directly compare with levels obtained during cold exposure.

In Vitro Studies

Adipocyte Culture

Thermogenic effects of FNDC5/FGF21 were tested on primary adipocytes established from human cervical, subcutaneous, and omental fat, as previously described (Lee et al., 2013c).

Gene/Protein Expression and Thermogenesis

Standard techniques were used for RNA/protein extraction and analysis by semiquantitative real-time PCR and immunoblotting. Cellular respiration was measured by XF24-3 extracellular flux analyzer (Seahorse Bioscience). Heat production was measured by IRT (FLIR Systems), as previously described (Lee et al., 2013c).

Additional clinical/laboratory experimental details and PET-CT scanning analytical methods are available in the Supplemental Information.

Statistical Analysis

Statistical analysis was performed using SPSS 20.0 (SPSS, Inc.). Data are expressed as mean ± SD. Comparisons between results during graded cold exposure (Main Study) and FGF21 time course (PET-CT Study) were performed using repeated-measure ANOVA with Bonferroni's correction. Paired t test was used for comparison of measurements at 24°C and 19°C. Data not normally distributed were log transformed before analysis but are presented in the text nontransformed. Pearson correlation coefficients were used to examine linear relations between variables. Areas under the curve were calculated using the trapezoidal rule. An α error of 0.05 was considered the threshold for statistical significance.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, one table, and three figures and can be found with this article at http://dx.doi.org/10.1016/j.cmet.2013.12.017.

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