Photoperiodic regulation of FGF21 production in the Siberian hamster

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Running head: seasonal FGF21 cycles in hamsters

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Abstract

FGF21 is an endocrine member of the fibroblast growth factor superfamily that has been shown to play an important role in the physiological response to nutrient deprivation. Food restriction

enhances hepatic FGF21 production, which serves to engage an integrated response to energy deficit. Specifically, elevated FGF21 levels lead to reduced gluconeogenesis and increased hepatic ketogenesis. However, circulating FGF21 concentrations also paradoxically rise in states of metabolic dysfunction such as obesity. Furthermore, multiple peripheral tissues also produce FGF21 in addition to the liver, raising questions as to its endocrine and paracrine roles in the control of energy metabolism. The objectives of this study were to measure plasma FGF21 concentrations in the Siberian hamster, a rodent which undergoes a seasonal cycle of fattening and body weight gain in the long days (LD) of summer, followed by reduction of appetite and fat catabolism in the short days (SD) of winter. Groups of adult male hamsters were raised in long days, and then exposed to SD for up to 12 weeks. Chronic exposure of LD animals to SD led to a significant increase in circulating FGF21 concentrations. This elevation of circulating FGF21 was preceded by an increase in liver FGF21 protein production evident as early as 4 weeks of exposure to SD. FGF21 protein abundance was also increased significantly in interscapular brown adipose tissue, with a positive correlation between plasma levels of FGF21 and BAT protein abundance throughout the experimental period. Epididymal white adipose tissue and skeletal muscle (gastrocnemius) also produced FGF21, but levels did not change in response to a change in photoperiod. In summary, a natural programmed state of fat catabolism was associated with increased FGF21 production in the liver and BAT, consistent with the view that FGF21 has a role in adapting hamsters to the hypophagic winter state.

Introduction

Fibroblast growth factor 21 (FGF21) was first identified in the liver [Nishimura et al., 2000], and later studies demonstrated its significant potential to regulate glucose homeostasis and metabolic function [Kharitonenkov et al., 2005]. Enhanced hepatic FGF21 production occurs during the adaptive response to starvation [Badman et al., 2007], where it is proposed to function in an autocrine fashion to regulate hepatic fatty acid breakdown, oxidation and subsequently ketone body production downstream of the master regulator of the hepatic fasting response, PPARα [Badman et al., 2007; Inagaki et al., 2007]. In addition, secreted FGF21 may also function in an endocrine manner targeting the brain to regulate reproductive behavior, appetite and locomotor activity [Owen et al., 2013; Bookout et al., 2013]. Production of FGF21 has subsequently been detected in multiple systemic tissues, including pancreas, brown adipose tissue (BAT), skeletal and cardiac muscle [Hondares et al., 2011; Planavila and et.al., 2013; Johnson et al., 2009], and appears to have a variety of additional physiological functions. For example, cold-exposure increases FGF21 production in BAT [Chartoumpekis et al., 2011; Hondares et al., 2011] where it stimulates the expression of several thermogenic genes [Fisher et al., 2012]. It is also reported to induce a BAT-like phenotype in white adipose tissue [Coskun et al., 2008; Fisher et al., 2012; Adams et al., 2013], a process termed 'browning' [Bartelt and Heeren, 2014].

Given the initial identification of enhanced FGF21 production as a protective mechanism during starvation, it is somewhat surprising that other studies have demonstrated increased FGF21 production in states of positive energy balance, for example in obese humans, and in genetic and dietary models of obesity in rodents [Fisher et al., 2010; Zhang et al., 2008; Dushay et al., 2010]. Such observations suggest that FGF21 may have multiple physiological and behavioral actions in addition to its roles in the adaptation to starvation, glucose homeostasis and cold exposure (Adams and Kharitonenkov, 2012). However, a recent publication has indicated that FGF21 is partially truncated in the plasma of human volunteers (Hager et al., 2013), an effect that has previously been reported to inactivate the protein in vitro (Kharitonenkov et al., 2008), calling into question the relevance of

circulating FGF21 to pathophysiological and physiological outcomes. New insights into these functions may be obtained by studying the function of FGF21 in seasonal mammals such as Siberian hamsters that display natural adaptations synchronized by changes in photoperiod, which stimulate systemic integrated modulation of energy balance, for example catabolism of abdominal fat depots resulting in body weight loss, reduced appetite, and increased thermogenic capacity [Warner et al., 2010; Heldmaier et al., 1982]. Our recent studies in this species demonstrated that exogenous treatment with recombinant FGF21 reduced appetite, increased energy expenditure and promoted fat oxidation, thus significantly decreased body weight [Murphy et al., 2013]. These effects were more pronounced in hamsters in the summer long-day (LD) state when the hamsters maintain a high body weight than in hamsters exposed to short days (SD) that promotes the winter-adaptive state. This implies that there may be an underlying change in FGF21-sensitivity across the seasonal cycle. Moreover, we also observed a significant increase in endogenous plasma levels of FGF21 in Siberian hamsters maintained in SD [Murphy et al., 2013]. However, it is not known which tissues are responsible for this elevation in plasma FGF21, nor when during the LD to SD transition plasma levels of FGF21 begin to increase. Since FGF21 appears to play an important role in the adaptive response to starvation and cold exposure, the primary objective of the present investigation was to determine the temporal profile of FGF21 levels in the liver, white adipose tissue (WAT), brown adipose tissue (BAT), skeletal muscle and plasma during the LD to SD transition.

Materials and methods

Animal housing and experimental design

Adult male animals were obtained from a colony of Siberian hamsters (*Phodopus sungorus*) maintained at the University of Nottingham Biomedical Services Unit [Ebling, 1994]. All studies were carried out in accordance with the UK Animals (Scientific Procedures) Act of 1986 (project licence: PPL

40/3604) and approved by the University of Nottingham Ethical Review Committee. Hamsters were group housed and maintained at approximately 21^{o}C and 40% humidity, and were allowed *ad libitum* access to water and standard laboratory chow comprising of 19% protein, 45% carbohydrate, 9% fat (Teklad 2019, Harlan, UK). Animals were housed from birth in long day conditions (LD) of 16 hours light: 8 hours dark with lights off at 11:00 GMT. Groups of hamsters (n=6/group) that were aged 3-4 months at the start of the study were transferred at 4 week intervals to short days (Fig. 1, top), thus were exposed to 8 hours light: 16 hours dark (SD) with lights off maintained at 11:00 GMT. 24 hamsters were used for the main study such that after 12 weeks groups had been exposed to 0, 4, 8 and 12 weeks of SD (Fig. 1). Food intake and body weight were recorded every two weeks. After 12 weeks of SD animals were euthanized, blood samples were collected into EDTA tubes on ice by cardiac puncture under terminal anesthesia, and plasma collected after centrifugation and stored at -80°C until required for assay. Samples of liver, interscapular BAT, epididymal WAT, and skeletal muscle (gastrocnemius) were collected for tissue specific FGF21 analysis.

Hormone measurement

An ELISA kit (Millipore, MA, USA) was used to measure circulating levels of FGF21 (rat/mouse kit EZRMFGF21-26K) in the plasma samples; the detection limit was 49 pg/mL. All samples were assayed in duplicate within a single assay.

Western blotting

Protein extraction

Protein was extracted from the organic phase of the RNA extraction homogenate solution. 1.5ml of isopropanol per ml of Trizol originally used was added to each sample. Samples were mixed, and left at room temperature for 10 minutes to allow for protein precipitation. Samples were centrifuged at 12,000g for 10 minutes, 2ml of wash solution was added and samples were mixed on a daisy wheel

for 20 minutes at room temperature. Samples were centrifuged at 7,500g for 5 minutes at 4°C, then pellets were vortexed in 2ml of 100% EtOH and left to stand at room temperature for 20 minutes. Samples were then centrifuged at 7,500g for 5 minutes at 4°C. Protein pellets were then re-dissolved in 400µl, of protein re-suspension solution and stored at -80°C. Quantification of protein concentration in the supernatant from liver, white and brown adipose and muscle tissue was conducted using the Pierce Bovine Serum Albumin (BSA) Protein Assay.

Western blotting

Protein separation was carried out using SDS-PAGE, using 5-20% gradient gels and then transferred overnight onto a hydrophobic polyvinylidene difluoride (PVDF) membrane (GE Healthcare). Membranes were then incubated in blocking buffer (e.g., BSA or milk) on a shaker for 1 hour. Primary antibodies for FGF21 (Eli Lilly) and β-actin (Cell Signalling) were diluted in TBS and blocking buffer (1-5%), applied to membranes that were then incubated overnight at 4°C on a shaker. Following the incubation period, membranes were incubated with rabbit anti-mouse HRP (Amersham Biosciences, UK) secondary antibodies at ratio of 1:2000 diluted in TBS-T containing 1-2% blocking buffer, for 1hr at room temperature. Protein bands were visualised by soaking membranes with either ECL Plus for 5 minutes and exposing membranes to Amersham Hyperfilm ECL (GE Health Care). All immunoreactive proteins were visualized using ECL plus (Amersham Biosciences, UK) and quantified by densitometry using the Quantity One 1-D Analysis Software version 4.5 (Bio-Rad Laboratories, Inc., USA).

Statistical analysis

All data analysis was carried out using Prism v5.0 (GraphPad, San Diego, CA). Longitudinal home cage measures of body weight and food intake were analyzed using a two factor repeated measures model. Subsequent comparisons of group means at specific time points were made by t-tests using Bonferroni corrections as appropriate. Cross-sectional measures were analyzed using a one-factor

ANOVA, with further comparisons of mean values at different stages of SD vs the LD control group made using Dunnett's tests. In all cases $p<0.05$ was considered statistically significant.

Results

Food intake, body and organ weights

As expected, there was a progressive decrease in body weight when hamsters were transferred from LD to SD (Fig. 1, middle). Post-hoc analysis revealed that body weight was significantly decreased by up to 20% in the groups exposed to SD for 8 or 12 weeks (Fig. 2A; effect of photoperiod $F=25.84$, $p<0.0001$). Daily food intake was decreased by approximately 25% following 12 weeks SD exposure when compared to that of animals maintained in LD (Fig. 1 bottom; effect of photoperiod $F=6.60$, $p<0.01$). There was approximately a 50% decrease in mean testis weight in animals maintained in SD for 12 weeks (Fig. 2B; overall effect of photoperiod, $p=0.07$, $F=2.26$). There was a progressive decrease in fat mass as hamsters were exposed to increasing periods of SD (Fig. 2D). Epididymal white adipose tissue (WAT) was approximately decreased by 18%, 50% ($p<0.0001$) and 60% ($p<0.0001$) following 4, 8 and 12 weeks of SD exposure respectively when compared to animals maintained in LD (Fig. 2D; effect of photoperiod, $p<0.0001$). Throughout the 12-week experimental period, animals maintained in LD had a summer pelage score of 4. However, following 8 and 12 weeks of SD exposure a winter pelage had begun to develop, as indicated by a decrease in pelage score (Figure 2C; effect of photoperiod, $p<0.0001$, $F=23.14$).

Plasma FGF21

There was a significant increase in plasma FGF21 levels following exposure to SD (Fig. 2E, $p<0.05$). Plasma concentrations of FGF21 were increased by 4.5-fold ($p<0.05$) and 2.9-fold ($p<0.05$) following 8 and 12 weeks of SD respectively (with no difference between those two time points) when compared to those of animals maintained in LD.

FGF21 abundance in tissues

In order to identify which tissues may be responsible for the increased plasma levels of FGF21 when exposing Siberian hamsters to SD, we measured protein levels in discrete tissues. In the liver and BAT there was a progressive increase in FGF21 protein abundance when switching animals from LD to SD (Fig. 3A). In liver, FGF21 protein content was increased by 1.4 fold relative to LD samples following 4 weeks of SD, and continued to increase by 1.8 and 2.4 fold following 8 and 12 weeks of SD respectively when compared to that of animals maintained in LD (Fig. 3A; effect of photoperiod, $p<0.01$). Similarly, there was a significant increase in FGF21 protein abundance in BAT following 8 (2.7 fold) and 12 (2.1 fold) weeks of SD when compared to that of animals maintained in LD (Fig. 3A; effect of photoperiod, $p<0.01$). There was a strong positive correlation between plasma FGF21 levels and BAT FGF21 protein abundance throughout the 12-week experimental period ($r=-0.97$ $r^2=0.94$; $p<0.05$). There were also high levels of FGF21 detected in skeletal muscle and WAT (Fig. 3B), but no significant effects of photoperiod on FGF21 protein content expression was observed in these tissues (Fig. 3B).

Discussion

The primary objective of the present investigation was to determine the effects of short-day photoperiod on tissue specific protein abundance of FGF21, in order to identify the tissues responsible for the temporal changes in plasma FGF21 levels induced by photoperiod in a seasonal model of adiposity. The main findings were a significant increase in plasma levels of FGF21 following 8 and 12 weeks of SD, associated with increased FGF21 protein abundance in liver and BAT. Characteristic of their natural progression into SD, there was a significant decrease in body weight and epididymal white adipose tissue throughout the 12-week experimental period in hamsters transferred to SD when compared to that of animals maintained in LD. These reductions in body weight and fat mass were associated with a reduction in daily food intake following 8-weeks of SD. The increased systemic availability of FGF21 appears to be accounted for initially by increased

hepatic production of FGF21, which was evident by 4 weeks of SD exposure. Increased production of FGF21 in BAT may also contribute to the increase in plasma as FGF21 content in this tissue was significantly increased after 8 weeks exposure to SD. Two other tissue samples, the epididymal white fat pad and the gastrocnemius leg muscle also contained substantial amounts of FGF21, but we found no evidence for photoperiodic regulation of FGF21 content in these tissues, suggesting that the seasonal increase in FGF21 reflects a tissue-specific mechanism rather that an generic response associated with decreased appetite and loss of body weight.

The effects of photoperiod on body weight and food intake have been well characterised across a range on mammalian species and the underlying central mechanisms are well understood [Hanon et al., 2008; Ebling and Barrett, 2008]. The reduction in body weight and daily food intake that occurs when switching Siberian hamsters from their LD fat state to that of their SD lean state is primarily associated with a reduction in hypothalamic thyroid hormone availability [Murphy et al., 2012]. Hepatic production of FGF21 was increased after just 4 weeks of SD exposure, so it is tempting to speculate that this response is also centrally mediated. Support for this notion is provided by studies in rats indicating that hypothalamic thyroid hormone signalling pathways are capable of regulating hepatic metabolic gene expression and glucose homeostasis via sympathetic out-flow [Klieverik et al., 2009; Fliers et al., 2010]. Further evidence suggesting interplay between the thyroid hormone signalling and hepatic production of FGF21 is provided by [Adams et al., 2010] who reported that peripherally administered thyroid hormone is capable of stimulating the production of FGF21 in a PPARα dependent manner in the liver of rodents [Adams et al., 2010]. Thus, it may be that in contrast to peripherally acting thyroid hormone, in the Siberian hamster reduced hypothalamic thyroid hormone signalling is sufficient to stimulate hepatic FGF21 production. Hepatic FGF21 may function in an autocrine manner to regulate locally hepatic fatty acid metabolism, but may also function in an endocrine manner targeting other peripheral and central tissues to facilitate the SD state. In support of a central mode of action, the starvation-induced increase in circulating FGF21 is reported to function centrally to suppress reproduction in mice [Owen et al., 2013]. FGF21 has been

reported to be capable of crossing the blood brain barrier [Hsuchou et al., 2007] and ICV infusion of FGF21 in obese rats increases energy expenditure and improves insulin sensitivity [Sarruf et al., 2010]. Therefore, it will be important to determine whether enhanced FGF21 production in SD contributes to the reduction in activity of the hypothalamo-pituitary-gonadal axis in hamsters.

In addition to the increased FGF21 content in liver, there was also a significant increase in the abundance of FGF21 in BAT following 8 weeks of SD exposure, which may also contribute to the increased systemic availability of FGF21 in SD. Hamsters were maintained at a constant ambient temperature in the current study, suggesting that this is an adaptive response in preparation for anticipated cold-exposure in winter. Studies in rats reveal that BAT also responds directly to thermogenic activation via the secretion of FGF21 into the circulation, due to adrenergic activation of the cAMP dependent PKA and p38 MAPK pathway [Hondares et al., 2011]. In line with these data, noradrenergic activation of β3 receptors is reported to stimulate the expression of several genes associated with BAT thermogenesis in the Siberian hamster exposed to SD [Demas et al., 2002; Bowers et al., 2005] . Bowers et al., [2005] have reported that the SD-induced decrease in whole-body fat mass is partly due to increased sympathetic out-flow to WAT and BAT after 5 and 10 weeks of SD. Demas et al., [2002] have reported that in response to SD photoperiod there is a significant upregulation of the mRNA content of several downstream targets of FGF21 including PGC1α and UCP1. Thus, taken together it seems likely that in response to sympathetic stimulation, BAT increases the production of FGF21 in order to aid in the regulation of BAT thermogenesis during the LD to SD transition. Following prolonged exposure to SD (~ 12 weeks) hamsters will enter short daily bouts of torpor, in order to conserve energy, which are characterised by a reduced physical activity, body temperature and metabolic rate [Heldmaier et al., 1999], and are dependent on the SD-induced reduction in hypothalamic thyroid hormone availability [Murphy et al., 2012]. After these brief bouts of torpor there is a need for hamsters to rapidly increase body temperature via BAT-induced thermogenesis [Heldmaier and Buchberger, 1985; Cannon and Nedergaard, 2004]. It has recently been proposed that FGF21 produced by the liver plays a crucial role in the induction of thermogenic

activity of BAT in ground squirrels following brief periods of torpor [Nelson et al., 2013]. Thus, FGF21 may also function in BAT of the Siberian hamster in an autocrine manner to regulate the production of heat via BAT-induced thermogenesis following brief bouts of torpor.

We conclude that increased hepatic and BAT production of FGF21 are likely to underlie the increased plasma levels of FGF21 in Siberian hamsters exposed to short photoperiods. In line with previous observations, the seasonal functions of FGF21 may be both locally in liver to regulate fatty acid metabolism and in BAT to regulate thermogenesis. Secreted FGF21 could also function centrally promote short day adaptations in this species, for example the reduction in appetite.

Acknowledgements

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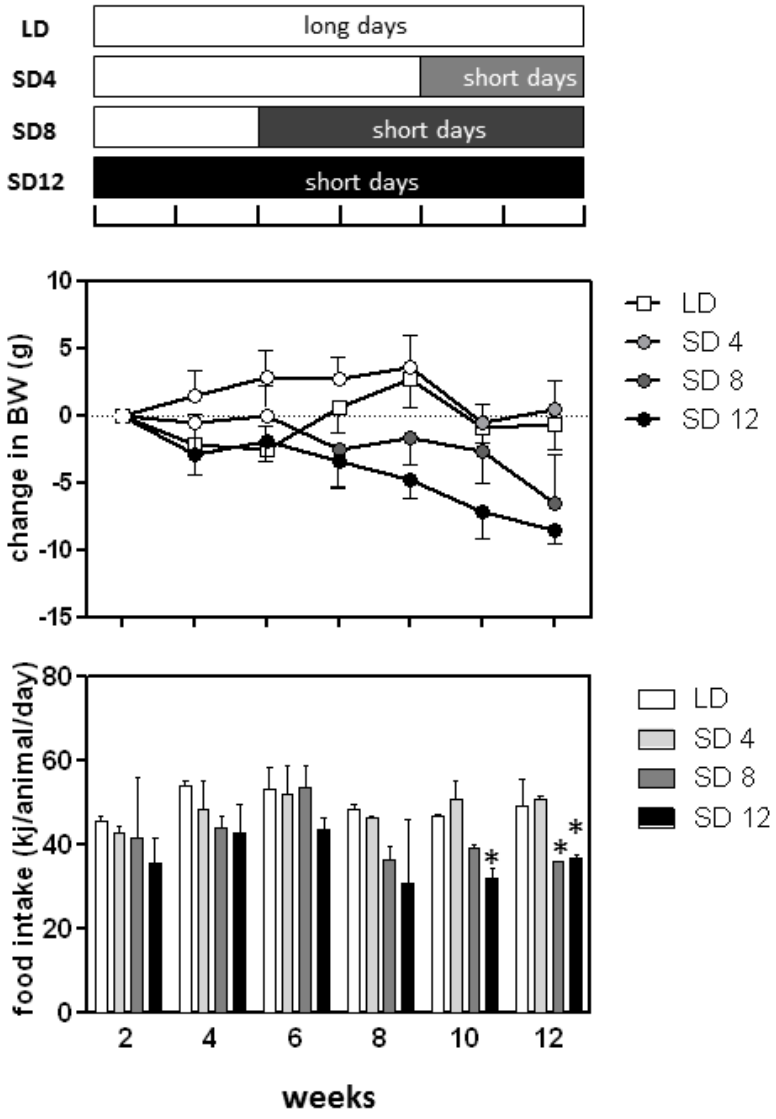

Figure 1. Top: experimental design: groups (n=6) of adult male hamsters initially maintained in long-days (LD, 16 hours light/8 hours dark) were transferred to short-days (SD, 8 hours light/16 hours dark) at 4 week intervals. Middle: change in body weight (g) and bottom: mean food intake (kj/animal/day) assessed at 2-week intervals. Values are group mean ± SEM. *p<0.05 vs LD group.

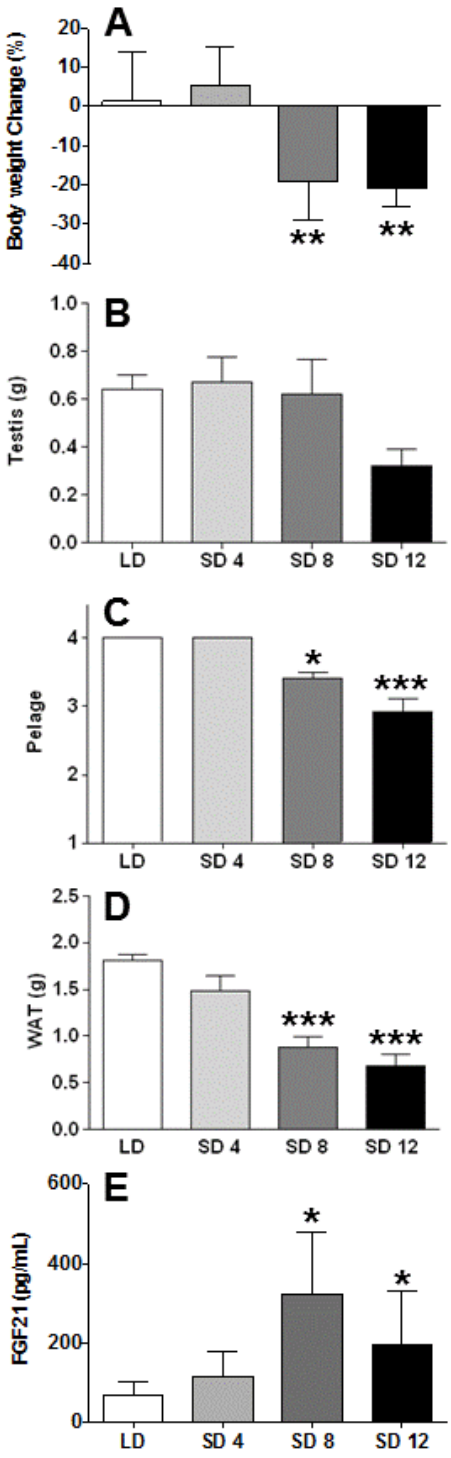

Figure 2. The effects of short photoperiod on **A**: net change in body weight, **B**: paired testis weight, **C**: pelage score (4=summer agouti, 1=winter white), **D**: epididymal white adipose tissue pad weight, and **E** plasma FGF21 concentrations. Values are group mean ± SEM, n=6 per group. *p<0.05, **p<0.01, ***P<0.001 vs LD group.

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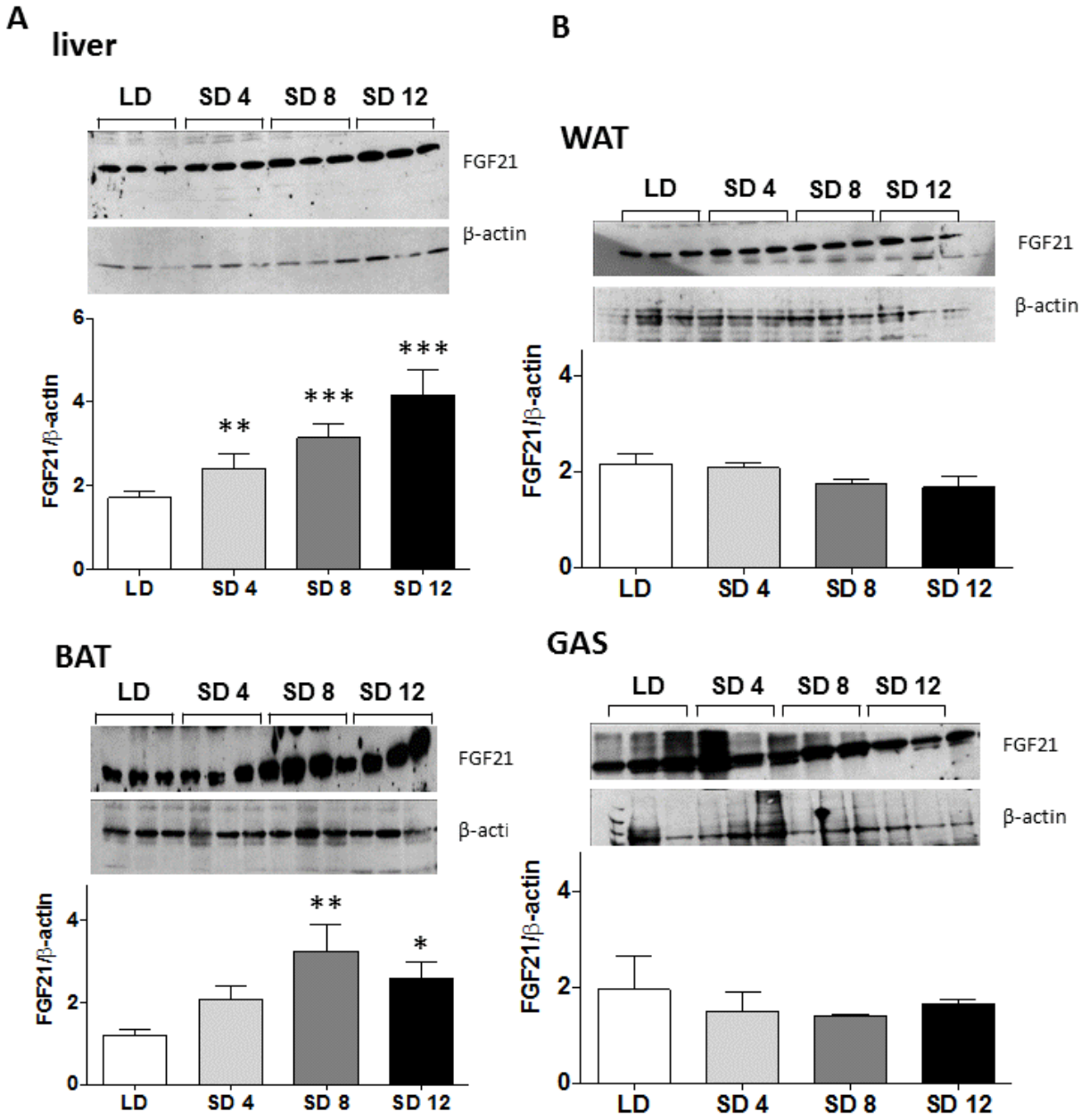

Fig. 3. The effect of photoperiod on FGF21 protein abundance in A liver (top) and brown adipose tissue (BAT, bottom) and B white adipose tissue (WAT, top) and gastrocnemius muscle (GAS, bottom) at the end of the 12-week experimental period (n = 6 in each group). Representative blots are shown above the group mean values (±SEM). *p b 0.05, **p b 0.01, ***p b 0.001 vs LD group.

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