Journal of Reproduction and Development, Vol. 66, No 2, 2020

—Original Article—

Short-term but not long-term high-fat diet induces an increase in gene expression of gonadotropic hormones and GPR120 in the male mouse pituitary gland

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Abstract. High-fat diet (HFD) is associated with the regulation of reproductive functions. This study aimed to investigate the effects of short-term HFD on the mRNA expression levels of follicle-stimulating hormone β subunit (FSHβ), luteinizing hormone β subunit (LHβ), gonadotropin-releasing hormone receptor, and long-chain fatty acid receptor, GPR120, in the matured male mouse pituitary gland. Adult male mice were fed either control chow or HFD for 1, 2, 5, 10, 30 and 150 days. *Fshb* and *Gpr120* mRNA expression levels in the pituitary glands were significantly increased during 2 to 30 days of HFD feeding. *Gnrh-r* mRNA in the 30 days HFD fed group and body weight in the 30 and 150 days HFD fed groups were higher than control. However, there were no significant differences in plasma non-esterified fatty acids or glucose levels during the 150 days of HFD feeding. These results suggest that male mice feeding a short-term HFD induces FSHβ synthesis and GPR120 expression in their pituitary gonadotropes.

Key words: Follicle-stimulating hormone (FSH), GPR120, High-fat diet (HFD), Luteinizing hormone (LH), Pituitary

(**J. Reprod. Dev. 66:** 143–148, 2020)

High-fat diet (HFD) feeding is known to affect various physiological functions. In particular, long-term HFD feeding is thought to be one of the main causal factors of obesity [1], type 2 diabetes, and hyperglycemia [2]. However, multiple studies have shown that short-term HFD feeding can also modulate physiological responses, such as the induction of hepatic insulin resistance [3], acceleration of protein degradation in muscles [4], elevation of autophagy flux in the liver [5], and activation of inflammatory, ER stress and apoptotic signals in the hippocampus [6]. Thus, it is possible that both long-term and short-term HFD feeding are involved in diverse physiological functions.

HFD feeding also affects the regulation of reproductive functions. Long-term HFD feeding is associated with abnormal reproductive functions, such as disrupting the estrus cycle in female rats [7] and suppressing the luteinizing hormone (LH) and follicle-stimulating hormone (FSH) surges in female mice [8]. Short-term HFD feeding has positive effects on reproductive functions, such as inducing early sexual maturation (including early vaginal opening) in prepubertal animals and increasing the LH pulse frequency in puberty-advanced female rats [9]. Although there is a strong association between HFD feeding and reproductive functions, the mechanisms still remain unclear. In particular, the effect of short-term HFD feeding on mature male animals is not yet well understood.

Received: November 8, 2019
Accepted: December 23, 2019
Advanced Epub: December 29, 2019

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The GPR120, a long chain fatty acid receptor, is a recognized lipid sensor expressed in various organs and tissues, including taste buds [10], muscles [11], the pancreas [12], liver [13], intestine [14], brain [15], and pituitary glands [16]. The physiological roles of GPR120 (discovered using knockout mice) include the induction of glucagon release from islets [17], the elevation of insulin resistance [18], and the regulation of adipocyte differentiation and lipogenesis [19]. Additionally, GPR120 mediates the secretion of both free fatty acid (FFA)-induced cholecystokinin and glucagon-like peptide 1 from enteroendocrine cell lines [14, 20]. It has also been suggested that GPR120 is an important inflammatory regulator in the brain [21], bone [22], and intestinal epithelial cells [23]. Thus, GPR120 plays a key role as an FFA sensor for controlling various physiological functions in numerous organs.

FFA signaling is considered a potential regulator of the hypothalamic-pituitary-gonadal (H-P-G) axis at the level of pituitary gonadotropes as the *Gpr120* mRNAs and protein expression were specifically observed in the gonadotropes of mouse anterior pituitary glands [16]. Furthermore, *Gpr120* mRNA expression is elevated by 24 h fasting [16] and suppressed by estrogen in a dose-dependent manner [24], suggesting that this receptor has a specific functionality in the mouse gonadotrope under conditions of malnutrition and may be involved in the estrogen feedback regulation of reproductive functions in the pituitary. Although previous studies have shown that HFD feeding elevates plasma FFA levels in mice [25], its effects on pituitary gonadotropes is unknown.

In this study, we investigated the effects of HFD feeding on the gonadal functions at the pituitary in mature male mice and its association with the role of GPR120 in the gonadotropes. To this end, we measured the expression levels of *Lhb*, *Fshb, Gnrh receptor* (*Gnrh-r*), and *Gpr120* mRNAs in matured male mice after 1, 2, 5,

10, 30 (as short-term) and 150 (as long-term) days of HFD feeding.

Materials and Methods

Animals

8-week-old ICR male mice were obtained from Japan SLC (Hamamatsu, Japan) and individually housed in a controlled environment (12 h light and 12 h dark; lights on at 0700 h; temperature, 24 ± 2°C). All the animals had free access to food (Labo-MR stock, Nihon Nosan Kogyo, Yokohama, Japan) and water until the feeding regimes of either an HFD with 60% fat (D12492, Research Diets) or a control diet with 10% fat (D12450B, Research Diets) were started. These feeding regimes continued for up to 1, 2, 5, 10, 30 or 150 days. Each animal's body weight was measured every day for a week before the HFD start day (day 0) and, subsequently, after 1, 2, 10, 20, 30 and 150 days. Results of body weight change in Fig. 1 showed the mean body weight of 30 and 150 days of HFD or control food animals until 30 days, and the mean body weight of 150 days were used as the results of 150 days. In the present study, we categorized less than 30 days of HFD feeding period as short-term HFD following previous studies [3–6, 26, 27]. The Committee on Animal Experiments of Kindai University approved the study. The experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Total RNA extraction and cDNA synthesis

The mice were killed by decapitation, and the pituitary glands were quickly removed and homogenized with TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA) in 1.5 ml tubes for total RNA extraction. The pituitary homogenate was treated with RNase-free DNase I to remove genomic DNA contamination, and cDNA was synthesized using a Superscript II kit with an oligo$(dT)_{12\text{-}18}$ primer (all reagents were purchased from Life Technologies, Carlsbad, CA, USA).

Real-time PCR

Lhb, *Fshb, Gnrh-r*, *and Gpr120* mRNA expression levels in the pituitary were determined by real-time PCR, using the SYBR Premix Ex *Taq* II master mix (TaKaRa Bio, Shiga, Japan) containing SYBR Green I and run on a 7500 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany). The following conditions were used: denaturation at 95°C (30 sec), amplification for 40 cycles with denaturation at 95°C (5 sec) and annealing and amplification at 60°C (34 sec). Data were analyzed using the standard curve method [28] and normalized to the L19 ribosomal protein gene (*L19*), which was used as a reference gene. The forward (F) and reverse (R) primers (Nippon EGT, Toyama, Japan) used for the mouse genes were as follows (Table 1): *Lhb* (NM_008497), *Fshb* (NM_008045), *Gnrh-r* (NM_010323), *Gpr120* (NM_181748) and *L19* (NM_009078). The expression levels of target genes were normalized with *L19* housekeeping gene expression levels. The presence of only a single peak for the dissociation curve was checked in every real-time PCR cDNA amplification sample.

Plasma NEFA and glucose assay

Blood samples were obtained from decapitated animals using heparinized 1 ml syringes, collected in 1.5 ml tubes and immediately put on ice. Plasma samples were obtained by centrifuging the blood at 15,000 rpm for 10 min and collecting the supernatants. Plasma concentrations of non-esterified fatty acids (NEFAs) and glucose were determined via enzymatic assays using the NEFA C-test Wako and glucose CII-test Wako, respectively. Detectable values of NEFA C-test and glucose CII-test were 0.05–2 mEq/l and 3.8–700 mg/dl

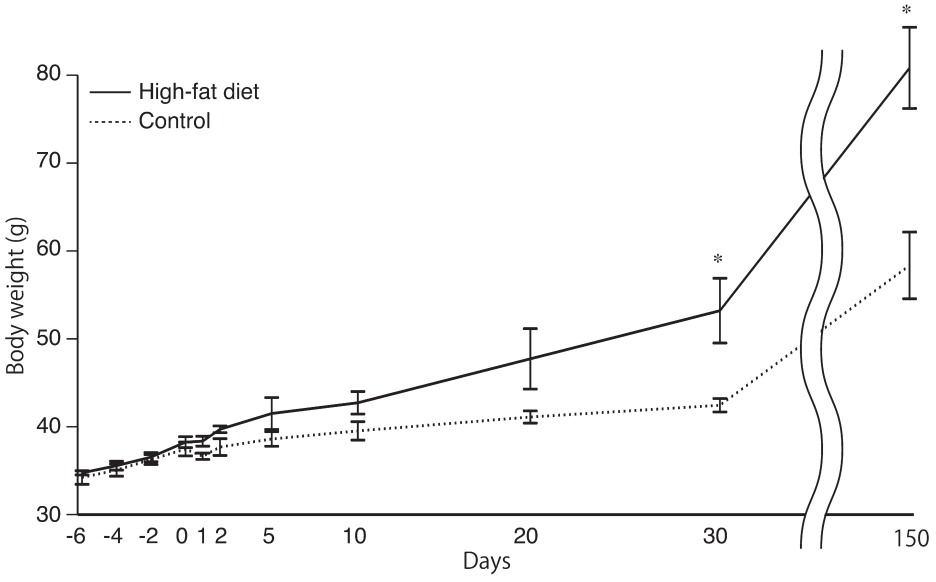

Fig. 1. Cumulative body weights of mice fed high-fat diet (HFD) (black line) or control (dotted line) diets. * $P < 0.05$ HFD *vs*. control diet on the same day. n = 4–5 per group.

Table 1. Primer used for qPCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)	Accession No.
Fshb	CTGCTACACTAGGGATCTGG	TGACATTCAGTGGCTACTGG	156	NM_008045
Lhb	CTAGCATGGTCCGAGTACTG	CCCATAGTCTCCTTTCCTGT	136	NM_008497
Gnrh-r	CAGGATGATCTACCTAGCAG	GCAGATTAGCATGATGAGGA	154	NM_010323
Gpr120	TCGCTGTTCAGGAACGAATG	CACCAGAGGCTAGTTAGCTG	130	NM_181748
L19	CCAAGAAGATTGACCGCCATA	CAGCTTGTGGATGTGCTCCAT	101	NM_009078

respectively. All test kits were obtained from the Fujifilm Wako Pure Chemical, Osaka, Japan.

Statistical analysis

All values were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using the student t-test to analyze the effect of HFD feeding. P-values < 0.05 were considered significant.

Results

Changes in body weight

The body weights of the mice after 30 and 150 days of HFD feeding were significantly higher than those of the control animals (Fig. 1). After 1 day of treatment, the body weight of the control animals was slightly lower, while that of the HFD mice stayed the same. Nevertheless, there were no significant changes in body weight in the groups from 1 week before the start of the experiment up to 20 days of treatment.

Effect of HFD feeding on plasma NEFA and glucose concentrations

There was no significant difference in plasma NEFA or glucose concentrations between the treatment group with 150 days of HFD feeding and the control group (Fig. 2).

Effect of HFD feeding on gonadotropic hormone b subunits and Gnrh-r mRNA expression in the mouse pituitary gland

8-week-old ICR male mice were fed either an HFD or a control diet for various durations (1 to 150 days). *Fshb* mRNA expression levels in the pituitary glands were significantly higher in the mice after 2 days of HFD feeding compared to that of the control animals, and this increase was maintained until 30 days of HFD feeding (Fig. 3). *Gnrh-r* mRNA expression levels in the pituitary glands were elevated after 30 days of HFD feeding compared to the control group and tended to decrease in 150 days. However, there was no significant difference in *Lhb* mRNA expression levels even after 150 days of HFD feeding.

Effect of HFD feeding on Gpr120 mRNA expression in the mouse pituitary gland

Gpr120 mRNA expression levels in the pituitary glands were significantly elevated after 2 days of HFD feeding compared to that of the control group, and this increase was maintained until after 30 days of treatment (Fig. 4). 150 days is not marked as significant.

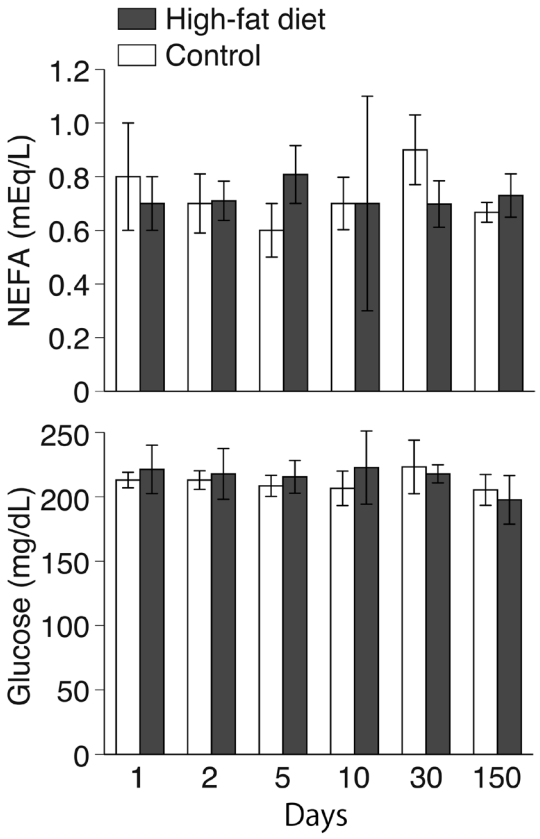

Fig. 2. Plasma concentrations of non-esterified fatty acid (NEFA) and glucose of mice fed high-fat diet (HFD) (gray columns) or control (open columns) diet for 1, 2, 5, 10, 30 and 150 days. n = 4–5 per group.

Discussion

In this study, we determined the effects of short-term HFD feeding on the expression levels of gonadotropin hormone β subunits in the anterior pituitary glands of mature mice. In previous studies, these effects were examined after a few months of HFD feeding [8, 29]. However, a short-term HFD (< 6 weeks) can also elicit various physiological responses [3, 26, 27]. We, therefore, examined the effects of HFD treatment for 30 days as short term and 150 days as the long-term control in this study. We found that just 2 days of HFD feeding induced an increase in *Fshb* mRNA expression levels in the mouse pituitary glands, although *Lhb* mRNA expression levels did not

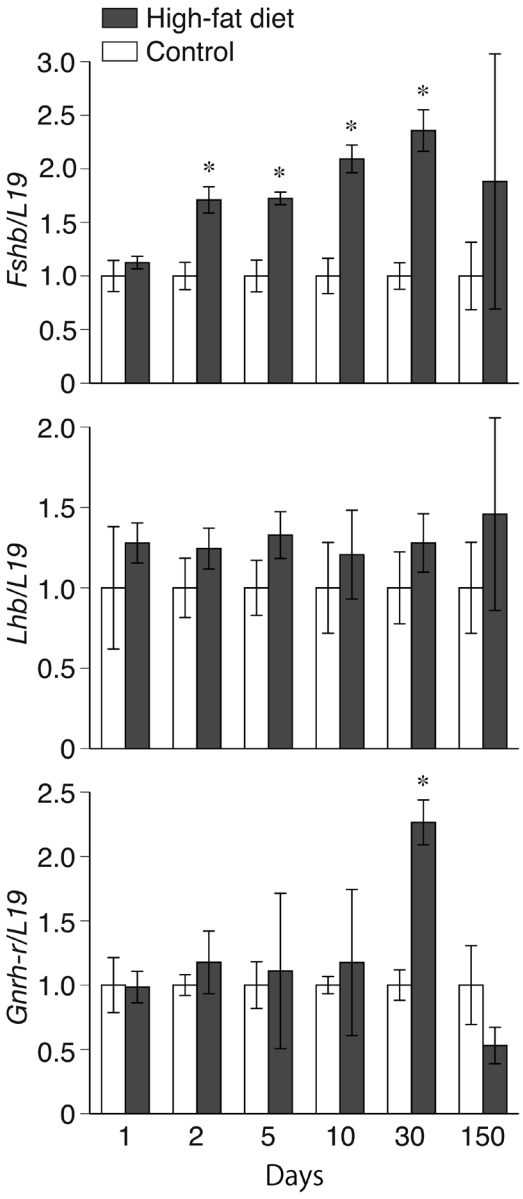

Fig. 3. mRNA expression levels of follicle-stimulating hormone β subunit (*Fshb*), luteinizing hormone β subunit (*Lhb*), and gonadotropin-releasing hormone receptor (*Gnrh-r*) in the pituitary glands of the mice fed high-fat diet (HFD) (gray columns) or control (open columns) diet for 1, 2, 5, 10, 30 and 150 days. * P < 0.05 HFD *vs*. control diet on the same day. n = 4–5 per group. *L19*, ribosomal protein L19.

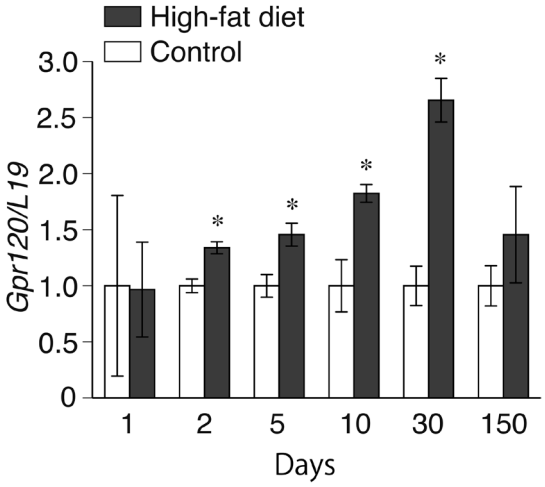

Fig. 4. mRNA expression levels of *Gpr120* in the pituitary glands of the mice fed high-fat diet (HFD) (gray columns) or control (open columns) diet for 1, 2, 5, 10, 30 and 150 days. * P < 0.05 HFD *vs*. control diet on the same day. n = 4–5 per group. *L19*, ribosomal protein L19.

change significantly in this short-term HFD schedule (Fig. 1). These findings suggest that the transcription of the *Fshb* gene might be more sensitive to FFAs than that of *Lhb*. This hypothesis is supported by our previous finding that the transcription of *Fshb* is more sensitive to long-chain unsaturated fatty acids compared to the transcription of *Lhb* in the mouse gonadotrope cell line, LβT2 [30]. Furthermore, it is possible that the animals had not eaten enough on the start day of HFD (day 0) in this study, because the body weight of control animals was lower on day 1, while that of the HFD animals stayed the same. Therefore, only 1 day of HFD feeding may be enough to regulate the transcription of *Fshb*. In addition, previous studies have shown that changes in *Gnrh-r* gene expression levels in the pituitary were homologous with the changes in the pattern of hypothalamic GnRH secretion [31, 32]. Therefore, we believe that hypothalamic Kisspeptin and GnRH secretion will be upregulated in animals fed a HFD for 30 days compared with control animals and downregulated in animals fed a HFD for 150 days. Indeed in several recent studies, the consumption of a HFD for more than 14 weeks in adult rodents was found to decrease hypothalamic *Kiss1* mRNA and kisspeptin levels and subsequent GnRH secretion via leptin resistance [33, 34]. Taken together, these results suggest that short-term HFD feeding, at least within 30 days, has a positive effect on the mature rodent H-P-G axis. Conversely, long-term HFD induced obesity would have a negative effect on the H-P-G axis through leptin resistance. Furthermore, short-term HFD feeding within 10 days may have a direct effect on pituitary gonadotropes, as we observed an increase in *Gnrh-r* mRNA expression levels in the pituitary glands after 10 days of HFD feeding in this study. However, in this study, only matured male mice were used in the experiments to examine the effects of HFD feeding on the gonadal functions at the pituitary level, despite male and female mice having different responsiveness to a HFD [35, 36]. Therefore, our data are still insufficient to conclude the definitive role of a short-term HFD in the transcription of matured murine gonadotropin hormone β subunit genes.

Gpr120 mRNA expression levels increased significantly after 2 days of HFD feeding and continued for up to 30 days of HFD feeding, though there was no significant increase in *Gpr120* mRNA expression levels after 150 days of HFD feeding. This upregulation may be induced by FFAs, as the GPCRs are often internalized after binding to the ligand, and this is followed by mRNA transcription and protein synthesis of the receptors, which maintains the number of

receptors in the cell membrane [37]. Conversely, long-term activation of GPR120 may downregulate the receptor mRNA level after 150 days of HFD feeding. In fact, GPR120 protein internalization has been observed after FFA stimulation of gut cells [14]. HFD-induced elevations in *Gpr120* mRNA expression levels have also been reported in mouse adipose tissue after 3 weeks of HFD feeding [38], and in rat cardiac tissue and extensor digitorum longus skeletal muscles after 12 weeks of HFD feeding [39]. Our results suggest that GPR120 is functional within 2 days of HFD feeding, at least in the mature male mouse pituitary. Interestingly, blood NEFA levels did not change significantly in the HFD groups compared to the control group with over 150 days of treatment in this study. This is in contrast to other studies where high concentrations of blood NEFA levels were reported in mice fed a HFD for more than 24 weeks [25, 40]. Pituitary gonadotropes may be sensitive to subtle blood FFA changes via the action of GPR120. Combined with the fact that 2 days of HFD feeding elevated the *Fshb* mRNA expression levels, it is possible that GPR120 acts as a fuel lipid sensor to regulate FSH production in the pituitary gonadotropes. Future studies are needed to clarify the role of *GPR120* in the gonadotropes after HFD feeding.

GPR120 is reported to engage multiple signal pathways through Gq/11, Gi family, or β-arrestin [41]. It is well known that Gi proteins decrease activity of cAMP-dependent protein kinases, such as protein kinase A (PKA). Based on the above and studies showing FSH synthesis by GnRH is mediated by cAMP-dependent PKA [42, 43], the *Fshb* gene would be activated either by the Gq/11 family or the β-arrestin signal pathway following expression of GPR120. Furthermore, previous studies demonstrated that the long-chain fatty acid receptor agonist, GW9508, induced the phosphorylation of mitogen-activated protein kinase (MAPK) containing ERK1/2 in TSC-1 cells and cultured brown adipocytes [44, 45], and GnRH-induced *Fshb* promoter activity was blocked by ERK1/2 inhibitors in LβT2 cells [46]. Collectively, these studies support the idea that HFD induced transcription of *Fshb* mRNA may be mediated by the phosphorylation of ERK1/2 through either the Gq/11 family or β-arrestin following expression of GPR120 in the gonadotropes. Further studies are needed to determine the signaling pathway involved downstream of GPR120 in gonadotropes.

In conclusion, short-term HFD feeding induces an increase in *Fshb* mRNA expression in matured male mice pituitary glands, whereas a long-term HFD does not increase *Fshb* mRNA levels. These results suggest that short-term HFD positively affects gonadal functions by upregulating FSH production in the pituitary. In addition, it is possible that FFA signaling via GPR120 in pituitary gonadotropes mediates this effect.

Acknowledgments

We thank to Mr Koichi Iwamoto, Shota Kumano, Ryoma Okamoto and Hiroaki Sato for technical assistance. This work was supported in part by the Japan Society for the Promotion of Science KAKENHI Grants 20780202 and 19K06446, and by a Kindai University grant RKS28-057 awarded to RM.

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