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—Original Article—

Long-chain unsaturated fatty acids reduce the transcriptional activity of the rat follicle-stimulating hormone β-subunit gene

Ryutaro MORIYAMA[1)], Tsubasa YAMAZAKI[1)], Takako KATO[2)] and Yukio KATO[2)]

[1)]*Laboratory of Environmental Physiology, Department of Life Science, School of Science and Engineering, Kinki University, Osaka 577-8502, Japan*

[2)]*Laboratory of Molecular Biology and Gene Regulation, Department of Life Science, School of Agriculture, Meiji University, Kanagawa 214-8571, Japan*

Abstract. Here, we assessed the effects of long-chain fatty acids (LCFAs) and the LCFA receptor agonist GW9508 on the transcription of the gonadotropin subunit genes *Cga*, *Lhb* and *Fshb* because LCFA receptor GPR120 was observed in mouse gonadotropes in our recent study. A transcription assay using LβT2 cells demonstrated that LCFAs, oleic acid, α-linolenic acid, docosahexaenoic acid and palmitate, repressed the expression of *Cga*, *Lhb*, and *Fshb* at concentrations between 50 and 100 μM. On the other hand, treatment with 10 μM unsaturated LCFAs, oleic acid, α-linolenic acid and docosahexaenoic acid, repressed only *Fshb* expression, while the same dose of a saturated LCFA, palmitate, had no effect on the expression of gonadotropin subunit genes. Furthermore, GW9508 did not affect promoter activity. Next, we examined deletion mutants of the upstream region of *Fshb* and found that the upstream regulatory region (-2824 to -2343 bp) of *Fshb* was responsible for the notable repression by 10 μM unsaturated LCFAs. Our results suggest that the upstream region of *Fshb* is susceptible to unsaturated LCFAs. In addition, unsaturated LCFAs play a role in repressing *Fshb* expression through the distal -2824 to -2343 bp region, which might be independent of the LCFA receptor GPR120 pathway.

Key words: Follicle-stimulating hormone (FSH), Free fatty acid, Gonadotropin, Luteinizing hormone (LH), Pituitary

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Gonadal function is regulated mainly at the hypothalamic level of the hypothalamic-pituitary-gonadal axis. However, increasing evidence suggests that gonadal function is controlled at the pituitary level. For example, cortisol has been reported to directly suppress pulsatile luteinizing hormone (LH) secretion from the pituitary in sheep [1, 2]. Moreover, insulin, leptin, adiponectin, and other hormones are known to directly regulate LH secretion via gonadotropes [3–5]. These previous reports suggest that the synthesis and secretion of gonadotropic hormones can be directly regulated at the pituitary level by peripheral signals such as hormones. The pituitary gonadotropic hormones, LH and follicle-stimulating hormone (FSH), exist as heterodimers, comprised of a common glycoprotein α-subunit (Cga) and their specific β-subunits, LHβ and FSHβ, respectively. Genes encoding these three subunits are expressed in the pituitary gonadotropes, and several extracellular signals including GnRH, progesterone, estrogen, activin, and inhibin have been reported to regulate their expression via a specific upstream response element [6–14]. Therefore, characterization of response elements of gonadotropin subunit genes will help to determine the mechanisms underlying gonadotropin regulation at the pituitary level.

Long-chain fatty acids (LCFAs) may act as metabolic signals in the regulation of reproductive functions. This is supported by findings that peripheral or central lipoprivation suppresses pulsatile LH secretion [15, 16], and central exposure to linoleic acid increases *Lhb* mRNA expression levels in rats [17]. Interestingly, LCFAs are supposed to have some influence on the synthesis and secretion of gonadotropic hormones at the pituitary level, since a free fatty acid (FFA) cocktail directly increases Lhb mRNA levels and suppresses Fshb mRNA levels in the gonadotropic cell line LβT2 [18] and linoleic acid increases LH release and Lhb mRNA levels in both LβT2 cells and rat primary cultured pituitary cells [19]. Furthermore, LCFAs may induce these phenomena through LCFA receptor GPR120, which is known to be expressed in the gonadotropes of the mouse pituitary [20] and LβT2 cells [19]. However, in previous studies with LβT2 cells, the role of LCFAs in the gene expression and secretion of gonadotropic hormones has been examined using concentrations of LCFAs of several hundred micromolar. Therefore, the effect of lower than 100 μM LCFAs on the gene expression and secretion of gonadotropic hormones is still unclear.

In the present study, we investigated the effects of concentrations of LCFAs below 100 μM and a LCFA receptor agonist, GW9508, on the transcription of gonadotropin subunit genes, *Cga*, *Lhb* and *Fshb,* in LβT2 cells and identified the gene regulatory region responsive to LCFAs.

Materials and Methods

Cell culture

The gonadotropic cell line LβT2 (kindly provided by Dr PL Mellon) was maintained in monolayer cultures in Dulbecco's modified Eagle's

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©2016 by the Society for Reproduction and Development
Correspondence: R. Moriyama (e-mail: mory@life.kindai.ac.jp)
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medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin solution (Sigma-Aldrich, St. Louis, MO, USA) in a humidified 5% CO_2/95% air incubator at 37°C.

At the time of the reporter assay, cells (2×10^4) were seeded in 100 µl Opti-MEM (Life Technologies) per well of a 96-well plate, 24 h prior to transfection and maintained in a humidified 5% CO_2/95% air incubator at 37°C.

LCFA stock and working solutions

The LCFAs, oleic acid, α-linolenic acid, docosahexaenoic acid (DHA) and palmitate, were dissolved in 50% ethanol at 65°C to prepare 500 mM stock solutions. Stock solutions and fatty acid-free bovine serum albumin (Sigma-Aldrich) in Opti-MEM were vortexed for 40 min and filtered to obtain a 10 mM homogenous mixture in Opti-MEM containing 2% bovine serum albumin.

Reporter assay

Upstream regions of the rat *Cga* (NC_005104.4), *Lhb* (NC_005100.4) and *Fshb* (NC_005102.4) genes were amplified using specific primer sets. The fragments were ligated into the secreted alkaline phosphatase (SEAP) plasmid vector pSEAP2-Basic (Clontech Laboratories, Palo Alto, CA, USA) as described previously [21–23]. The resulting reporter vectors contained the following gonadotropin subunit upstream regions: -3793/+37 (*Cga*); -2930/+17 (*Lhb*); and -2824/+28, -2342/+28, -1718/+28, -1499/+28, -1219/+28, -970/+28, -650/+28, -346/+28, -43/+28 (*Fshb*).

Transfection was performed using 200 ng DNA and 0.3 µl FuGENE HD (Roche Diagnostics, Basel, Switzerland) per well according to the protocol described in a previous study [24]. The cells were treated with an LCFA solution (10 µl per well) or the LCFA receptor agonist GW9508 at 7–8 h after transfection. After another 72 h of incubation, 5 µl of culture medium from each well was assayed for SEAP activity using the Phospha-Light Reporter Gene Assay System (Applied Biosystems, Foster City, CA, USA) and Powerscan H1 microplate luminometer (DS Pharma Biomedical, Osaka, Japan) according to the manufacturers' protocols.

Statistical analysis

All values were expressed as the mean ± SEM. Dunnett's method was used to analyze the effect of LCFAs or GW9508 on SEAP activity. P-values of < 0.05 were considered significant.

Results

Effect of LCFAs or GW9508 on transcriptional activation of gonadotropin subunit genes

The promoter activity of *Fshb* (-2824/+28) was significantly repressed by not only treatment with both 100 µM and 50 µM DHA, oleic acid, α-linolenic acid or palmitate but was also significantly repressed by 10 µM DHA, oleic acid or α-linolenic acid ($P < 0.05$; Fig. 1). On the other hand, the promoter activity of *Cga* (-3793/+37) was significantly repressed by treatment with 100 µM DHA and by both 100 µM and 50 µM of oleic acid, α-linolenic acid or palmitate, but it was not significantly repressed by 10 µM LCFAs. Similar to the results for *Cga*, the promoter activity of *Lhb* (-2930/+17) was significantly repressed by treatment with 100 µM DHA, oleic acid, α-linolenic acid or palmitate and by treatment with 50 µM palmitate.

Next, we determined whether LCFAs regulate the synthesis of gonadotropes through GPR120 at the pituitary level and found that the LCFA receptor agonist GW9508 did not affect promoter activity at any concentration of LCFA used in this study.

Deletion analysis for the Fshb upstream region

To confirm the marked repression of *Fshb* by 10 µM oleic acid, α-linolenic acid and DHA, deletion mutants of the upstream region of *Fshb* were examined using transfection and reporter gene assays. Oleic acid, α-linolenic acid and DHA significantly repressed promoter activity of the −2824 to +28 b region (Fig. 2). However, the -2342/+28, -1718/+28, -1499/+28, -1219/+28, -970/+28, -650/+28, -346/+28, and -43/+28 b regions lost the repression by oleic acid, α-linolenic acid and DHA.

Figure 3 shows the locations of putative binding sites for transcription factors between the -2824 to -2343 b region of the rat *Fshb* upstream promoter, which responded to unsaturated LCFAs. Among the transcription factors binding to these sites, FOXJ1, GLU1, MSX, PITX1, PRRX2, RUNX1 and SOX2 were identified as those with expression in the murine pituitary gland.

Discussion

In this study, we determined the effect of LCFAs and the LCFA receptor agonist GW9508 on the transcription of gonadotropin hormone subunit genes, including *Cga*, *Lhb* and *Fshb*, and identified the gene regulatory regions that were responsive to LCFAs. As shown in Fig. 1, unsaturated LCFAs, DHA, oleic acid and α-linolenic acid, markedly repressed the expression of *Fshb* at a low concentration (10 µM), while the saturated LCFA, palmitate, caused no apparent change in gonadotropin subunit gene expression at the same dose. Further, treatment with 50–100 µM LCFAs repressed expression of the gonadotropin subunit genes *Cga*, *Lhb* and *Fshb* (Fig. 1). In previous studies, the role of LCFAs on the transcription of gonadotropin hormone subunit genes has been examined using concentrations in the range of several hundred micromolars of LCFAs [17–19]. In this study, however, the low concentration of 10 µM unsaturated LCFAs repressed the expression of *Fshb*. The concentration of nonesterified fatty acids in the rodent peripheral blood is in the hundreds of micromolar range [25, 26]; therefore, the concentration of LCFAs used in this study is similar to the physiological level or even slightly lower than that recorded in rodent peripheral blood. These findings suggest that the transcription of *Fshb* is more sensitive or susceptible to unsaturated LCFAs compared with the previously reported concentration and that unsaturated LCFAs may be involved in the repression of *Fshb* transcription. Reproductive aging in women is characterized by shortening of the estrous cycle length [27], increased incidences of oocyte spindle aberrations or aneuploidy [28] and declining fertility [29]. Similar to humans, female rodents also show similar age-related physiological changes in reproductive function [30, 31]. These changes are associated with elevated baseline levels of serum FSH in humans [32] and mice [33]. Interestingly, dietary administration of omega-3 polyunsaturated fatty acids such as DHA decreased serum baseline FSH levels and FSH response to GnRH without changing serum LH

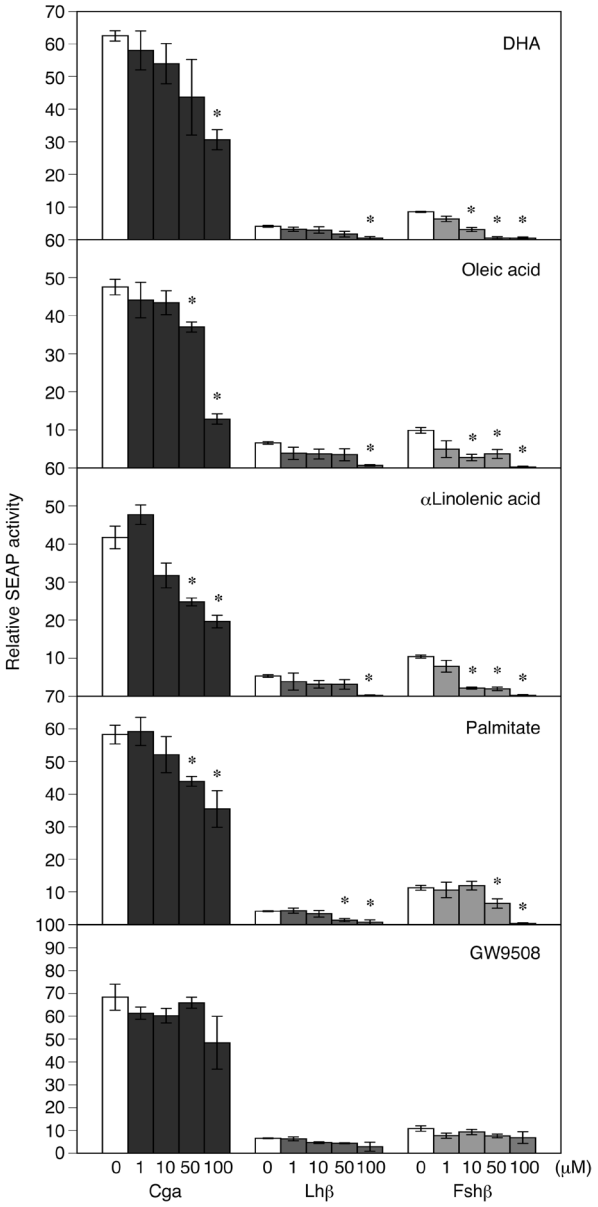

Fig. 1. Transient transfection assay of the rat gonadotropin subunit gene promoters in LβT2 cells with or without treatment with oleic acid, α-linolenic acid, docosahexaenoic acid (DHA), palmitic acid or the long-chain fatty acid (LCFA) receptor agonist GW9508. Reporter constructs containing *Cga* (-3793/+37 b), *Lhb* (-2930/+17 b) or *Fshb* (-2824 to +28 b) promoters fused with the secreted alkaline phosphatase (SEAP) gene in the pSEAP-Basic vector were transfected into LβT2 cells. The cells were exposed to oleic acid, α-linolenic acid, DHA, palmitic acid, or GW9508 (1, 10, 50 or 100 μM) 7–8 h after transfection. An aliquot of the culture medium was used for the SEAP assay. SEAP activities are presented as the activity relative to that of the basic vector. Values are the mean ± SEM of four independent experiments. * $P < 0.05$ *vs*. pSEAP2-Basic (Dunnett's method).

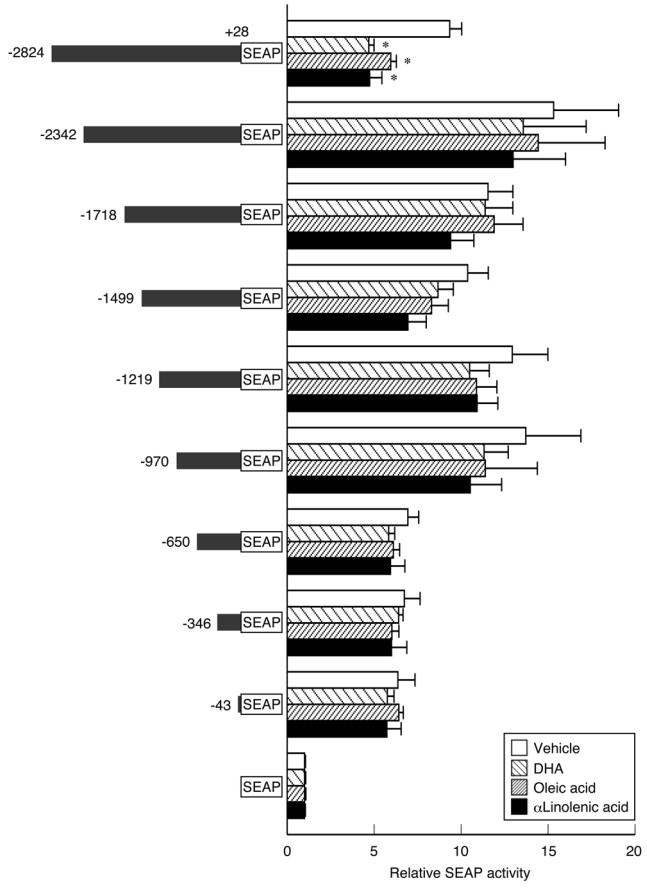

Fig. 2. Deletion analysis of the rat *Fshb* promoter region (-2824 to +28 b regions) in LβT2 cells with or without treatment with 10 μM unsaturated LCFAs oleic acid, α-linolenic acid, or docosahexaenoic acid (DHA). On the left, reporter constructs are shown containing serial deletion mutants of the *Fshb* promoter fused with the secreted alkaline phosphatase (SEAP) gene in the pSEAP2-Basic vector that were transfected into LβT2 cells. On the right, SEAP activities are represented as the activity relative to that of the basic vector. Values are the mean ± SEM for four independent experiments. * $P < 0.05$ *vs*. pSEAP2-Basic (Dunnett's method).

levels in reproductive-age women with normal body weights [34]. Furthermore, Nehra *et al*. [35] reported that dietary treatment with a diet rich in DHA prolonged reproductive lifespan and improved oocyte quality in mice. Thus, the effect of a low concentration of unsaturated LCFAs on the transcription of *Fshb* in this study may have identified a part of the mechanism of unsaturated LCFAs in the delay of female reproductive aging and improvement of oocyte quality.

A transfection assay using serial deletion mutants of the -2824 to +28 b upstream region of *Fshb* revealed that the 10 μM unsaturated LCFA-responsive region for reducing *Fshb* transcription is located in the -2824 to -2343 b region (Fig. 2). Previous studies have shown that an FFA cocktail or linoleic acid reduces basal *Fshb* mRNA levels by disrupting the effects of activin on transcription of *Fshb* in LβT2 cells [17, 18]. LβT2 cells have been reported to produce activin endogenously to maintain basal *Fshb* expression [36]. These previous results imply that the reduction of *Fshb* transcription caused by unsaturated LCFAs via the -2824 to -2343 b region in the present study may also be mediated by the disruption of activin effects on

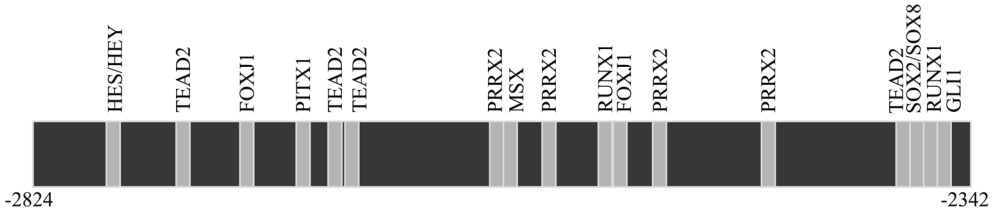

Fig. 3. Locations of gene transcription factor binding sites in the -2824 to -2343 b region upstream of the *Fshb* promoter that was responsive to unsaturated LCFAs. Abbreviations of transcription factors: Forkhead box protein J1 (FOXJ1), glioma-associated oncogene homolog 1 (GLI1), hairy and enhancer of split (HES)/split-related with YRPW motif (HEY), Msh homeobox 1-like protein (MSX), paired-like homeodomain 1 (PITX1), paired related homeobox 2 (PRRX2), runt-related transcription factor 1 (RUNX1), sex-determining region Y-box 2 (SOX2)/sex-determining region Y-box 8 (SOX8), and TEA domain family member 2 (TEAD2).

Fshb expression. However, Suszko *et al*. [9] reported that the most activin-responsive domain of the rat *Fshb* promoter within the proximal promoter region was between -230 and -199 b. Therefore, the -2824 to -2343 b region is a novel region for the transcriptional control with unsaturated LCFAs. It is not clear whether this region is an activin-responsive domain leading to the disruption of *Fshb* transcription by unsaturated LCFAs. Interestingly, the -2824 to -2343 b region may contain plural putative regulatory elements for diverse transcription factors, including Forkhead box protein J1 (FOXJ1), glioma-associated oncogene homolog 1 (GLI1), hairy and enhancer of split/split-related with YRPW motif, Msh homeobox 1-like protein (MSX), paired-like homeodomain 1 (PITX1), paired related homeobox 2 (PRRX2), runt-related transcription factor 1 (RUNX1), sex-determining region Y-box 2 (SOX2)/sex-determining region Y-box 8, and TEA domain family member 2 (Fig. 3). In particular, FOXJ1, GLI1, MSX, PITX1, PRRX2, RUNX1, and SOX2 are expressed in the murine pituitary gland [37–42]. Therefore, unsaturated LCFAs may directly and/or indirectly modulate the binding of any of these factors. Indeed, in a previous study [43], PITX1 was observed to regulate murine and human *Fshb* transcription. Nevertheless, the role of the -2824 to -2343 b upstream region of rat *Fshb* and its molecular mechanism for the suppression by unsaturated LCFAs will need to be clarified by further investigations.

We hypothesized that LCFAs directly regulate the synthesis of gonadotropes through GPR120 at the pituitary level, because GPR120 expression has been observed in the gonadotropes of the mouse pituitary [20]. To test our hypothesis, the LCFA receptor agonist GW9508 was used. However, treatment with GW9508 did not affect gonadotropin subunit gene expression at the LCFA doses used in our study, although LCFAs repressed the promoter activity of gonadotropin subunit genes in LβT2 pituitary gonadotropic cells (Fig. 1). The dose of GW9508 used in this study was similar to that used in a previous study in which LβT2 cells were exposed to GW9508 [19] and induced an agonistic effect on GRP120 [44]. These results indicate that the reduction of transcription of *Fshb* is likely mediated by the β-oxidation products of unsaturated LCFAs and/or by the direct or indirect action of unsaturated LCFAs on the *Fshb* transcriptional regulatory mechanisms.

The present results showing that LCFAs repressed the transcription of *Cga* and *Lhb* (Fig. 1) contradict the results of previous studies. Sharma *et al*. [18] reported that an FFA cocktail induced an increase in *Lhb* mRNA levels in LβT2 cells. Garrel *et al*. [17, 19] reported that linoleic acid treatment induced an increase in the *Lhb* mRNA levels, while it had no effect on *Cga* mRNA levels in LβT2 cells or rat anterior pituitary cells. The inconsistency may depend on the difference in experimental methods used. Previous studies have demonstrated the effect of LCFAs on gonadotropin hormone subunit mRNA levels using real-time PCR, whereas we investigated the function of the enhancer/promoter elements of the gonadotropin genes using the SEAP reporter gene assay system. We only examined the region ~3.8 kb upstream of the rat gonadotropic subunit genes. In the SEAP reporter gene assay system, it may be necessary to analyze additional upstream regions to determine the gonadotropin transcription control mechanism by LCFAs. The upstream regions of *Cga* and *Lhb* examined in this study may lack the specific response regions for LCFAs, in contrast to the results measured for *Cga* and *Lhb* mRNA in the previous studies. The evidence concerning the LCFA response in the transcription of *Cga and Lhb* is not conclusive.

In conclusion, the promoter assay using reporter vectors revealed that the transcription of *Fshb* was susceptible to unsaturated LCFAs compared with the transcription of *Cga* and *Lhb*. Furthermore, the unsaturated LCFAs, DHA, oleic acid and α-linolenic acid, exhibited repressive regulation of rat *Fshb* expression at -2824 and -2343 b of the 5′-flanking region and might be independent of the GPR120 pathway.

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