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—Original Article—

AMP-activated protein kinase activation reduces the transcriptional activity of the murine luteinizing hormone β-subunit gene

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Abstract. Malnutrition is one of the factors that induces reproductive disorders. However, the underlying biological processes are unclear. AMP-activated protein kinase (AMPK) is an enzyme that plays crucial role as a cellular energy sensor. In the present study, we examined the effects of AMPK activation on the transcription of the murine gonadotropin subunit genes *Cga*, *Lhb*, and *Fshb*, and the gonadotropin-releasing hormone receptor *Gnrh-r*. Real-time PCR and transcription assay using LβT2 cells demonstrated that 5-amino-imidazole carboxamide riboside (AICAR), a cell-permeable AMP analog, repressed the expression of *Lhb*. Next, we examined deletion mutants of the upstream region of *Lhb* and found that the upstream regulatory region of *Lhb* (–2527 to –2198 b) was responsible for the repression by AICAR. Furthermore, putative transcription factors (SP1, STAT5a, and TEF) that might mediate transcriptional control of the *Lhb* repression induced by AICAR were identified. In addition, it was confirmed that both AICAR and a competitive inhibitor of glucose metabolism, 2-deoxy-D-glucose, induced AMPK phosphorylation in LβT2 cells. Therefore, the upstream region of *Lhb* is one of the target sites for glucoprivation inducing AMPK activation. In addition, AMPK plays a role in repressing *Lhb* expression through the distal –2527 to –2198 b region.

Key words: 5-Amino-imidazole carboxamide riboside (AICAR), AMP-activated protein kinase (AMPK), Glucoprivation, Luteinizing hormone (LH), Pituitary

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The hypothalamic-pituitary-gonadal (H-P-G) axis plays a pivotal role in the mammalian reproductive system. Kisspeptin/Gonadotropin-releasing hormone (GnRH) secreted from the hypothalamus acts as a signal to regulate the secretion of gonadotropic hormones, luteinizing hormone (LH), and follicle stimulating hormone (FSH) via the GnRH-receptor (GnRH-R) in pituitary gonadotropes. LH and FSH act on the gonads to regulate gonadal function. The function of the gonads is believed to be regulated by the kisspeptin-GnRH signal pathway in the hypothalamus, which is the uppermost component of the H-P-G axis. However, the pituitary gland is also thought to be involved in the mechanism that controls gonad function, since the synthesis and secretion of gonadotropic hormones are reportedly directly regulated by peripheral signals at the pituitary level. It has been reported that cortisol can suppress pulsatile LH secretion at the pituitary level in sheep [1], and insulin, leptin, adiponectin, and molecules directly regulate LH secretion *in vitro* [2–4]. In our previous study, a transcription assay using LβT2 gonadotropic cells demonstrated that unsaturated long-chain fatty acids, such as oleic acid, α−linolenic acid, and docosahexaenoic acid, markedly repressed basal *Fshb* gene expression [5]. Thus, the gonadotropes might directly sense peripheral signals and regulate the synthesis and secretion of the LH and FSH gonadotropic hormones to control the function of gonad at the pituitary level.

Pituitary gonadotropic hormones, LH and FSH, exist as heterodimers. They are composed of a common glycoprotein α-subunit (Cga) and a specific β-subunit, LHβ and FSHβ, respectively. Genes encoding these three subunits are expressed in pituitary gonadotropes, whereas several extracellular signals including GnRH, progesterone, estrogen, activin, and inhibin have been reported to regulate their expression via a specific upstream response element [6–11]. Therefore, the characterization of the response elements of gonadotropin subunit genes would help determine the mechanisms underlying gonadotropin regulation at the pituitary level.

Malnutrition is one of the factors that induce reproductive disorders. However, the underlying biological processes, such as the lower energy sensing system, that regulates reproductive functions are not clearly understood. AMP-activated protein kinase (AMPK) is a heterotrimeric complex formed by αβγ subunits. AMPK is thought

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to be an intracellular sensor that is activated by an energy deficiency, such as hypoglycemia, or by several hormones that are secreted during malnutrition. AMPK plays a pivotal role in the regulation of peripheral energy homeostasis, since AMPK is activated by the intracellular AMP/ATP ratio when ATP levels decrease [12]. Therefore, AMPK might be involved in reproductive control as a sensor of the peripheral energy status at several points along the H-P-G axis [13]. AMPK stimulation inhibits LH and FSH secretion at the pituitary level, *Lhb* and *Fshb* mRNA levels in rat pituitary cell cultures [14], and LH secretion in LβT2 cells [3]. However, the effect of AMPK on the response elements of gonadotropin subunit genes at the pituitary level is not well understood.

This study examined whether intracellular energy depletion regulates the transcription of the murine gonadotropin subunit genes *Cga*, *Lhb*, *Fshb,* and *Gnrh-r* via AMPK activation, and sought to confirm the gene regulatory region that is responsive to AMPK activation *in vitro*. We used 5-amino-imidazole carboxamide riboside (AICAR), a cell-permeable AMP analog, to mimic intracellular energy depletion [15].

Materials and Methods

Cell cultures

The LβT2 mouse gonadotropic cell line provided by Dr PL Mellon (Department of Reproductive Medicine, University of California, San Diego, CA, USA) was maintained in monolayer cultures in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Waltham, MA, 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. To perform the reporter assay, cells (2×10^4) were seeded in wells of a 96-well plate containing 100 µL Opti-MEM (Thermo Fisher Scientific) 24 h prior to transfection and were maintained in a humidified 5% CO_2/95% air incubator at 37°C.

Animals

For RT-PCR experiment, 8-week-old male ICR mice obtained from Japan SLC (Hamamatsu, Japan) were maintained in a temperature-controlled room under a 12 h light/dark cycle. The protocols for the care and use of the mice was approved by the Committee on Animal Experiments of Kindai University. Experiments were conducted in accordance with the NIH Guidelines for Animal Care and Use of Laboratory Animals. For microarray analysis, Wistar-Imamichi rats were housed individually in a temperature-controlled room under a 12 h light/darkn cycle. All animal experiments were performed after receiving approval from the Institutional Animal Care and Use Committee, Meiji University, and were conducted in accordance with the aforementioned NIH guidelines.

AICAR treatment

AICAR (Sigma-Aldrich) was dissolved in water and the 250 mM stock solution was stored at –20°C until use.

RNA extraction and cDNA synthesis

Total RNA was prepared from LβT2 cells and pituitary glands of decapitated mice using TRI Reagent (Sigma-Aldrich) and treated with RNase-free DNase I to remove any genomic DNA contamination. Then, cDNA was synthesized using a Superscript IV kit with an oligo$(dT)_{12\text{-}18}$ primer. All reagents were purchased from Thermo Fisher Scientific.

Real-time PCR

The mRNA levels of *Lhb*, *Fshb*, *Cga*, and *Gnrh-r* in LβT2 cells were determined by real-time PCR using SYBR Premix Ex Taq II (TaKaRa Bio, Shiga, Japan) containing SYBR Green I, in a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following conditions were used: denaturation at 95°C for 30 sec and amplification by cycling 40 times at 95°C for 5 sec and at 60°C for 34 sec. Data were analyzed using the standard curve method and normalized to TATA-box binding protein (Tbp) expression as the reference gene. The forward and reverse primer sets (Thermo Fisher Scientific) used for each gene are shown in Table 1. To test the effect of AICAR over the expression of tested genes, in some experiments LβT2 cells were exposed to 50, 100, or 200 µM AICAR for 48 h.

RT-PCR

The expression of the transcription factor genes was determined by RT-PCR. cDNAs were amplified by PCR using GoTaq DNA polymerase (Promega, Madison, WI, USA). The cycling protocol used was as follows: an initial denaturation step for 60 sec at 94°C;

Table 1. List of primer sequences for RT-PCR and real-time PCR

Gene	Forward primer	Reverse Primer
Tbp	5'-GATCAAACCCAGAATTGTTCTCC-3'	5'-ATGTGGTCTTCCTGAATCCC-3'
Cga	5'-GCTGTGTGGCCAAAGCATTT-3'	5'-CAGTGGCACTCCGTATGATTCTC-3'
Lhb	5'-CTAGCATGGTCCGAGTACTG-3'	5'-CCCATAGTCTCCTTTCCTGT-3'
Fshb	5'-CTGCTACACTAGGGATCTGG-3'	5'-TGACATTCAGTGGCTACTGG-3'
Gnrh-r	5'-CAGGATGATCTACCTAGCAG-3'	5'-GCAGATTAGCATGATGAGGA-3'
AP-2a	5'-GCCACAAAACAGATCTGCAA-3'	5'-TGGAGACCTGCATCGTAGT-3'
Sp1	5'-ATCTGGTGGTGTGGGATACA-3'	5'-GAGGCTCTTCCCTCACTGTCT-3'
Stat1	5'-GACCCTAAGCGAACTGGATAC-3'	5'-AGACATGGGAAGCAGGTTG-3'
Stat5a	5'-GAGTTTGACCTGGATGAGAG-3'	5'-TTCTAGCGGAGGTGAAGAG-3'
Tcf3	5'-TTCTCACCTAGCCCCTCAA-3'	5'-TGGAGACCTGCATCGTAGT-3'
Tef	5'-AAGGAGAACCAGATCACCA-3'	5'-CACGATGGTCTTGCACTT-3'

35 cycles of denaturation for 30 sec at 94°C, primer annealing for 30 sec at 56°C, and extension for 90 sec at 72°C; and 7 min at 72°C at the end of cycling to complete extension. The amplified products were then separated on 2% agarose gels containing 0.5 μg/ml ethidium bromide. The forward and reverse primers (Thermo Fisher Scientific) used are shown in Table 1.

Harr-plot analysis

Harr-plot analysis is a graphical method that allows the comparison of two nucleotide sequences and identification of regions of similarity between them. Nucleotide sequence homology was determined between the mouse and rat 5'-flanking regions, up to 4.0 kb from the transcription initiation sites of the *Cga* gene and up to 3.0 kb from the transcription initiation sites of *Lhb* and *Fshb* genes. Each dot represents > 85% identity in 20 nucleotides.

Reporter assay

Upstream regions of the rat *Cga* (NC_005104.4), *Fshb* (NC_005102.4), and *Lhb* (NC_005100.4) genes were amplified using specific primer sets. Fragments were ligated into the secreted alkaline phosphatase (SEAP) plasmid vector pSEAP2-Basic (Clontech Laboratories, Palo Alto, CA, USA) as described previously [16, 17]. Resulting reporter vectors contained the following gonadotropin subunit upstream regions: –3793 to +37 of *Cga*; –2824 to +28 of *Fshb*; and –2930 to +17, –2527 to +17, –2197 to +17, –1976 to +17, –1595 to +17, –1370 to +17, –1097 to +17, –718 to +17, and –433 to +17 of *Lhb*.

Transfection was performed using 200 ng of DNA and 0.3 μl FuGENE HD (Roche Diagnostics, Basel, Switzerland) per well according to a protocol described in a previous study [18]. Cells were treated with an AICAR solution (10 μl per well) 7 to 8 h after transfection and incubated for 48 h. Then, 5 μl of culture medium from each well was assayed for SEAP activity using a Phospha-Light Reporter Gene Assay System (Applied Biosystems) and a Powerscan H1 microplate luminometer (DS Pharma Biomedical, Osaka, Japan) according to the manufacturers' protocols.

Western blotting

Overnight serum-starved LβT2 cells were treated either with 25 mM 2-deoxy-*D*-glucose (2DG) for 0.5, 1 or 4 h, or with 100 or 200 μM AICAR for 24 h and then lysed in extraction buffer (50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) containing 1% protease inhibitors (Nakarai Tesque, Kyoto, Japan) and a phosphatase inhibitor cocktail (Sigma-Aldrich). Total cell lysates were centrifuged at 15000 *g* for 5 min. Supernatants were mixed with 4 × sodium dodecyl sulfate sample buffer, boiled, and separated in polyacrylamide gels. Proteins were then transferred to polyvinylidene difluoride membranes (Millipore, San Jose, CA, USA). The membranes were probed with primary antibodies at the following dilutions: anti-AMPKα polyclonal antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), anti-phospho-AMPKα (Thr172) polyclonal antibody (1:1000; Cell Signaling Technology), or anti-β-actin monoclonal antibody (1:2000; Sigma-Aldrich). Membranes were further incubated with anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (1:4000, Cell Signaling Technology) or HRP-conjugated anti-mouse IgG antibody (1:2000, Santa Cruz Biotechnology, Dallas, TX, USA), and developed with ImmunoStar Zeta (Fujifilm Wako Chemicals, Osaka, Japan). Chemiluminescence was recorded using an ImageQuant LAS 500 (GE Healthcare, Chicago, IL, USA).

Microarray analysis

Total RNAs were prepared from the whole pituitaries of embryonic day (E)12.5 (n = 26), E13.5 (n = 15), E14.5 (n = 10), E16.5 (n = 14), E18.5 (n = 11), E20.5 (n = 10), and postnatal day (P)0 (n = 9) rats, and from the anterior lobes of P5 (n = 9), P15 (n = 8), P30 (n = 4), P60 (n = 3), and P600 (n = 3) rats using ISOGEN (Nippon Gene, Tokyo, Japan). Microarrays were performed using Rat Genome 230 2.0 GeneChip (Affymetrix Japan, Tokyo, Japan) for total RNA samples. Data of microarrays were normalized by median normalization.

Statistical analyses

All values are expressed as the mean ± standard error of the mean (SEM). One-way ANOVA followed by Dunnett's multiple comparison test was used to analyze the effect of AICAR on SEAP activity. P-values < 0.05 were considered significant.

Results

Effect of AICAR on mRNA expression levels of mouse gonadotropin subunit genes and Gnrh-r

After 48 h of treatment, AICAR induced significant decreases in *Lhb* mRNA levels in LβT2 cells (Fig. 1). In contrast, mRNA levels of *Fshb*, *Cga*, and *Gnrh-r* were not significantly different.

Homology sequence between the mouse and rat 5′-flanking regions of gonadotropin subunit genes

Harr-plot analyses were performed to confirm the sequence homology between the mouse and rat 5′-flanking regions of the *Cga*, *Lhb*, and *Fshb* genes, since a reporter assay was performed using each rat gene in the mouse pituitary-lineage cell line, LβT2. At a maximum upstream region of 4.0 kb from the transcription initiation site of *Cga* gene and 3.0 kb from that of *Lhb* and *Fshb* genes, mice and rats shared a high degree of identity in their nucleotide sequences (Fig. 2).

Effect of AICAR on the transcriptional activation of rat gonadotropin subunit genes

The promoter activity of rat *Lhb* (–2930 to +17) was significantly repressed by treatment with 100 and 200 μM AICAR ($P < 0.05$; Fig. 3). Conversely, the promoter activities of rat *Cga* (–3793 to +37 bp) and *Fshb* (–2824 to +28) genes were not significantly repressed by either AICAR concentration. Furthermore, 50 μM of AICAR treatment did not affect the promoter activity of rat *Cga* (–3793 to +37), *Lhb* (–2930 to +17), and *Fshb* (–2824 to +28).

Deletion analysis for the Lhb upstream region

To confirm the repression of *Lhb* by 100 μM AICAR, deletion mutants of the upstream region of *Lhb* were examined using transfection and reporter gene assays. AICAR significantly repressed the promoter activity of the –2930 to +17 and the –2527 to +17 regions (Fig. 4). However, the –2197 to +17, –1976 to +17, –1595 to +17, –1370 to +17, –1097 to +17, –718 to +17, and –433 to +17 regions

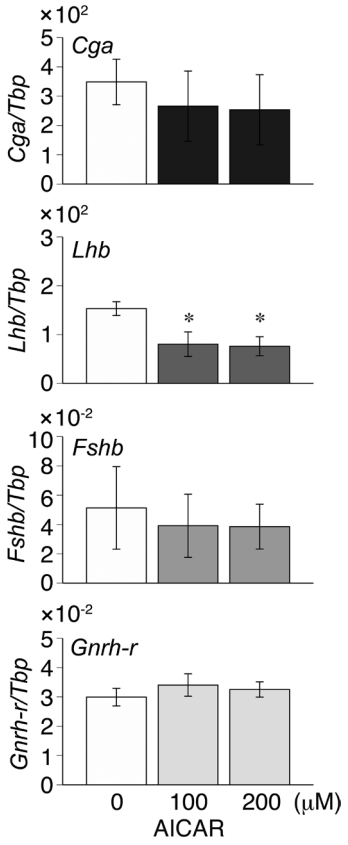

Fig. 1. Effect of 48-h treatment with 100 or 200 μM 5-amino-imidazole carboxamide riboside (AICAR) on mouse glycoprotein α-subunit (*Cga*), luteinizing hormone β-subunit (*Lhb*), follicle-stimulating hormone β-subunit (*Fshb*), and gonadotropin-releasing hormone receptor (*Gnrh-r*) mRNA levels in LβT2 cells. Data are expressed as the mean ± SEM. Each mRNA value was normalized using the TATA-box binding protein (*Tbp*) mRNA levels as a reference. There were four independent experiments/group. * $P < 0.05$ *vs.* 0 μM AICAR (control).

were not significantly repressed by AICAR.

Nucleotide sequence homology between –2527 and –2198 b promoter regions of the rat and mouse Lhb genes

The nucleotide sequences of the –2527 to –2198 b regions upstream of the mouse and rat *Lhb* genes shared 90.3% homology (Fig. 5a). Fig. 5a and 5b show the locations of putative binding sites for transcription factors, along the –2527 to –2198 b regions of both the mouse and rat *Lhb* genes that responded to AICAR induced AMPK activation. Among the putative transcription factors that bind to these sites, activating enhancer binding protein 2α (AP-2α), specificity protein 1 (SP1), signal transducer and activator of transcription 1 (STAT1), signal transducer and activator of transcription 5A (STAT5a), transcription factor 3 (TCF3), and thyrotroph embryonic factor (TEF) were identified as those expressed in the rodent pituitary gland. Table 2 shows the expression pattern of these transcription factors in the rat pituitary examined by microarray analysis. All these transcription factors, except AP-2α, were constitutively expressed in the rat pituitary gland from fetal to adult stages.

Effect of AICAR on mRNA expression levels of transcription factors that bind to the Lhb upstream region in LβT2 cells

Mouse *Sp1*, *Stat1*, *Stat5a*, *Tcf3*, and *Tef* mRNA expression was detected by RT-PCR (Fig. 6a) in mouse pituitary and LβT2 cells, whereas *AP-2α* expression could not be found. After 48 h of exposure to either 100 or 200 μM AICAR, mRNA levels of mouse *Sp1*, *Stat5a*, and *Tef*, but not *Stat1* and *Tcf3*, tended to decrease, although not significantly, in contrast with those from untreated LβT2 cells (Fig. 6b).

Effect of 2DG or AICAR on AMPK activation in LβT2 cells

Glucoprivation induced by 25 mM 2DG enhanced the phosphorylation of AMPK (Thr172) in LβT2 cells (Fig. 7). Furthermore, artificial AMPK activation by 0.1 or 0.2 mM AICAR, an AMP analog, also enhanced phosphorylation of AMPK (Thr172). Both 2DG and AICAR induced a significant increase in phosphorylated AMPK.

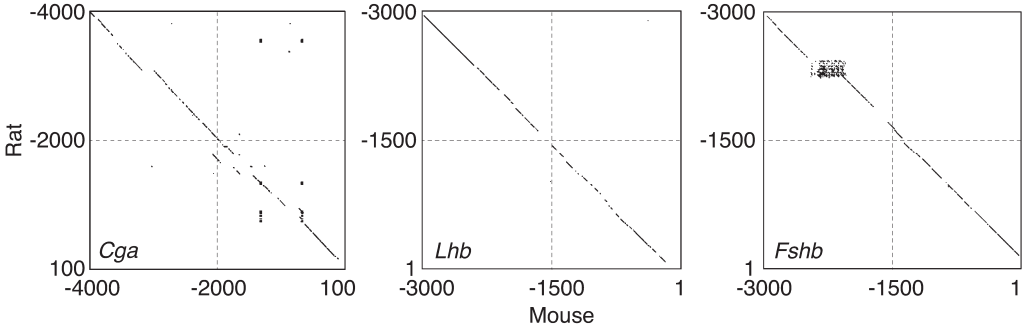

Fig. 2. Nucleotide sequence homology between the mouse and rat 5'-flanking regions, up to 4.0 kb from the transcription initiation sites of the glycoprotein α-subunit (*Cga*) gene and up to 3.0 kb from the transcription initiation sites of luteinizing hormone β-subunit (*Lhb*) and follicle-stimulating hormone β-subunit (*Fshb*) genes determined by Harr-plot analysis. Each dot represents > 85% identity in 20 nucleotides. The vertical scale indicates the 5'-flanking region of the rat gene and the horizontal scale that of the mouse gene.

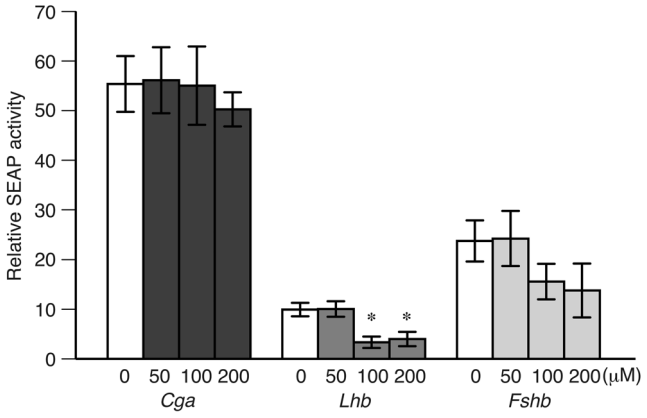

Fig. 3. Transient transfection assay of rat gonadotropin subunit gene promoters with or without treatment with the AMP-activated protein kinase (AMPK) activator 5-amino-imidazole carboxamide riboside (AICAR) in LβT2 cells. Reporter constructs containing *Cga* (–3793 to +37), *Lhb* (–2930 to +17), or *Fshb* (–2824 to +28) promoters fused with the secreted alkaline phosphatase (SEAP) gene in the pSEAP-Basic vector were transfected into LβT2 cells. The cells were exposed to 50, 100, or 200 μM AICAR. The reported activities are presented as the SEAP activity relative to that of the basic vector. Values are the mean ± SEM of four independent experiments. * $P < 0.05$ *vs*. pSEAP2-Basic.

Discussion

The present study demonstrated that AICAR down-regulated *Lhb* transcription in LβT2 cells. The result indicates that the AMPK signaling pathway directly or indirectly inhibits *Lhb* transcription, to regulate the H-P-G axis at the pituitary level. This inhibition seems to be independent of GnRH-R, because AICAR did not alter the mRNA level of *Gnrh-r* in this study. Considering that protein synthesis requires the recruitment of mRNA, it would have a close correlation between transcription and translation rates in general. Thus, the AMPK signaling pathway might down-regulates not only *Lhb* transcription but also LHβ protein translation and subsequent LH synthesis in LβT2 cells. In fact, it has been reported that AICAR induces not only suppression of *Lhb* transcription but also LH secretion in LβT2 cells [3]. On the other hand, FSH would ordinarily be synthesized in sufficient quantity in LβT2 cells despite the administration of AICAR, given the abundant Cga. In addition, the regulatory mechanism of transcription of gonadotropin hormone subunit genes induced by AMPK activation may be at least partly similar or the same among murine species, especially between rats and mice, because rats and mice share a high degree of identity in their nucleotide sequences in 5′-flanking regions until approximately 4.0 kb upstream from the transcription initiation site of *Cga* gene and approximately 3.0 kb of *Lhb*, and *Fshb* genes. Indeed, the results from the mouse mRNA expression assay were almost the same as those from the rat promoter assay in this study. Tosca *et al.* [14] reported

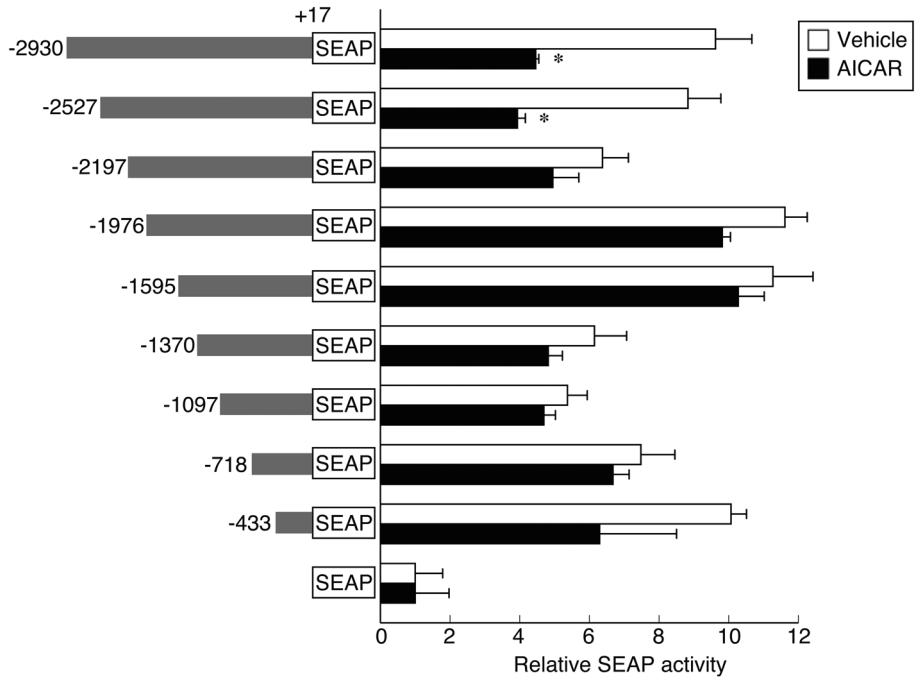

Fig. 4. Deletion analysis of the rat luteinizing hormone β-subunit (*Lhb*) promoter region (–2930 to +17 b regions) with or without treatment with the AMP-activated protein kinase (AMPK) activator 5-amino-imidazole carboxamide riboside (AICAR) in LβT2 cells. The left portion shows reporter constructs containing serial deletion mutants of the *Lhb* promoter fused to the secreted alkaline phosphatase (SEAP) gene in the pSEAP2-Basic vector that were transfected into LβT2 cells. The right portion shows SEAP activities relative to that of the basic vector. Values are the mean ± SEM for four independent experiments. * $P < 0.05$ *vs*. pSEAP2-Basic.

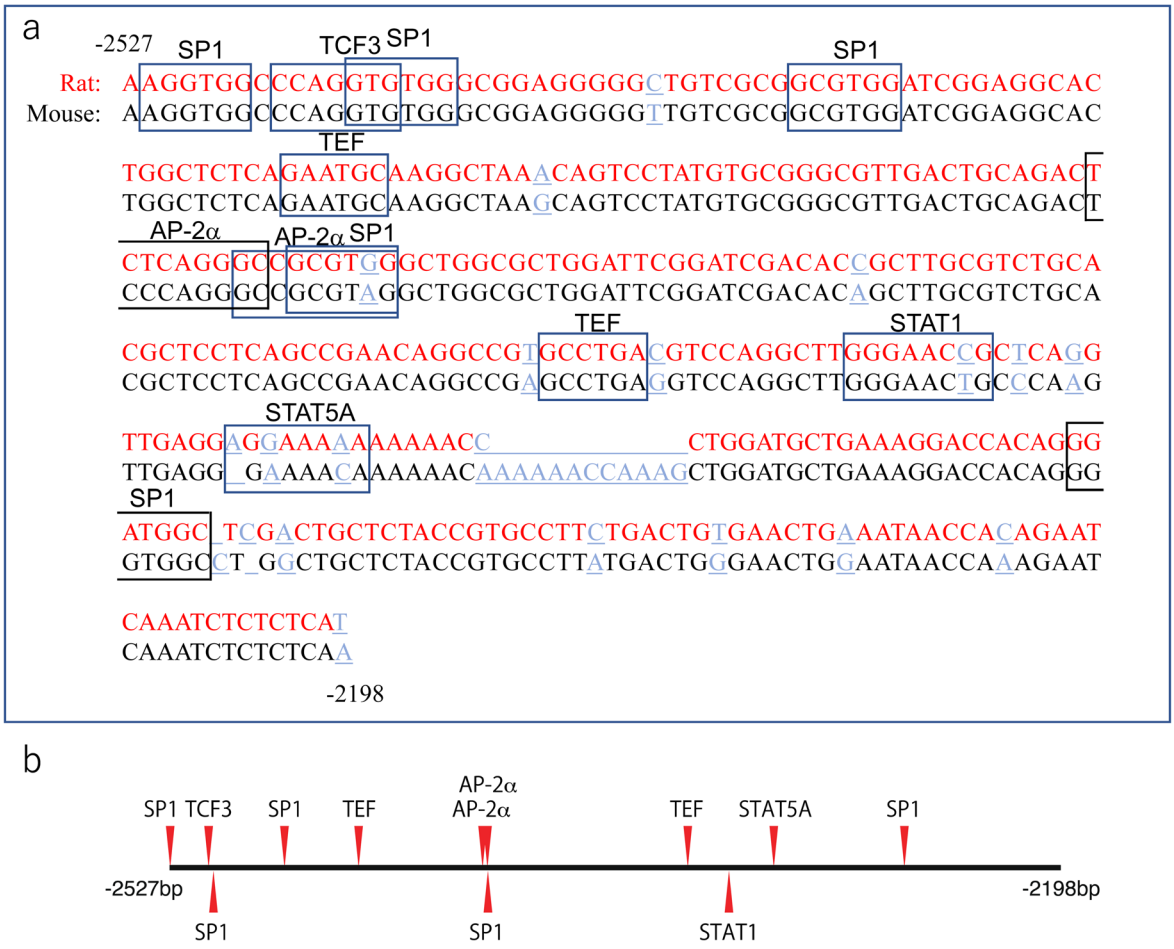

Fig. 5. Nucleotide sequence alignment (a) and location of gene transcription factor binding sites (a, b) in the –2527 to –2198 b region upstream *Lhb* promoter that was responsive to the AMP-activated protein kinase (AMPK) activator 5-amino-imidazole carboxamide riboside (AICAR). The rat luteinizing hormone β-subunit (*Lhb*) promoter was compared with that of the mouse. Ap-2α, Activating enhancer binding protein 2α; SP1, specificity protein 1; STAT1, signal transducer and activator of transcription 1; STAT5A, signal transducer and activator of transcription 5A; TCF3, transcription factor 3; TEF, thyrotroph embryonic factor.

Table 2. Expression pattern of transcription factor genes that can bind to upstream site (–2527/–2198) of rat luteinizing hormone β-subunit (*Lhb*) in the rat pituitary

Gene title	Gene symbol	Ratio per each median											
		E12.5	E13.5	E14.5	E16.5	E18.5	E20.5	P0	P5	P15	P30	P60	P600
Activating enhancer binding protein-2, alpha	*AP-2a*	0	0	0	0	0	0	0	0	0	0	0	0
Specificity protein 1	*Sp1*	11	12	11	9	9	8	8	8	7	7	7	7
Signal transducer and activator of transcription 1	*Stat1*	3	3	4	4	4	5	6	7	10	12	14	11
Signal transducer and activator of transcription 5A	*Stat5a*	1	2	2	1	1	1	2	2	1	2	2	2
Transcription factor 3	*Tcf3*	5	4	4	2	2	2	2	1	1	1	1	1
Thyrotroph embryonic factor	*Tef*	2	1	2	2	3	3	4	5	5	4	3	5

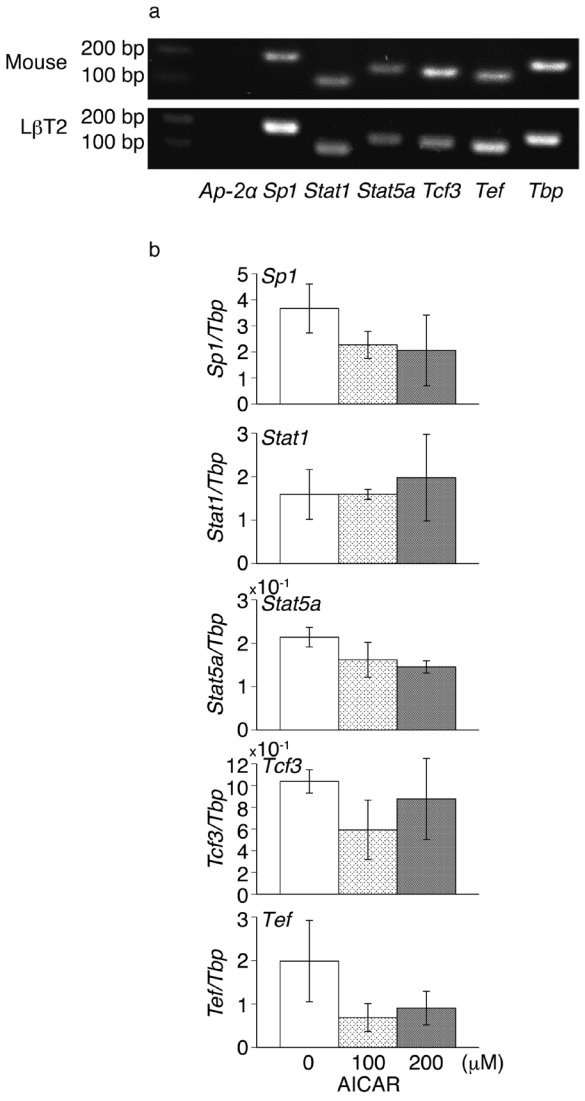

Fig. 6. a) Gene expression, determined by RT-PCR, of the transcription factors, activating enhancer binding protein 2α (*Ap-2α*), specificity protein 1 (*Sp1*), signal transducer and activator of transcription 1 (*Stat1*), signal transducer and activator of transcription 5A (*Stat5a*), transcription factor 3 (*Tcf3*), and thyrotroph embryonic factor (*Tef*), that might bind to the –2527 to –2198 b region upstream of the rat luteinizing hormone β-subunit (*Lhb*) promoter in mouse and LβT2 cells. b) Real-time PCR of the same transcription factors, except for *Ap-2α*, after a 48-h treatment with 100 or 200 μM 5-amino-imidazole carboxamide riboside (AICAR). Data are expressed as the mean ± SEM for four independent experiments. Each mRNA value was normalized using the TATA-box binding protein (*Tbp*) mRNA level as a reference. * $P < 0.05$ *vs*. 0 μM AICAR (control).

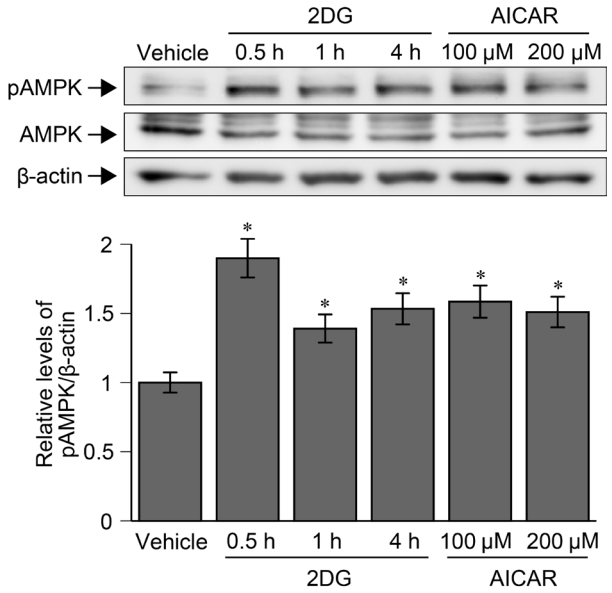

Fig. 7. AMP-activated protein kinase (AMPK) phosphorylation is stimulated by 2-deoxy-*D*-glucose (2DG) and 5-amino-imidazole carboxamide riboside (AICAR). LβT2 cells were treated with either 25 mM 2DG for 0.5, 1, or 4 h, or with 100 or 200 μM AICAR for 24 h. Total cell lysates were extracted and subjected to immunoblotting using anti-phospho-AMPKα (Thr172), anti-AMPKα, and anti-β-actin antibodies. Densitometric analysis was performed in three experiments, and phospho-AMPKα (pAMPK) was normalized for β-actin. Data are expressed as the mean ± SEM. * $P < 0.05$ *vs*. Vehicle.

that metformin, an indirect activator of AMPK, inhibited not only *Lhb* mRNA expression but also *Fshb* in rat pituitary cell cultures. The different in results between the prior and the present studies may reflect the different cell types and drugs used. In the present study, we showed the effect of AICAR on a mouse gonadotrope cell line. Cell lines often alter their phenotype, native functions, and their responsiveness to stimuli [19]. Indeed, the altered responsiveness to stimuli between gonadotrope and rodent pituitary cell cultures was previously reported [20]. AICAR is recognized as an AMP mimetic activator of the AMPK that directly activates AMPK [15], whereas metformin is recognized as an indirect activator of the AMPK that induces AMPK activation through inhibition of complex I of the respiratory chain in mitochondria [21]. These differences in cell types and drugs would result in a different outcome. Therefore, it is necessary to keep in mind that the murine gonadotropin transcription control mechanism under the physiological condition may differ somewhat from the present results. Indeed, the present study showed that the promoter activities of *Fshb* tended to be repressed. The results concerning the AMPK response in the transcription of the murine gonadotropin hormone subunit gene are still not conclusive.

The –2527 to –2198 b region of the *Lhb* gene was identified as a novel region for the transcriptional control by AMPK. This region may contain several putative regulatory elements for diverse transcription factors, including SP1, STAT1, STAT5a, TCF3, and TEF. The previous studies showed that AMPK activation decreases the protein level of SP1 and STAT1 in many types of cells [22–25]. In this study, however, we observed the decreasing trend in *Sp1*, *Stat5a,* and *Tef* mRNA, but not in *Stat1*. Therefore, further investigations of the suppression by AMPK activation should clarify the role of molecules and their mechanisms through the –2527 to –2198 bp upstream region of the *Lhb* gene.

Metabolic disorders suppress pulsatile LH secretion in mammals

[26–28]. Several studies have also reported that an energy sensor regulating the H-P-G axis exists in the brain [29–31]. AMPK is recognized as a fuel gauge that is activated in response to fasting or glucoprivation. Presently, 2DG-induced glucoprivation induced AMPK phosphorylation in LβT2 cells. This result suggests that mouse gonadotropic cells directly sense glucoprivation to regulate the H-P-G axis. Furthermore, we previously described that LβT2 cells directly responded to long-chain fatty acid levels to regulate the transcription of gonadotropic hormones [5] and the expression of the long-chain fatty acid receptor GPR120 in mouse pituitary gonadotropes [32]. Thus, gonadotropes may sense not only blood glucose levels but also peripheral free fatty acid levels in the pituitary gland. Lu *et al.* [3] reported that adiponectin decreased LH secretion in the pituitary gonadotropes in an AMPK-dependent manner. Therefore, both the synthesis and secretion of gonadotropic hormones may be directly regulated by peripheral signals, such as hormones and nutrition, at the pituitary level.

In conclusion, the present study reveals that the transcription of murine *Lhb* is inhibited by AMPK activation. Furthermore, AMPK repressive regulation of the murine *Lhb* gene expression involves the 5′-flanking region between –2527 and –2198 b.

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