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The cathepsin L gene is a direct target of FOXO1 in skeletal muscle

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FOXO1 (forkhead box O1), a forkhead-type transcription factor whose gene expression is up-regulated in the skeletal muscle during starvation, appears to be a key molecule of energy metabolism and skeletal muscle atrophy. Cathepsin L, a lysosomal proteinase whose expression is also up-regulated in the skeletal muscle during starvation, is induced in transgenic mice overexpressing FOXO1 relative to wild-type littermates. In the present study, we conducted *in vivo* and *in vitro* experiments focusing on FOXO1 regulation of *Ctsl* (cathepsin L gene; *CTSL1* in humans) expression in the skeletal muscle. During fasting and refeeding of C57BL/6 mice, *Ctsl* was regulated in parallel with FOXO1 in the skeletal muscle. Fasting-induced *Ctsl* expression was attenuated in transgenic mice overexpressing a dominant-negative form of FOXO1 or in skeletal-muscle-specific *Foxo1*-knockout mice relative to respective wild-type controls. Using C2C12 mouse myoblasts overexpressing a constitutively active form of FOXO1, we showed that FOXO1 induces *Ctsl* expression. Moreover, we found FOXO1-binding sites in both the mouse *Ctsl* and human *CTSL1* promoters. The luciferase reporter analysis revealed that the mouse *Ctsl* and human *CTSL1* promoters are activated by FOXO1, which is abolished by mutations in the consensus FOXO1-binding sites. Gel mobility-shift and chromatin immunoprecipitation assays showed that FOXO1 is recruited and binds to the *Ctsl* promoter. The present study provides *in vivo* and *in vitro* evidence that *Ctsl* is a direct target of FOXO1 in the skeletal muscle, thereby suggesting a role for the FOXO1/cathepsin L pathway in fasting-induced skeletal muscle metabolic change and atrophy.

Key words: atrophy, cathepsin L, forkhead box O1 (FOXO1), forkhead transcription factor, muscle metabolism, starvation.

INTRODUCTION

The skeletal muscle is the largest organ in the human body, with important roles in exercise, glucose uptake and energy expenditure. Skeletal muscle metabolism is changed by the supply of nutrients and circulating hormones [1,2]. Starvation and disease states (such as diabetes and cancer cachexia) lead to a rapid reduction in skeletal muscle mass (atrophy) [2]. What is the physiological role of muscle atrophy? As the brain mainly uses glucose as an energy source, during starvation it needs to be supplied with glucose. Thus, for short periods of fasting, skeletal muscle increases utilization of lipids instead of glucose. On the other hand, for longer periods of fasting or starvation resulting in muscle atrophy, skeletal muscle protein is degraded and mobilized as a source of amino acids for gluconeogenesis that occurs mainly in the liver [3].

The FOXO (forkhead box O) members FOXO1, FOXO3a and FOXO4 belong to a subfamily of the forkhead transcription factors [4,5]. The FOXO family regulates a variety of biological processes such as metabolism, cell proliferation, apoptosis, stress response and longevity [6–9]. FOXO1 activates gluconeogenic enzyme genes in the liver, such as those for PEPCK (phosphoenolpyruvate carboxykinase) and G6Pase (glucose-6-phosphatase). A dominant-negative form of FOXO1 (DN-FOXO1), which contains the DNA-binding domain, but lacks the transcriptional activation domain, suppressed the fasting-induced increase of *Pepck* and *G6Pase* expression in liver cells [10]. We showed previously that energy-deprived conditions in mice, such as fasting and diabetes, up-regulated expression of *Foxo1* in skeletal muscle of mice [11]. Several FOXO1 target genes have been identified in skeletal muscle. For instance, FOXO1 up-regulates *PDK4* (pyruvate dehydrogenase kinase 4), a kinase that suppresses glycolysis [12], and *LPL* (lipoprotein lipase), an enzyme that increases lipid incorporation [11], and down-regulates *SREBP1c* (sterol-regulatory-element-binding protein 1c), a master regulator of lipogenesis [13]. The FOXO1 target genes may be involved in the utilization of lipids instead of glucose in the skeletal muscle. On the other hand, forced expression of FOXO1 or FOXO3a up-regulates the expression of a variety of atrophy-related genes including the *MuRF1* and *atrogin/MAFbx* ubiquitin ligases [14,15], as well as *Bnip3* and *LC3*, important molecules for autophagy [16,17], thus inducing skeletal muscle atrophy *in vitro* and *in vivo*. We have created transgenic mice that overexpress FOXO1 in skeletal muscle (FOXO1 mice) and found

Abbreviations used: ChIP, chromatin immunoprecipitation; DBE, DAF16 (decay-accelerating factor 16)-binding element; DMEM, Dulbecco's modified Eagle's medium; DN-FOXO1, dominant-negative forkhead box O1; ER, oestrogen receptor; FBS, fetal bovine serum; FOXO, forkhead box O; GADD45α, growth-arrest and DNA-damage-inducible protein 45α; G6Pase, glucose-6-phosphatase; HEK, human embryonic kidney; PEPCK, phosphoenolpyruvate carboxykinase; PLSD, protected least-significant difference; TAM, tamoxifen.

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that they exhibit skeletal muscle atrophy [18]. Moreover, FOXO1 has been shown to induce *Gadd45a* (growth-arrest and DNA-damage-inducible protein 45α) [19], a suppressor of the cell cycle, thereby facilitating skeletal muscle atrophy. Thus identification and functional analysis of FOXO1 target genes will help facilitate a better understanding of skeletal muscle metabolism.

Cathepsin L is a lysosomal proteinase, whose expression is up-regulated during various forms of skeletal muscle atrophy including starvation [20–22]. In the skeletal muscle of the FOXO1 mice, *Ctsl* expression was markedly increased [18]. Earlier findings showed that lysosomal proteolysis is activated upon skeletal muscle atrophy [23,24]. Although circumstantial evidence suggests that cathepsin L is involved in skeletal muscle atrophy, to our knowledge, there are no reports on the regulation of *Ctsl* by FOXO1. In the present study, we provide *in vivo* and *in vitro* evidence that *Ctsl* is a direct target of FOXO1 in skeletal muscle.

EXPERIMENTAL

Genetically modified animals

The human skeletal muscle α-actin promoter [25] was kindly provided by Dr E.C. Hardeman and Dr K. Guven (Children's Medical Research Institute, Westmead, NSW, Australia). DN-FOXO1, a mutant version of FOXO1 containing amino acid residues 1–256, has been described previously [26]. Transgenic plasmid containing the cDNA for DN-FOXO1 (see Figure 2A) was excised and purified for injection (at $2\,ng \cdot \mu l^{-1}$) [18]. Fertilized eggs were recovered from C57BL/6 females crossed with C57BL/6 males and microinjected at Japan SLC Inc. (Hamamatsu, Japan). To obtain skeletal-muscle-specific *Foxo1*-knockout mice, we inactivated *Foxo1* expression in the skeletal muscle by crossing mice homozygous for a floxed *Foxo1* allele with myogenin-cre transgenics. Myogenin-cre and *Foxo1*^{flox} mice were as described previously [27]. The mice were maintained at a constant temperature of 24°C with fixed artificial light (12 h light/12 h dark). All animal experiments were conducted in accordance with the guidelines of Tokyo Medical and Dental University Committee on Animal Research (No. 0090041) and National Institute of Health and Nutrition (No. 0706).

C2C12 cells and cell cultures

C2C12 mouse myoblasts (RIKEN Cell Bank, Tsukuba, Japan) were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) FBS (fetal bovine serum) until the cells reached confluence. The medium was then replaced with DMEM containing 2% (v/v) horse serum (differentiation medium) and incubated for 4 days to induce the formation of myotubes before each experiment. C2C12 myoblasts stably expressing FOXO1–ER (oestrogen receptor) fusion proteins were obtained as described previously [28]. In brief, C2C12 cells were stably transfected with the empty pBABE retrovirus or pBABE vectors expressing fusion proteins containing a constitutively active form of human FOXO1 [FOXO1(3A)] [26] [where three Akt phosphorylation sites (Thr^{24}, Ser^{256} and Ser^{319}) are replaced by alanine residues] in-frame with a modified TAM (tamoxifen)-specific version of the murine ER-ligand-binding domain. FOXO1–ER plasmid was provided by Dr Terry G. Unterman (Department of Medicine, University of Illinois at Chicago, U.S.A.) Cells were selected with puromycin and colonies were pooled for studies, as reported previously [13]. The fusion proteins are restricted to the cytoplasmic space until activation by treatment with TAM [28].

Quantitative real-time PCR

Quantitative real-time PCR was performed as described previously [13]. Total RNA was prepared using Sepazol. cDNA was synthesized from 5 μg of total RNA using ReverTra Ace® (TOYOBO) with random primers. Gene expression levels were measured with an ABI PRISM 7700 Sequence Detection System using SYBR Green PCR Core Reagents (Applied Biosystems). Levels of mRNA were normalized to those of *36B4* mRNA. The primers used were as follows. Cathepsin L: forward, 5′-TCTCACGCTCAAGGCAATCA-3′, reverse, 5′-AAGCAAAATCCATCAGGCCTC-3′; GADD45α: forward, 5′-CGTAGACCCCGATAACGTGGTA-3′, reverse, 5′-CGGATGAGGGTGAAATGGAT-3′; FOXO1: forward, 5′-ATTCGGAATGACCTCATGGA-3′, reverse, 5′-GTGTGGGAAGCTTTGGTTGG-3′; DN-FOXO1 (transgene specific): forward, 5′-GACTACAAGGACGACGATGA-3′, reverse, 5′-AGCGGCTCGAAGTCCGGGTC-3′, FOXO3a: forward, 5′-TCTGCGGGCTGGAAGAACT-3′, reverse, 5′-CTCTTGCCCGTGCCTTCAT-3′; FOXO4: forward, 5′-ATGGATGGTCCGCACGGTG-3′, reverse, 5′-CTTGCCAGTGGCCTCGTTG-3′; and 36B4: forward, 5′-GGCCCTGCACTCTCGCTTTC-3′, reverse 5′-TGCCAGGACGCGCTTGT-3′.

Cloning of the mouse *Ctsl* and human *CTSL1* promoters

The mouse *Ctsl* promoter has been described previously [29]. The 4-kb mouse *Ctsl* promoter region was excised with BamHI from pMEPCAT3 and cloned into a pGL3-basic luciferase vector (Promega Corporation). The 4-kb mouse promoter was sequenced. The human *CTSL1* promoter [30,31] was obtained by PCR from genomic DNA of HEK (human embryonic kidney)-293 cells. The PCR primers used were 5′-GTGGTGCGCGCCTGTAGTCC-3′ and 5′-GGCGCACTCCACGGATGCCG-3′. Mutations in the promoter sequences were introduced using a QuikChange® site-directed mutagenesis kit (Stratagene). Primers used were human DBE1: 5′-CTGGGACAGTCAGTGGGCAAGCCACGAACC-3′; human DBE2: 5′-GGGACAGTCAGTGGGCAAGCCACGAACC-3′; and mouse DBE: 5′-GTGATAGACTGAGTGGGCAAACATACAAAG-3′. DBE is DAF16 (decay-accelerating factor 16)-binding element, to which FOXO1 binds [32].

Transfection and luciferase assay

HEK-293 cells were plated at a density of 10^5 cells/12-well plate in DMEM containing 10% (v/v) FBS. Luciferase gene constructs containing a *Ctsl* promoter fragment with or without mutations of putative FOXO1-binding sites were prepared. The luciferase reporter plasmid (0.8 μg), the expression plasmid [pCAG-FOXO1(3A) or empty pCAG, 0.8 μg], and a phRL-TK vector (25 ng; Promega) as an internal control for transfection efficiency, were transfected into HEK-293 cells using Lipofectamine™ 2000 (Invitrogen). After an overnight transfection period, cells were lysed and assayed for luciferase activity using the dual-luciferase assay kit (Promega). The activity was calculated as the ratio of firefly luciferase activity to *Renilla* luciferase activity (internal control) and expressed as the average of triplicate experiments.

Gel mobility-shift assay

The gel mobility-shift assay was performed as described previously [33]. *In-vitro*-translated human FOXO1 was generated from pCMX-FOXO1, using the TNT® T7

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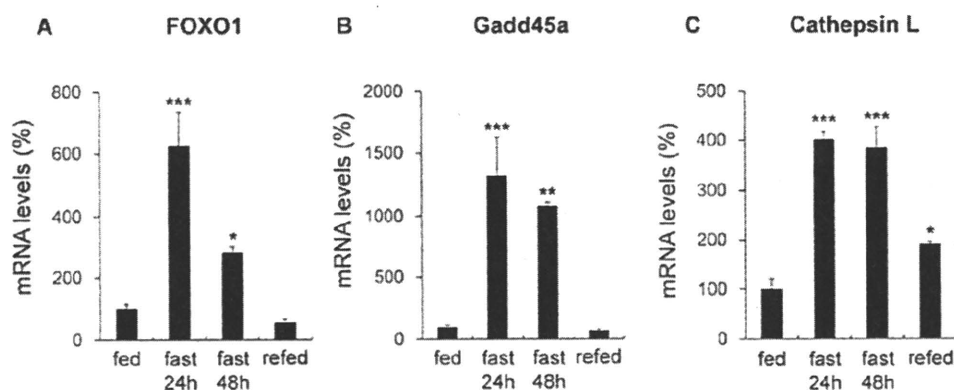

Figure 1 ***Foxo1*, *Gadd45a* and *Ctsl* expression in skeletal muscle of fasted and refed mice**

Mice (C57BL6, male, 8 weeks of age) were divided into four experimental groups of four mice each. They were either allowed to eat freely (fed), or subjected to a 24 or a 48 h fast. Others were subjected to a 48 h fast followed by 8 h of feeding (refed). Mice were killed, and relative mRNA levels of *Foxo1* (endogenous), *Gadd45a* and *Ctsl* in skeletal muscle (gastrocnemius) were analysed by quantitative real-time PCR. Levels of mRNA were normalized to those of *36B4* mRNA. Values of fed samples were set at 100. $^{*}P < 0.05$, $^{**}P < 0.01$; and $^{***}P < 0.001$ compared with the fed group.

Quick Coupled Transcription/Translation System (Promega) according to the manufacturer's instruction. Double-stranded oligonucleotide probes used in gel mobility-shift assays were prepared by annealing both strands of each putative FOXO1-binding site in the human *CTSL1* promoter (DBE1: 5′-ATCTCCAAAATAGTAAACAAATTCCTGCAG-3′, −145 to −152, numbering the first nucleotide of exon 1 as +1; DBE2: 5′-GGGACAGTCAGTAAACAAGCCACGAACC-3′, −1400 to −1407, numbering the first nucleotide of exon 1 as +1; DBE1 mutant: 5′-ATCTCCAAAATAGTGGGCAA-ATTCCTGCAG-3′; and DBE2 mutant: 5′-GGGACAGTC-AGTGGGCAAGCCACGAACC-3′; underlining indicates sites of mutation) and labelling with [γ-^{32}P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Roche Applied Science). The labelled probes (50000 d.p.m.) were incubated with extracts containing *in-vitro*-translated FOXO1 in a mixture (total volume of 25 μl) containing 10 mM Tris/HCl (pH 7.5), 50 mM NaCl, 1 mM DTT (dithiothreitol), 1 mM EDTA and 4.4 % glycerol with 1 mg of poly(dI-dC)·(dI-dC) for 30 min on ice and then separated by electrophoresis on a 6 % polyacrylamide gel in 45 mM Tris/HCl (pH 8.0), 45 mM borate and 1 mM EDTA. After electrophoresis, gels were dried and analysed with a BAS-2500 (Fuji Film).

ChIP (chromatin immunoprecipitation) assay

ChIP was carried out using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's guidelines [13,34]. Briefly, C2C12 myoblasts stably expressing FOXO1(3A)–ER were incubated for 24 h with or without 1 μM TAM. Proteins were cross-linked to DNA with the addition of formaldehyde (1 % final concentration). Cells were washed and lysed in SDS lysis buffer, sonicated for 10 s and allowed to recover for 30 s over ice (this was repeated seven times). Lysates were cleared with Protein A–agarose for 30 min, pelleted and incubated overnight with an anti-FOXO1 antibody (sc-11350; Santa Cruz Biotechnology). Before the incubation, input samples were removed from the lysate and stored at 4 °C until extraction. Following incubation with the antibody, protein–DNA complexes were eluted (1 % SDS and 0.1 M $NaHCO_3$), and the cross-links were reversed. DNA was purified by phenol/chloroform extraction. PCR primers were designed to locate DBE of the *Ctsl* promoter: forward, 5′-AAAAGACAAGAGGATGCCTT-3′, and reverse, 5′-CTGGTGTTCTCAGGTTAGTC-3′. The amplified region was −3670 to −3339, numbering the first nucleotide of exon 1 as +1. PCR primers were also designed to locate non-DBE of the *Ctsl* promoter: forward, 5′-CCACGAAAAGAATTTCTACCA-3′ and reverse, 5′-AGTTGTAGATTAAAATGTGCAG-3′. The amplified region was −439 to −289, numbering the first nucleotide of exon 1 as +1.

Statistical analysis

All results are expressed as means ± S.E.M. Statistical comparisons of data from experimental groups were made with a one-way ANOVA, and groups were compared using Fisher's PLSD (protected least-significant difference) test (Statview 5.0; Abacus Concepts, Berkeley, CA, U.S.A.). When differences were significant, groups were compared using Fisher's PLSD test. Statistical significance was defined as $P < 0.05$.

RESULTS

Co-ordinate regulation of *Foxo1* and *Ctsl* expression in the mouse skeletal muscle during fasting and refeeding

To analyse the *in vivo* relationship between *Foxo1* and *Ctsl* expression, we first examined their gene expression in the skeletal muscle of mice subjected to fasting and refeeding. Expression of mRNAs for *Foxo1* and *Gadd45a*, a *bona fide* FOXO1-target gene [19], was increased in skeletal muscle after 24 and 48 h of fasting (Figures 1A and 1B). *Ctsl* mRNA expression was also markedly increased in the skeletal muscle during fasting (Figure 1C). The effect of fasting was reversed by refeeding. *Foxo1* and *Ctsl* mRNA levels were increased in various regions of skeletal muscles, such as gastrocnemius (Figure 1), soleus, extensor digitorum longus and tibialis anterior (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/427/bj4270171add.htm). In other tissues, such as the brain, kidney and adipose tissue, fasting did not markedly alter *Foxo1* and *Ctsl* mRNA expression (Y. Kamei, unpublished work). These observations indicate that the expression of *Foxo1* and *Ctsl* is co-ordinately regulated in the skeletal muscle during fasting and refeeding.

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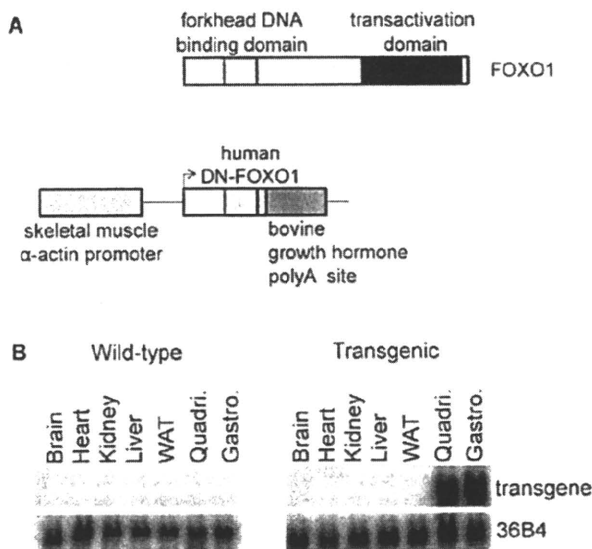

Figure 2 Creation of DN-FOXO1 transgenic mice

(**A**) The structure of FOXO1 and DN-FOXO1. DN-FOXO1 is described in the map of the 4.3-kb construct used for transgenic microinjection. The transgene was under the control of the human skeletal muscle α-actin promoter and included exon 1 and the intron of the human skeletal muscle α-actin gene as well as the bovine growth hormone polyadenylation (polyA) site. (**B**) Tissue distribution of transgene expression in DN-FOXO1 mice. RNA samples were prepared from various tissues in DN-FOXO1 and wild-type mice (male, 10 weeks of age). Northern blot analyses were conducted using the DN-FOXO1 probe and 36B4 reblotting was used as the loading control. Gastro., gastrocnemius; Quadri., quadriceps; WAT, white adipose tissue.

Transgenic mice overexpressing DN-FOXO1

Previously, we generated transgenic mice with skeletal-muscle-specific overexpression of human FOXO1 using the α-actin promoter (FOXO1 mice) [18]. Skeletal muscle in these FOXO1 mice showed an increase in *Ctsl* mRNA levels [18]. To examine the possible *in vivo* regulation of *Ctsl* by FOXO1, we also generated transgenic mice with skeletal-muscle-specific overexpression of DN-FOXO1 (Figure 2A), which suppresses FOXO1-mediated transcription. DN-FOXO1 contains the DNA-binding domain, but lacks the transcription activation domain, of FOXO1 [10,27,35]. DN-FOXO1 transgene expression was observed specifically in skeletal muscle (Figure 2B). Histologically, there was no appreciable difference in skeletal muscle between DN-FOXO1 and wild-type mice (results not shown).

Fasting-induced *Ctsl* expression is suppressed in the skeletal muscle of DN-FOXO1 mice

We used 16 DN-FOXO1 mice and 16 gender- and age-matched wild-type mice. Eight mice each were allowed to eat freely (fed) or were fasted for 24 h. *Foxo1*, *Gadd45a* and *Ctsl* expression was increased in the skeletal muscle from wild-type mice (Figure 3). In DN-FOXO1 mice, fasting-increased endogenous *Foxo1* expression was attenuated compared with wild-type mice (Figure 3A), suggesting that FOXO1 up-regulates its own gene expression. Moreover, induction of *Ctsl* as well as *Gadd45a* expression by fasting was markedly diminished in the DN-FOXO1 mice (Figures 3B and 3C). These observations indicate that FOXO1 significantly contributes to the up-regulation of *Ctsl* expression during fasting *in vivo*.

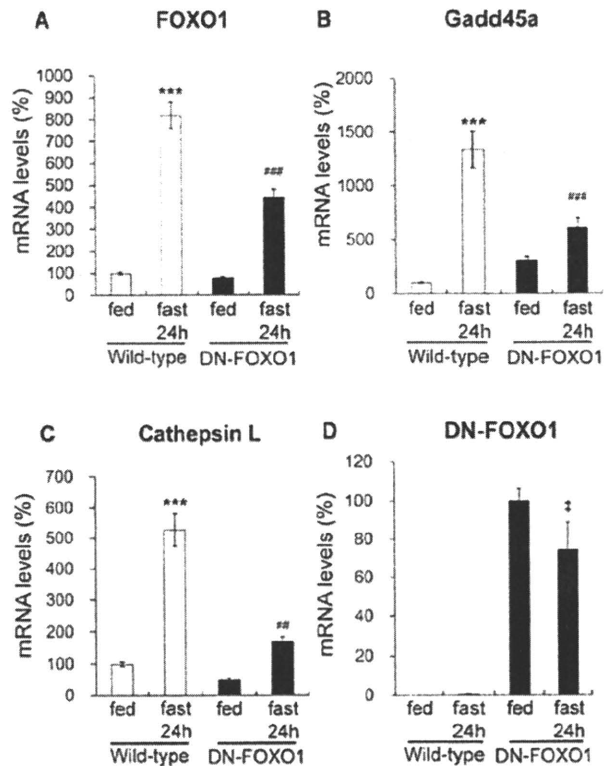

Figure 3 Gene expression in skeletal muscle of fed or fasted DN-FOXO1 mice

DN-FOXO1 mice (12 weeks of age) or age- and gender-matched wild-type mice were allowed to eat freely (fed) or subjected to a 24 h fast. The number of animals used in each group was eight. Expression of (**A**) *Foxo1* (endogenous), (**B**) *Gadd45a*, (**C**) *Ctsl* and (**D**) DN-FOXO1 (transgene) in skeletal muscle (gastrocnemius). (**A–C**) Values of wild-type mice with fed samples were set at 100. (**D**) Values of DN-FOXO1 mice with fed samples were set at 100 for DN-FOXO1 transgene. The transgene expression level was slightly decreased by fasting. Levels of mRNA were normalized to those of *36B4* mRNA. $^{***}P < 0.001$ compared with samples of wild-type fed mice. $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with samples of wild-type fasted mice. $^{\ddagger}P < 0.05$ compared with samples of transgenic fed mice. Results are representative of three independent experiments with similar results.

Fasting-induced *Ctsl* expression is suppressed in the skeletal muscle of skeletal-muscle-specific *Foxo1*-knockout mice

To examine whether the induction of *Ctsl* expression in the muscles of fasting animals is dependent on FOXO1 or not, we used muscle-specific *Foxo1*-knockout mice (myogenin-cre/*Foxo1*^{flox}) [27]. Knockout and control mice were fed or were fasted for 24 h, and expression of FOXO family members (*Foxo1*, *Foxo3a* and *Foxo4*), *Ctsl* and *Gadd45a* was examined (Figure 4). In control mice, *Foxo1*, *Foxo3a*, *Ctsl* and *Gadd45a* expression was markedly up-regulated. The *Foxo1* expression in the knockout mice that were fed was much lower than that in the control mice that were fed. We did not observe marked induction of *Foxo1* expression in the fasted knockout mice relative to the fed knockout mice. In the knockout mice, the induction of *Ctsl* expression was suppressed (Figure 4C). Expression of *FOXO4* did not differ among groups (results not shown). These observations indicate that FOXO1 is important for the up-regulation of *Ctsl* expression during fasting. However, since *Ctsl* expression during fasting was not completely suppressed, there may be additional factor(s). FOXO3a may be such a factor, as its expression was up-regulated during fasting both in control and knockout mice (Figure 4D).

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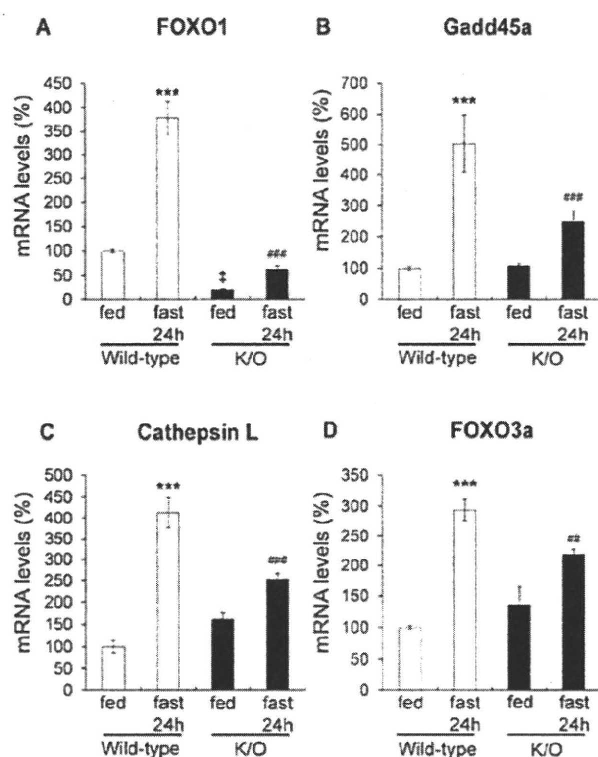

Figure 4 Gene expression in the skeletal muscle of fed or fasted muscle-specific *Foxo1*-knockout mice

Knockout (K/O) mice (4 weeks of age) or age-matched wild-type mice were allowed free access to standard chow (fed) or subjected to a 24 h fast. The number of animals used was: K/O fed, $n = 3$; K/O fast, $n = 3$; control fed, $n = 5$; control fast, $n = 4$. Expression of (**A**) *Foxo1* (endogenous), (**B**) *Gadd45a*, (**C**) *Ctsl* and (**D**) *FOXO3a* in the skeletal muscle (gastrocnemius). Values obtained in wild-type mice that were fed were set at 100. Levels of mRNA were normalized to those of *36B4* mRNA. $^{***}P < 0.001$, relative to wild-type fed mice. ## $P < 0.01$ and ###$P < 0.001$ relative to wild-type fasted mice. ‡ $P < 0.05$ relative to wild-type fed mice.

Activation of FOXO1 in C2C12 myocytes promotes *Ctsl* expression

To study the effects of FOXO1 on *Ctsl* expression in muscle cells, we first employed C2C12 cells stably expressing a constitutively active form of FOXO1 [FOXO1(3A)] in-frame with a modified form of the ER ligand-binding domain that responds selectively to TAM [28]. Previous studies with these cells have shown that fusion proteins are restricted to the cytoplasmic space in the absence of ligand and then rapidly translocate to the nucleus upon treatment with TAM [28]. Each mRNA signal in Figure 5(A) is the sum of endogenous *Foxo1* mRNA and retrovirus-derived FOXO1(3A)–ER mRNA. The endogenous *Foxo1* mRNA was very low in C2C12 cells. As expected, treatment with TAM did not change FOXO1(3A)–ER mRNA levels. Treatment with TAM resulted in a marked induction of *Gadd45a* expression, confirming that it successfully promoted the transcriptional activity of our FOXO1(3A)–ER-C2C12 myotubes. As shown in Figure 5, treatment with TAM also markedly increased the mRNA abundance of *Ctsl* as well as *Gadd45a*. No changes in *Ctsl* mRNA expression were observed in FOXO1(3A)–ER cells in the absence of TAM or in control C2C12 cells stably transfected with empty vector (Mock) (Figure 5C). These results suggest that the expression of *Ctsl* is up-regulated directly by the activation of FOXO1 in muscle cells.

The mouse *Ctsl* promoter is activated by FOXO1

The above data suggest that *Ctsl* is a direct transcriptional target of FOXO1 in muscle cells. FOXO1 is known to bind the sequence GTAAACAA or DBE [32]. We therefore examined using a transient transfection-reporter assay whether the mouse *Ctsl* and human *CTSL1* promoters are activated by FOXO1. The mouse *Ctsl* promoter has been cloned previously [29]. We sequenced the 4-kb mouse *Ctsl* promoter and found a single consensus FOXO1-binding site (GTAAACAA) (−3528 to −3535, numbering the first nucleotide of exon 1 as + 1). Plasmid constructs linking the mouse *Ctsl* promoter including the putative FOXO1-binding site to the luciferase reporter gene were analysed. FOXO1 increased the mouse *Ctsl* promoter (−4000 to + 10)-driven reporter activity (Figure 6A). Furthermore, mutation in the consensus FOXO1-binding site abolished the FOXO1-induced luciferase activity (Figure 6A). Consistent with *in vivo* transgenic mice data (Figure 3), in the *in vitro* transfection reporter assay, DN-FOXO1 dose-dependently suppressed the FOXO1(3A)-induced transcriptional activity of the mouse *Ctsl* promoter (Figure 6B). These observations suggest that FOXO1 up-regulates the mouse *Ctsl* expression via the DBE sequence in its promoter.

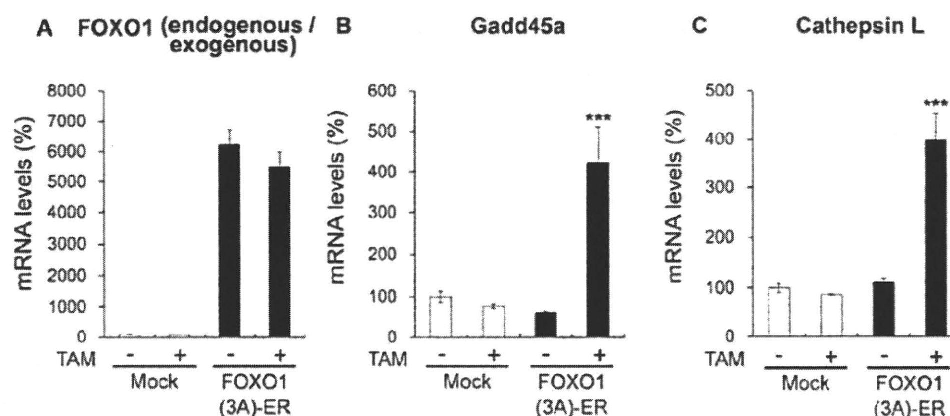

Figure 5 *Ctsl* expression by FOXO1 in C2C12 muscle cells

The abundance of mRNA transcripts for (**A**) *Foxo1*, (**B**) *Gadd45a* and (**C**) *Ctsl* in control (mock, open bars) and FOXO1(3A)–ER C2C12 cells (closed bars) treated with (+) or without (−) TAM for 24 h was analysed by quantitative real-time PCR. Levels of mRNA were normalized to those of *36B4* mRNA. $^{***}P < 0.001$ compared with samples without TAM.

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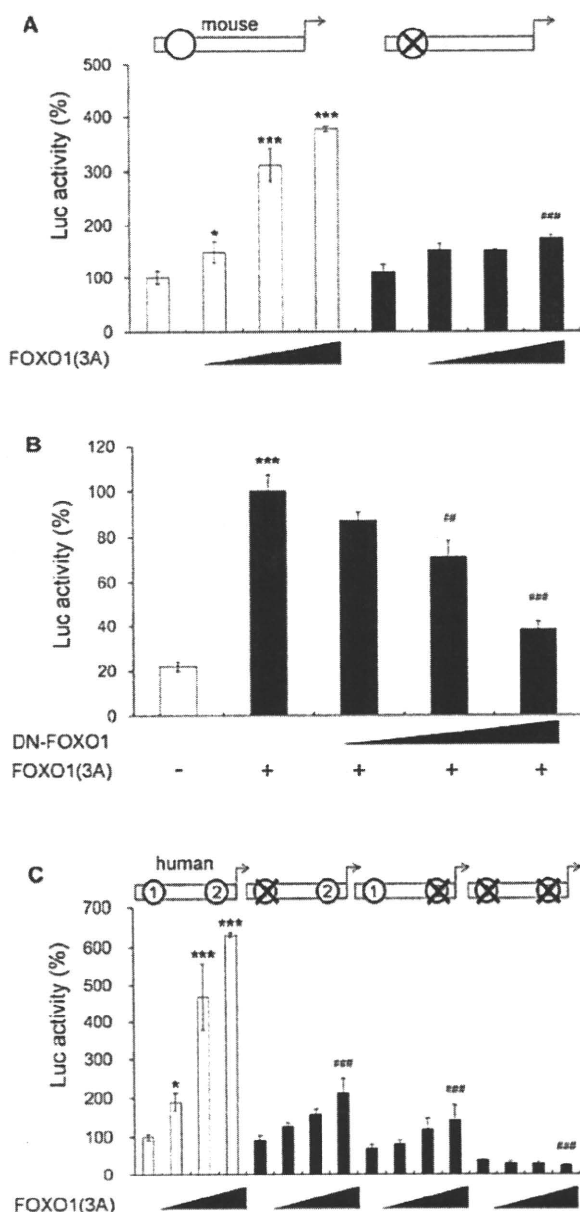

Figure 6 Transient transfection-reporter assay of the effect of FOXO1 on *Ctsl* promoters

The effect of increasing the amount of FOXO1 was examined by co-transfection with the reporter plasmids in HEK-293 cells. **(A)** The mouse *Ctsl* promoter (−4 kb) with mutations in putative FOXO1-binding sites. **(C)** The human *CTSL1* promoter (−1.6 kb) with mutations in the putative FOXO1-binding site. Activation of the luciferase reporter gene was measured in relative light units and was normalized to the dual-luciferase activity. Mean values from triplicate experiments are shown as the fold-induction, where the activity in the absence of FOXO1 is the reference value (set at 100). Schematic representations of *Ctsl* and *CTSL1* promoter constructs are shown above the histograms. Circles denote the putative FOXO1-binding sites and crosses denote mutations in the FOXO1-binding sites. Number in the circles: 1 is DBE1 (−1400 to −1407) and 2 is DBE2 (−145 to −152, numbering the first nucleotide of exon 1 as +1). $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the value of wild-type promoter in the absence of FOXO1(3A). $^{\#\#\#}P < 0.001$ compared with the values of wild-type promoter with the largest amount of FOXO1(3A). **(B)** Transient transfection assay of the DN-FOXO1 construct used to suppress FOXO1-mediated transactivation *in vitro*. The effect of DN-FOXO1 was examined by co-transfection of the reporter plasmid containing the mouse *Ctsl* promoter with or without pCAG-FOXO1(3A). An increasing amount of DN-FOXO1 suppressed FOXO1(3A)-induced *Ctsl* promoter activity. Activation of the luciferase reporter gene was measured in relative light units and normalized to the dual-luciferase activity. Mean values from triplicate experiments are shown as the fold-induction, where the activity in the absence of DN-FOXO1 and in the presence of FOXO1(3A) is the reference value (set at 100). $^{***}P < 0.001$ compared with the value in the absence of DN-FOXO1 and FOXO1(3A). $^{\#\#}P < 0.01$; and $^{\#\#\#}P < 0.001$ compared with the value in the absence of DN-FOXO1 and in the presence of FOXO1(3A).

The human *CTSL1* promoter is bound and activated by FOXO1

In the human *CTSL1* promoter [30,31], we also found two potential FOXO-binding sites; there are two perfect DBEs (−145 to −152 and −1400 to −1407, numbering the first nucleotide of exon 1 as +1). FOXO1 increased the human *CTSL1* promoter (−1600 to +10)-driven reporter activity in a transfection assay (Figure 6C). In addition, mutations in the consensus DBEs abolished the FOXO1-induced luciferase activity. Thus the results of the luciferase assay with the human *CTSL1* promoter were similar to those with the mouse *Ctsl* promoters.

We also examined the binding of FOXO1 to the DBEs in the human *CTSL1* promoter with a gel mobility-shift assay. FOXO1 that was synthesized *in vitro* clearly bound to oligonucleotides containing the putative FOXO1-binding sites of the human *CTSL1* promoter, and did not bind to oligonucleotides with mutations in the consensus DBEs (Figure 7A). Moreover, we performed a ChIP analysis using C2C12 cells expressing FOXO1(3A)–ER (as used in Figure 5), and found that FOXO1 was recruited to the mouse *Ctsl* promoter containing the DBE (Figure 7B). These observations, taken together, suggest that FOXO1 up-regulates the mouse *Ctsl* and human *CTSL1* expression via the DBE sequences of their promoters; *CTSL1* is a direct target of FOXO1 in the skeletal muscle.

DISCUSSION

FOXO1 signalling is important in linking nutritional and hormonal cascades to the regulation of skeletal muscle atrophy. As a transcriptional factor and/or cofactor, FOXO1 regulates many genes in a variety of biological processes. Identification and molecular analysis of FOXO1 target genes should help facilitate a better understanding of skeletal muscle metabolism. In the present study, we showed that FOXO1 directly activates *Ctsl* expression.

In the present study, we first conducted *in vivo* experiments focusing on FOXO1 regulation of *Ctsl* expression in skeletal muscle in the context of physiological nutritional change. During fasting and refeeding of C57BL6 mice, *Ctsl* was regulated in parallel with FOXO1 in skeletal muscle (Figure 1). Fasting-induced *Ctsl* expression was attenuated in DN-FOXO1 mice (Figure 3) and in skeletal-muscle-specific *Foxo1*-knockout mice (Figure 4) relative to respective wild-type controls. In this regard, we observed previously that *Ctsl* expression is markedly increased in skeletal muscle of FOXO1 mice [18]. Taken together, our results suggest that FOXO1 activates *Ctsl* expression *in vivo*. The increase in *Ctsl* mRNA is delayed compared with that of *Foxo1* (Figure 1). This could be explained as follows: (i) *Ctsl* mRNA may have a long half life, or (ii) *Ctsl* is activated by different transcription factors as well as FOXO1 during fasting. Indeed, it has been reported that addition of glucocorticoid, whose blood level is increased during fasting, has been reported to increase the level of *Ctsl* mRNA [20]. Cathepsin L is considered to play a major role in the terminal degradation of proteins delivered to lysosomes by endocytosis or autophagy [24,36]. A previous study shows that pharmacological inhibition in rats of both cathepsin L and calpain, an intracellular Ca^{2+}-dependent

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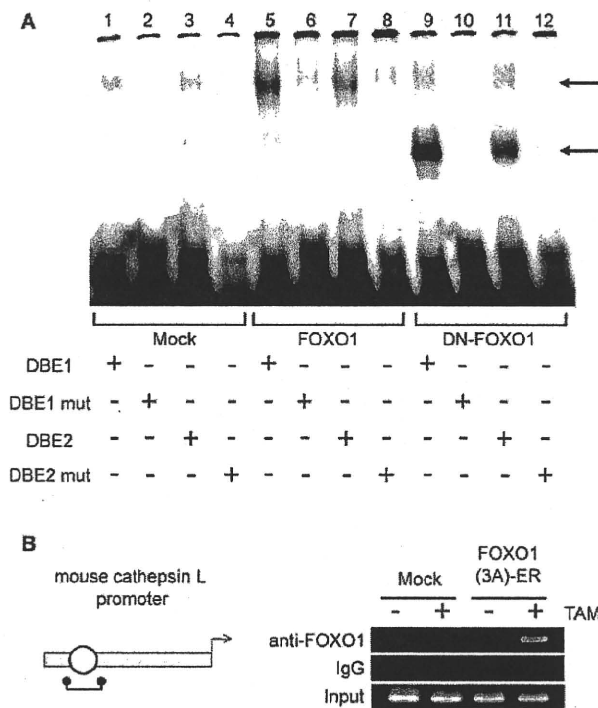

Figure 7 Recruitment of FOXO1 to the putative FOXO1-binding sites of the *Ctsl* promoter

(**A**) Gel mobility-shift assay. Synthetic double-stranded oligonucleotides containing putative FOXO1-binding sites of the human *CTSL1* promoter were used. *In vitro* synthesized FOXO1 protein was incubated with ^{32}P-labelled double-stranded oligonucleotides (DBE1, −1400 to −1407; DBE2, −145 to −152, numbering the first nucleotide of exon 1 as +1; or DBE1 mut and DBE2 mut, oligonucleotides with mutation in the consensus sequence) for 30 min on ice. The protein–DNA complexes were resolved on an 8% non-denaturing gel. FOXO1 and DN-FOXO1 were able to bind labelled *CTSL1* oligonucleotides (FOXO1, lanes 5 and 7; DN-FOXO1, lanes 9 and 11), but not mutated oligonucleotides (FOXO1, lanes 6 and 8; DN-FOXO1, lanes 10 and 12). The arrows indicate the specific protein–DNA complex. (**B**) ChIP assay. Primers specific to the region of the mouse *Ctsl* promoter containing the DBE were used for PCR analysis. FOXO1(3A)–ER was recruited to the *Ctsl* promoter in the presence of TAM. No signals were detected using control IgG. Primers corresponding to the region of non-DBE did not give any signals (not shown).

protease, prevents sepsis-induced bulk protein degradation [37] and suggests a role for cathepsin L in the degradation of various skeletal muscle proteins. It is therefore conceivable that FOXO1-induced transcriptional activation of *Ctsl* plays a role in fasting-induced autophagy and proteolysis. During fasting, a large number of genes show a change in their expression; some are changed directly as a physiological response, and others may be changed indirectly as secondary events. Nevertheless, *Ctsl* expression is likely to be regulated by FOXO1.

Using C2C12 myoblasts, we also showed that FOXO1 induces endogenous *Ctsl* expression *in vitro* (Figure 5). Moreover, we showed that FOXO1 can bind to and activate the *Ctsl* promoter (Figures 6 and 7). The promoter of *Ctsl* has been sequenced and analysed in humans, mice and rats. Transcription factors such as the specificity proteins Sp1/Sp3 have been reported to increase the basal activities of the promoter [31,38,39]. In the present study, we have provided the first evidence for transcriptional activation of the *Ctsl* promoter by an inducible transcription factor, FOXO1, thereby suggesting that *Ctsl* is a direct target gene of FOXO1. During fasting, among members of the cathepsin family, only *Ctsl* expression is markedly increased in skeletal muscle [21]. Indeed, there are no consensus DBEs in the putative promoter of other cathepsins (−1.5 kb from the transcription start sites of cathepsins B, C, D, E, G, H, J, K S and Z in humans and mice; Y. Yamazaki and Y. Kamei, unpublished work), indicating that FOXO1 specifically activates *Ctsl* during fasting. Therefore cathepsin L probably plays a role in fasting-induced adaptive responses including skeletal muscle atrophy.

FOXO1 has been shown to activate the expression of *Gadd45a*, *Pepck* and *G6Pase* via direct binding to DBE *in vitro* [19,40]. On the other hand, adenoviral introduction of DN-FOXO1 can suppress the gene expression of *Pepck* and *G6Pase* in the liver *in vivo* [10]. Previous studies suggest that FOXO1 can regulate gene expression in at least two different ways: (i) FOXO1 directly binds to and transactivates the promoter of its target genes [32], or (ii) FOXO1 interacts with other transcription factors via protein–protein interactions, without DNA binding, thereby regulating the expression of target genes [27,41]. Because FOXO1 interacts with nuclear receptors via its C-terminus [41], DN-FOXO1 can suppress the action of FOXO1 as a transcription factor without affecting its action as a transcriptional cofactor. Therefore FOXO1 appears to regulate *Ctsl* expression via a transcriptional mechanism.

We reported previously that FOXO1 mice have decreased skeletal muscle mass [18]. In the present study, there was no appreciable histological difference in skeletal muscle between DN-FOXO1 and wild-type mice. We also observed no marked difference in body weight and skeletal muscle mass between DN-FOXO1 and wild-type mice (results not shown). This may be because DN-FOXO1 does not suppress all the FOXO1 actions in a dominant-negative fashion, as described in the above. A detailed phenotypic analysis of DN-FOXO1 mice is ongoing in our laboratory.

In conclusion, the present study provides *in vivo* and *in vitro* evidence that *Ctsl* is a direct target of FOXO1 in skeletal muscle. The results provide important clues towards understanding the molecular mechanism underlying FOXO1-mediated transcriptional regulation of gene expression in skeletal muscle. Further studies will better clarify the physiological and pathophysiological implication of FOXO1-induced *Ctsl* expression in skeletal muscle.

AUTHOR CONTRIBUTION

Yasutomi Kamei, Tadahiro Kitamura, Takayoshi Suganami, Osamu Ezaki and Yoshihiro Ogawa led the design and overall implementation of the trial. Yasutomi Kamei wrote the initial draft of the paper in consultation with Yukio Hirata, Bruce Troen and Yoshihiro Ogawa. Yoshihiro Yamazaki, Satoshi Sugita, Fumiko Akaike, Sayaka Kanai, Shinji Miura and Ichizo Nishino were responsible for laboratory analyses. All authors contributed to interpretation of data and have seen and approved the final manuscript.

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Role of urocortin 2 secreted by the pituitary in the stress-induced suppression of luteinizing hormone secretion in rats

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Nemoto T, Iwasaki-Sekino A, Yamauchi N, Shibasaki T. Role of urocortin 2 secreted by the pituitary in the stress-induced suppression of luteinizing hormone secretion in rats. *Am J Physiol Endocrinol Metab* 299: E567–E575, 2010. First published July 27, 2010; doi:10.1152/ajpendo.00163.2010.—We have previously shown that urocortin 2 (Ucn 2), a member of the corticotropin-releasing factor (CRF) peptide family that binds to CRF type 2 receptor, is expressed in proopiomelanocortin (POMC) cells of rat pituitary and that its secretion and expression are increased by CRF in both the anterior and intermediate lobes and suppressed by glucocorticoids in the anterior lobe. We have also shown that Ucn 2 secreted by POMC cells acts on gonadotrophs expressing CRF type 2 receptors and inhibits the expression and secretion of gonadotropins. In the present study, we examined whether pituitary Ucn 2 is involved in stress-induced inhibition of gonadotropin secretion. A 90-min period of immobilization stress increased POMC mRNA expression without influencing Ucn 2 mRNA expression and suppressed luteinizing hormone (LH) β-subunit mRNA expression in the anterior lobe and plasma LH levels, while it increased both POMC and Ucn 2 mRNA expression in the intermediate lobe of the pituitary. Pretreatment with anti-CRF IgG blocked immobilization-induced increases in plasma ACTH and corticosterone and in POMC mRNA expression in both pituitary lobes and Ucn 2 mRNA expression in the intermediate pituitary. It also blocked immobilization-induced suppression of plasma LH and LH β-subunit mRNA expression. Pretreatment with anti-Ucn 2 IgG blocked immobilization-induced suppression of plasma LH and LH β-subunit expression without affecting immobilization-induced ACTH and corticosterone release and POMC or Ucn 2 mRNA expression. These results suggest that CRF suppresses the secretion and expression of LH probably through pituitary Ucn 2 in stress.

luteinizing hormone; corticotropin-releasing factor; pituitary

STRESS INHIBITS REPRODUCTIVE function (12, 13, 23). The hormones composing the hypothalamic-pituitary-adrenal (HPA) axis such as corticotropin-releasing factor (CRF), adrenocorticotropin (ACTH), β-endorphin, and corticosteroids reportedly play important roles in the suppressive influence of stress on reproductive function (41–43). CRF is a key stress mediator in the endocrine system, autonomic nervous system, emotion, and behavior (4, 19, 46). The various actions of CRF are mediated through two subtypes of CRF receptors (CRF-R), CRF-R1 and CRF-R2. CRF binds with a higher affinity to CRF-R1 than to CRF-R2 (4, 18). Urocortin 2 (Ucn 2) is a CRF peptide family and shows higher affinities to both CRF-R1 and CRF-R2 compared with CRF, in particular to CRF-R2 (40). Hypothalamic CRF inhibits gonadotropin-releasing hormone (GnRH) neuron activity either directly or indirectly through β-endorphin in the arcuate nucleus (42, 43). Intracerebroventricular infusion of CRF in the third ventricle inhibits the estrous cycle and ovulation and reduces immunoreactive GnRH stores in the median eminence (39). ACTH has been found to decrease the response of plasma luteinizing hormone (LH) to GnRH and inhibit the LH surge (38). Numerous GnRH neurons receive β-endorphinergic input, and β-endorphin suppresses the activity of GnRH neurons via the μ-opioid receptor (25, 47). Furthermore, several studies show that the secretion of gonadotropins is suppressed by peripheral administration of glucocorticoids in various species (16, 48). Glucocorticoids are thought to act at the hypothalamus and/or anterior pituitary to inhibit the secretion of gonadotropins (16).

We have previously demonstrated that Ucn 2 is expressed in proopiomelanocortin (POMC) cells of the anterior and intermediate lobes of rat pituitary (50) and that the expression of Ucn 2 mRNA is increased by CRF in the anterior and intermediate lobes and decreased by glucocorticoids only in the anterior lobe (31). CRF-R2 is expressed on gonadotrophs (22). We have recently reported that Ucn 2 suppresses the secretion and expression of gonadotropins and that a selective CRF-R2 antagonist and small-interfering RNA (siRNA) against CRF-R2 significantly increase the secretion and expression of gonadotropins in vitro (32). We also have shown that anti-Ucn 2 IgG significantly increases the secretion and mRNA expression of gonadotropins both in vitro and in vivo and that intraperitoneal injection of anti-Ucn 2 IgG in immature female rats induces a tendency toward earlier occurrence of menarche (32). These findings suggest that pituitary Ucn 2 is involved in the regulatory mechanism of the expression and secretion of gonadotropins. Because the amount of Ucn 2 secreted in culture media is much lower than that of ACTH, it appears that Ucn 2 secreted by POMC cells acts on gonadotrophs in a paracrine manner.

It is unclear whether Ucn 2 plays some role in stress-induced suppression of gonadotropin secretion, although the secretion of Ucn 2 by POMC cells is stimulated by CRF. We therefore attempted to clarify, in this study, whether Ucn 2 is involved in the mechanism by which stress suppresses the expression and secretion of gonadotropins. For this purpose, we examined the secretion of ACTH, corticosterone, and LH and follicle-stimulating hormone (FSH) and the expression of POMC, Ucn 2, LH, and FSH β-subunit mRNA in the pituitary in immobilization-exposed rats and tested the effect of intraperitoneal administration of anti-CRF IgG or anti-Ucn 2 IgG on the secretion and expression of these hormones in these rats.

MATERIALS AND METHODS

Animals. All procedures involving rats in this study were reviewed and approved by the Laboratory Animals Ethics Review Committee of Nippon Medical School. Male Wistar rats (7 wk old) were maintained at 23 ± 2°C on a 12:12-h light-dark cycle (lights on at 0800,

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off at 2000). They were allowed ad libitum access to laboratory chow and distilled water.

Stress exposure. For immobilization stress, rats were wrapped in a flexible wire mesh and immobilized for 30, 60, 90, or 120 min between 1300 and 1500 in an isolated room (3). Rats were killed in the adjacent room immediately after each period of immobilization; their trunk blood was collected to examine plasma ACTH, corticosterone, LH, and FSH levels; and their pituitary was divided into the anterior and posterior intermediate lobes for measurement of POMC, Ucn 2, LH, and FSH β-subunit mRNA expression. As controls, nonstressed rats were used, and they were killed 5–8 min before the decapitation of the rats exposed to 30 min of immobilization stress.

Primary culture of pituitary cells. Thirty male rats aged 6 wk were killed by decapitation, and their pituitary glands were removed under sterile conditions. The anterior pituitary lobes were collected and pooled together and then mechanoenzymatically dispersed as described previously with several modifications (32). Briefly, lobes were washed two times in PBS and then incubated at room temperature in the PBS containing 0.047 g/l $MgCl_2$, 0.1 g/l $CaCl_2$, and 0.01% dispase (Godoshusei, Tokyo, Japan) with constant stirring for 30 min. After dispersed cells were washed with PBS three times, a 1-ml aliquot of DMEM-F-12 containing 1.0×10^5 cells was placed in each well of three 48-well plates (total 144 wells). The cells were subsequently allowed to attach to the surface of the wells at 37°C with 5% CO_2 and 95% air in a humidified incubator for 4 days. On the day of the experiment, the culture medium was changed. After 4 h incubation with CRF at concentrations ranging from 1 to 10^3 pM, the culture medium from each well was collected. After centrifugation at 3,500 rpm for 10 min at 4°C to remove debris, the culture media were frozen and kept at −80°C until used. After 2, 4, and 8 h of incubation with CRF at a concentration of 10^2 pM, cells were harvested, and total RNA was extracted using a CellAmp Direct RNA Prep Kit (Takara Bio, Shiga, Japan).

Passive immunization. We had previously generated antisera against rat CRF and mouse Ucn 2. An IgG fraction was purified from each antiserum obtained after the fifth booster using a protein A sepharose column. The specificity of the anti-Ucn 2 antiserum has been described in our previous reports (32, 50). The cross-reactivity of the anti-Ucn 2 antiserum with rat CRF, Ucn 1, and Ucn 3 was 0.0, 0.014, and 0.023%, respectively. The specificity of the anti-CRF antiserum has been described in previous reports (10, 45), and the immunostaining results obtained using the anti-CRF antiserum were not influenced by 1 or 10 μg of rat Ucn 1, Ucn 2, or Ucn 3 (data not shown). Rats were injected intraperitoneally with anti-Ucn 2 IgG, anti-CRF IgG, or normal rabbit serum (NRS) IgG (1 mg/kg body wt dissolved in 1 ml of normal saline). Later (2 h), they were exposed to immobilization stress for 90 min. Rats were then killed immediately, and their trunk blood and pituitaries were collected for the hormone assays described in the following sections.

Plasma hormone assay. Trunk blood was collected into tubes containing EDTA 2Na (1 mg/ml blood) and centrifuged at 3,000 rpm for 20 min at 4°C. A 1-ml aliquot of plasma was transferred to 1.5-ml Eppendorf tubes and stored at −80°C for later measurement. Plasma ACTH, corticosterone, LH, and FSH concentrations were measured using an ACTH ELISA (M046006; MD Bioscience, St. Paul, MN), a corticosterone EIA kit (500651; Cayman Chemical, Ann Arbor, MI), a rodent LH ELISA test (ERKR7010; Endocrine Technologies, Newark, CA), and a rat FSH ELISA (AER004; Biocode-Hycel, Liege, Belgium), respectively. According to the manufacturers' literature supplied with the kits, the assay sensitivities were 5 pg/ml for ACTH ELISA, 16.4 pg/ml for corticosterone EIA, 0.5 ng/ml for LH ELISA, and 0.78 ng/ml for FSH ELISA.

RIA for plasma Ucn 2. For the extraction of Ucn 2, 0.5 ml of trunk plasma of nonstresssed or stress-exposed rats diluted with 3 ml of 5% acetic acid was applied to an Oasis HLB 60-mg column (Waters, Milford, MA) that had been washed with 2 ml of methanol and 2 ml of distilled water. The column was washed with 2 ml of methanol, and the bound fraction was eluted with 2 ml of 0.1% trifluroroacetic acid containing 60% acetonitrile. The samples were then applied to an evaporator and a lyophilizer and then dissolved in RIA buffer [0.1 M PBS containing 1 mM EDTA, 0.01% NP-40 (Nacalai Tesque, Kyoto, Japan), and 0.02% NaN_3, pH 7.4] for assay. Synthetic rat Ucn 2 (Yanaihara Institute, Shizuoka, Japan) was iodinated using the chloramine-T method and purified on a Sephadex G-50 column as previously described (50). Standard synthetic rat Ucn 2 or extracted plasma sample was incubated with anti-Ucn 2 antiserum in 5-ml plastic tubes for 24 h at 4°C. ^{125}I-labeled Ucn 2 was then added to each tube, and the reactions were incubated for another 24 h. Goat anti-rabbit γ-globulin was used to separate tracer bound to antiserum from free tracer. The anti-Ucn 2 serum was used for RIA at a final dilution of 1:350,000 to yield a maximum binding of ~30%. The intra-assay and interassay coefficients of variation were 4.0 and 9.4%, respectively. The sensitivity of Ucn 2 RIA was 0.1 ng/ml.

RNA extraction and real-time RT-PCR analysis. Total RNA was extracted from rat pituitaries using RNAiso Plus (Takara Bio). First-strand cDNA was synthesized using 0.5 μg of denatured total RNA at 37°C for 15 min, 84°C for 5 s, and 4°C for 5 min using a PrimeScript RT reagent kit (Takara Bio). PCR was performed by denaturation at 94°C for 5 s and annealing-extension at 60°C for 30 s for 40 cycles, using SYBR premix Ex Taq (Takara Bio) and specific primers for rat Ucn 2, POMC, LH and FSH β-subunit, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). To normalize each sample for RNA content, GAPDH, a housekeeping gene, was used. Diluted normal rat pituitary cDNA and the second derivative method (33) were used as the standard and for calculating C_t values.

Statistical analysis. Statistical analysis was performed using two-way ANOVA and an unpaired *t*-test using Prism 5.0 software (GraphPad Software, La Jolla, CA). Pituitary cell culture using the same experimental protocol was performed two times. For real-time RT-PCR data, all results are expressed as percent of controls. Statistical significance was established at the $P < 0.05$ level.

Table 1. *Primer sequences of the studied genes*

Genes	Primers	Length, bp	Accession No. (GenBank)
POMC	CCTCCATAGACGTGTGGAGCTG AAGGGCTGTTCATCTCCGTTG	151 (180–330)	NM_139326
Ucn 2	GAGCAACTCTAAAGCCAGCCCTTAC TGATTCCTGGCAGCCTTGTTC	112 (195–306)	NM_13385.2
LH β	TGAGCCCAAGTGTGGTGTG GGCAGCTGGCAGTACTCGAA	167 (48–214)	NM_012858
FSH β	TGAACTGACCAACATCACCATCTC CAGCCAGGCAATCTTATGGTCTC	189 (66–254)	NM_001007597
GAPDH	GGCACAGTCAAGGCTGAGAATG ATGGTGGTGAAGACGCCAGTA	143 (242–384)	NM_017008

POMC, proopiomelanocortin; Ucn 2, urocortin 2; LH, luteinizing hormone; FSH, follicle-stimulating hormone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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RESULTS

Effects of immobilization stress on pituitary hormone expression and plasma ACTH and corticosterone levels. Immobilization stress significantly increased plasma ACTH (4.41 ± 0.85-fold at 30 min and 2.42 ± 0.37-fold at 60 min, $P < 0.05$) and corticosterone (4.88 ± 1.07-fold at 30 min, 4.04 ± 0.66-fold at 60 min, and 2.26 ± 0.43-fold at 90 min, $P < 0.05$) levels compared with nonstressed controls (Fig. 1, *A* and *B*). It also significantly increased POMC mRNA expression (1.48 ± 0.03-fold at 60 min, 1.84 ± 0.19-fold at 90 min, and 1.37 ± 0.02-fold at 120 min, $P < 0.05$) in the anterior lobe of the pituitary, whereas it induced no significant change in Ucn 2 mRNA expression in the same lobe compared with nonstressed controls (Fig. 1, *C* and *D*). In contrast, immobilization stress significantly increased both POMC mRNA expression (1.70 ± 0.20-fold at 30 min, 1.88 ± 0.06-fold at 60 min, 2.20 ± 0.35-fold at 90 min, and 2.37 ± 0.10-fold at 120 min, $P < 0.05$) and Ucn 2 mRNA expression (2.71 ± 0.04-fold at 90 min and 1.84 ± 0.18-fold at 120 min, $P < 0.05$) in the intermediate lobe of the pituitary compared with that of nonstressed controls (Fig. 1, *E* and *F*). Immobilization stress significantly decreased plasma LH (46.7 ± 0.06% at 60 min, 58.3 ± 6.3% at 90 min, and 56.7 ± 3.1% at 120 min, $P < 0.05$) and pituitary LH β-subunit mRNA expression (25.0 ± 8.3% at 90 min and 75.0 ± 12.5% at 120 min, $P < 0.05$)

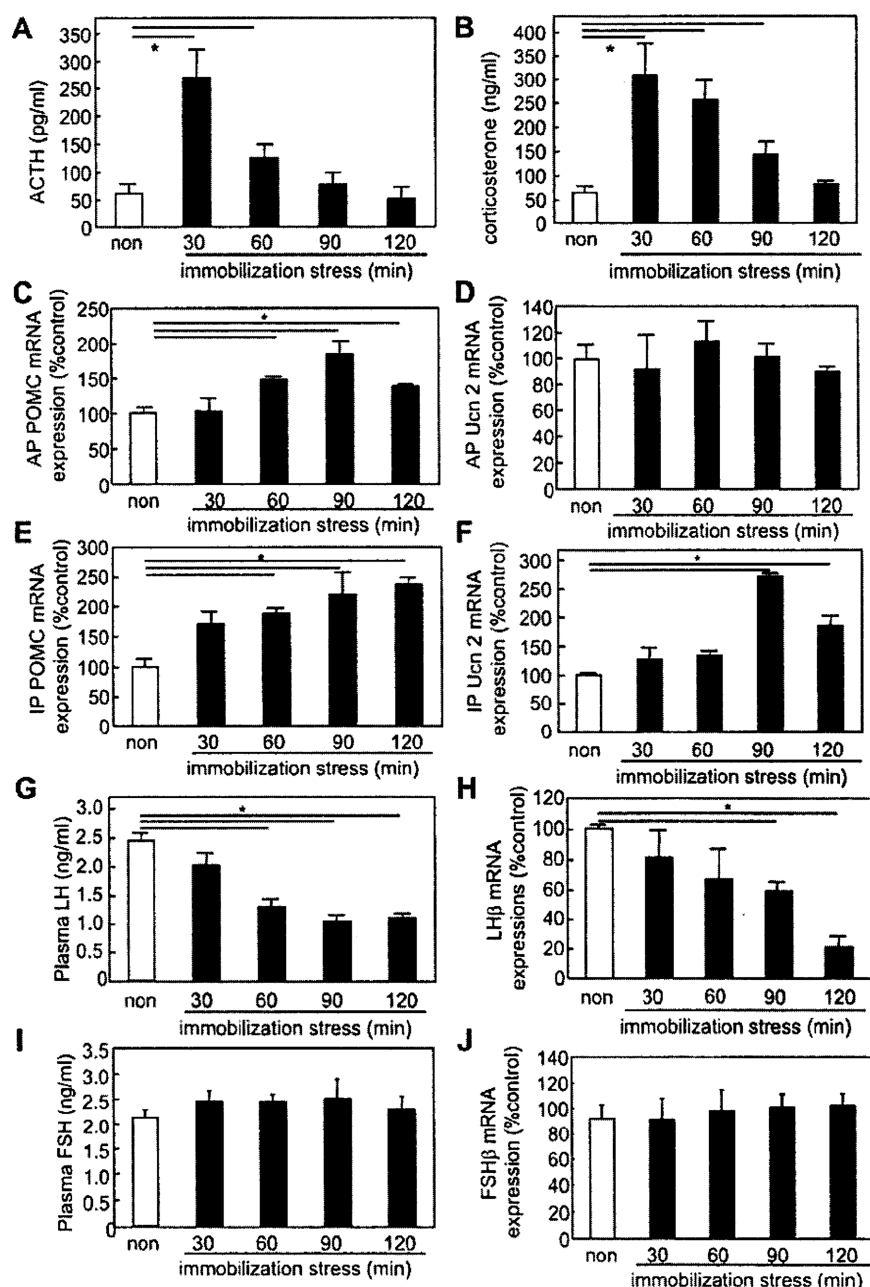

Fig. 1. Effects of 30-, 60-, 90- and 120-min immobilization stress on plasma adrenocorticotropin (ACTH), corticosterone, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) levels and on proopiomelanocortin (POMC), urocortin 2 (Ucn 2), LH β-subunit, and FSH β-subunit mRNA expression in rats. Male rats (7 wk old) were exposed to immobilization stress for 30, 60, 90, or 120 min. Their trunk blood was assayed for ACTH (*A*), corticosterone (*B*), LH (*G*), and FSH (*I*). Pituitaries were divided into anterior (AP) and intermediate (IP) pituitary, and pituitary mRNA was extracted to assay mRNA expression of POMC (*C* for anterior pituitary and *E* for intermediate pituitary), Ucn 2 (*D* for anterior pituitary and *F* for intermediate pituitary), and gonadotropin β-subunit (*H* for LH and *J* for FSH). Each mRNA expression level is shown as %nonstressed controls. The nos. of rats in each experimental group were 10 for *A* and *B* and 5 for *C–H*. *$P < 0.05$ compared with the nonstressed controls (non).

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without significant changes in plasma FSH or pituitary FSH β-subunit mRNA expression (Fig. 1, *G–J*). Plasma Ucn 2 levels did not change during the 60-min period of immobilization stress (Fig. 2).

CRF does not affect LH secretion and LH β-subunit mRNA expression in cultured anterior pituitary cells. Although incubation of monolayered anterior pituitary cells with CRF for 4 h significantly increased ACTH (1.52 ± 0.23-fold at 30 pM, 1.72 ± 0.25-fold at 10^2 pM, and 2.16 ± 0.32-fold at 10^3 pM, $P < 0.05$, $n = 8$) (Fig. 3*C*) and POMC mRNA at a concentration of 10^2 pM (1.39 ± 0.29-fold for 4 h and 1.54 ± 0.17-fold for 8 h, $P < 0.05$, $n = 8$) (Fig. 3*D*), it did not change LH secretion at concentrations ranging from 1 to 10^3 pM for 4 h and LH β-subunit mRNA expression at a concentration of 10^2 pM for 2, 4, and 8 h (Fig. 3, *A* and *B*).

Pretreatment with anti-CRF IgG or anti-Ucn 2 IgG blocks stress-induced suppression of LH secretion and expression. Although plasma ACTH levels at 30 and 90 min and plasma corticosterone levels at 90 min were significantly increased in the anti-CRF IgG-pretreated rats compared with those in the nonstressed anti-CRF IgG-pretreated rats, the plasma ACTH levels at 30 and 90 min in the anti-CRF IgG-pretreated rats were significantly lower than those at 30 and 90 min in NRS IgG-pretreated rats. Moreover, the plasma corticosterone level at 30 min in the anti-CRF IgG-pretreated rats was also significantly lower than that at 30 min in NRS IgG-treated rats (Fig. 4, *A* and *B*). Pretreatment with anti-CRF IgG also blocked immobilization stress-induced POMC mRNA expression in the anterior and intermediate lobes and Ucn 2 mRNA expression in the intermediate lobe (Fig. 4, *C*, *E*, and *F*). Furthermore, pretreatment with anti-CRF IgG blocked immobilization stress-induced suppression of plasma LH levels and LH β-subunit mRNA expression at 90 min (Fig. 4, *G* and *H*). Pretreatment with anti-Ucn 2 IgG blocked stress-induced suppression of plasma LH levels and LH β-subunit mRNA expression in the anterior lobe at 90 min without any significant changes in plasma ACTH and corticosterone levels, or in POMC and Ucn 2 mRNA expression, in either the anterior or intermediate lobe (Fig. 5). The lack of effect of pretreatment with anti-Ucn 2 IgG on the plasma ACTH and corticosterone levels or on POMC mRNA expression suggests that the IgG does not cross-react with CRF in vivo.

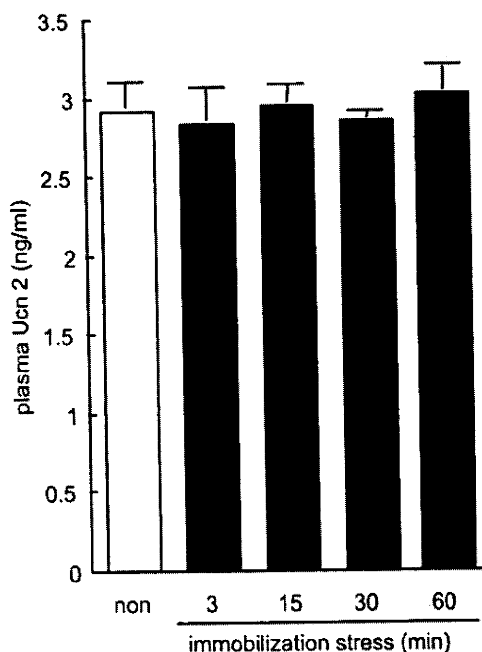

Fig. 2. Effect of 3-, 15-, 30-, and 60-min period of immobilization stress on plasma Ucn 2. Male rats (7 wk old) were exposed to immobilization stress for 3, 15, 30, or 60 min. Their trunk blood was assayed for Ucn 2. The no. of rats in each experimental group was 5.

DISCUSSION

We previously reported that Ucn 2 is biosynthesized by POMC cells of the anterior and intermediate lobes of rat pituitary and that the mRNA expression and secretion of Ucn 2 by POMC cells are increased by CRF in both the anterior and intermediate lobes (31, 50). We have also reported that the mRNA expression and secretion of Ucn 2 are suppressed by glucocorticoids in the anterior lobe, but not in the intermediate lobe (31). In the present study, we have demonstrated that immobilization stress increases the expression of the mRNA of both Ucn 2 and POMC in the intermediate lobe of the pituitary and POMC in the anterior lobe and plasma ACTH levels and that these immobilization stress-induced changes are significantly attenuated by anti-CRF IgG. These results suggest that immobilization stress increases the secretion and mRNA expression of POMC in the pituitary and the mRNA expression of Ucn 2 in the intermediate lobe through CRF released from the hypothalamus.

Because anti-CRF IgG or anti-Ucn 2 IgG did not affect basal ACTH and corticosterone levels in nonstressed rats compared with these levels in NRS IgG-pretreated nonstressed rats (Figs. 4 and 5), consistent with previous reports that intravenous injection of anti-CRF antibody does not alter basal ACTH levels but blocks the increase in ACTH resulting from stress (35, 44), we propose that anti-CRF IgG or anti-Ucn 2 IgG induces no feedback effect on the HPA axis.

The plasma ACTH and corticosterone response to immobilization stress in nontreated rats in Fig. 1 was lower than the responses to the same stress shown in NRS IgG-injected rats in Figs. 4 and 5, although the responses of POMC mRNA expression in the anterior pituitary were almost the same among those experiments. Several repetitions of these experiments using the same protocol yielded similar results. This has led us to the conclusion that the intraperitoneal IgG injection itself has acted as a stressor to affect the immobilization stress-induced ACTH secretion, since it has been reported that a prior stress facilitates the subsequent stress-induced ACTH response (1a, 11a, 47a). In other words, the intraperitoneal IgG injection affected the HPA axis as a prior stressor, although the precise mechanism involved is unknown.

Our previous in vitro study showed that Ucn 2 inhibits the secretion of LH and FSH and the mRNA expression of their β-subunits (32). Our study using anti-Ucn 2 IgG and siRNA against CRF-R2 has also demonstrated that Ucn 2 secreted by POMC cells in the anterior lobe tonically inhibits the expression and secretion of gonadotropins (32). The present study has shown that immobilization stress suppresses plasma LH levels and pituitary LH β-subunit mRNA expression, although plasma FSH and pituitary FSH β-subunit mRNA expression

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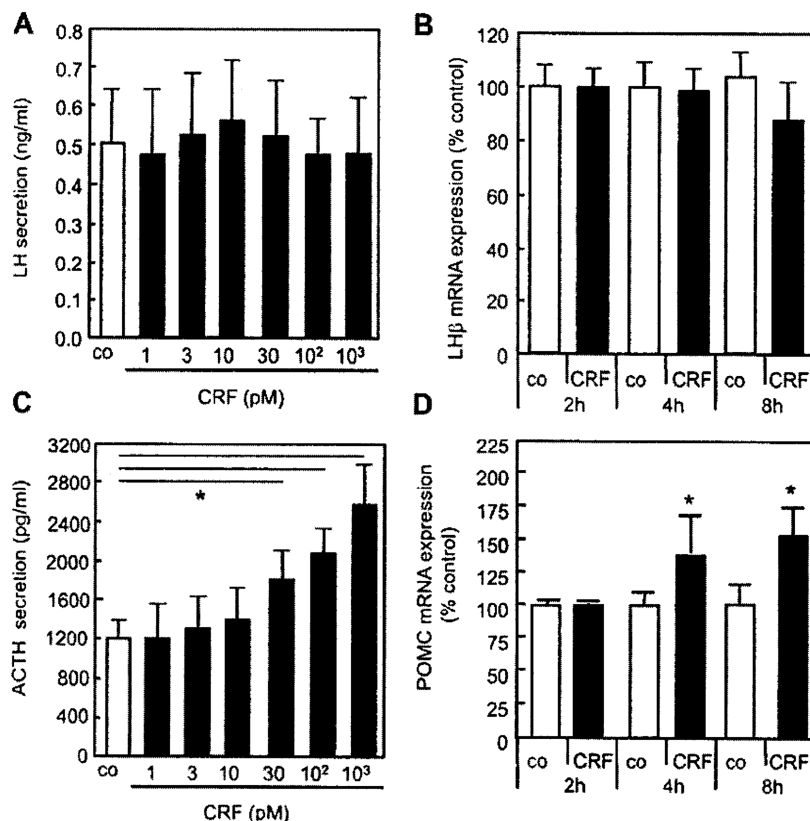

Fig. 3. Effects of corticotropin-releasing factor (CRF) on LH secretion and LH β-subunit mRNA expression. Anterior pituitary cells were treated with CRF at concentrations of 1, 3, 10, 30, 10^2, and 10^3 pM for 4 h. After incubation, culture media were collected for assays of LH (*A*) and ACTH (*C*). Anterior pituitary cells were also treated with CRF at a concentration of 10^2 pM for 2, 4, or 8 h. After incubation, total RNA was extracted from cells to measure LH β-subunit (*B*) and POMC (*D*) mRNA expression levels using the Thermal Cycler Dice Real-Time PCR System. Each mRNA expression levels is shown as %control. Data represent means ± SE. Eight wells were used for each treatment. $*P < 0.05$ compared with controls. co, Control.

are not affected. These differences between the LH and FSH responses to stress are consistent with other reports showing the absence of an FSH response to various stressors (11, 16, 48), although the precise mechanism involved is unclear. Because passive immunization with anti-Ucn 2 IgG blocked the immobilization stress-induced suppression of plasma LH levels and pituitary LH mRNA expression in the present study, we believe that the immobilization-induced suppression of secretion and expression of LH is mediated by Ucn 2.

In the present study, pretreatment with anti-Ucn 2 IgG 2 h before exposure to immobilization stress affected LH β-subunit expression, while in our previous study the effect of anti-Ucn 2 IgG on basal LH β-subunit mRNA expression was detected 24 h but not 8 h after anti-Ucn 2 IgG administration (32). This interval between the administration and effect of anti-Ucn 2 IgG on LH β-subunit mRNA expression appears to depend on the amount of secreted Ucn 2. In our previous study, anti-Ucn 2 IgG was administered to nonstressed rats whose secretion of Ucn 2 from the pituitary was probably much lower, thus causing a weaker inhibitory effect on LH β-subunit mRNA expression compared with that of immobilization stressed rats used in the present study. In contrast, in the present study, the 90-min immobilization stress, which would have elevated Ucn 2 secretion, significantly suppressed LH β-subunit mRNA expression in NRS IgG-treated rats, as shown in Figs. 4 and 5. In this case, the blocking effect of anti-Ucn 2 IgG on Ucn 2-induced inhibition of LH β-subunit gene expression appears to manifest in a shorter period with passive immunization. Thus it would have taken longer for the effect of anti-Ucn 2 IgG on the basal levels of LH β-subunit mRNA expression to become significantly different from that in the NRS IgG-treated nonstressed rats of our previous study.

We were unable to determine the origin of the Ucn 2 that inhibits the secretion and mRNA expression of LH during immobilization stress. Under normal conditions, Ucn 2 is expressed in various peripheral tissues, including the adrenal glands, lungs, skeletal muscles, and skin (8, 9, 20, 50). In the present study, we found that basal plasma Ucn 2 concentrations are a few nanograms per milliliter. These concentrations of Ucn 2 reflect the secretion of Ucn 2 by various tissues into the peripheral circulation, since basal plasma concentrations of Ucn 2 secreted from the pituitary are speculated to be <100 pg/ml, based on the ACTH and β-endorphin levels in the peripheral circulation. The secretion of Ucn 2 from POMC cells of both the anterior and intermediate lobes is stimulated by CRF (31), and the release of CRF from the hypothalamus would increase during immobilization stress since plasma ACTH increased during immobilization stress, as shown in the present study. Thus, the secretion of Ucn 2 from POMC cells most likely increases during immobilization stress. However, the plasma Ucn 2 levels did not change during immobilization stress. This could be explained by the difference in the amount of secreted Ucn 2 between the pituitary and other peripheral tissues, the former being much lower than the latter. In other words, the secretion pattern of Ucn 2 by the pituitary might be obscured by Ucn 2 secreted by various other peripheral tissues (8, 50). It is unlikely that CRF released from the hypothalamus into the pituitary portal vessels stimulates the secretion of Ucn 2

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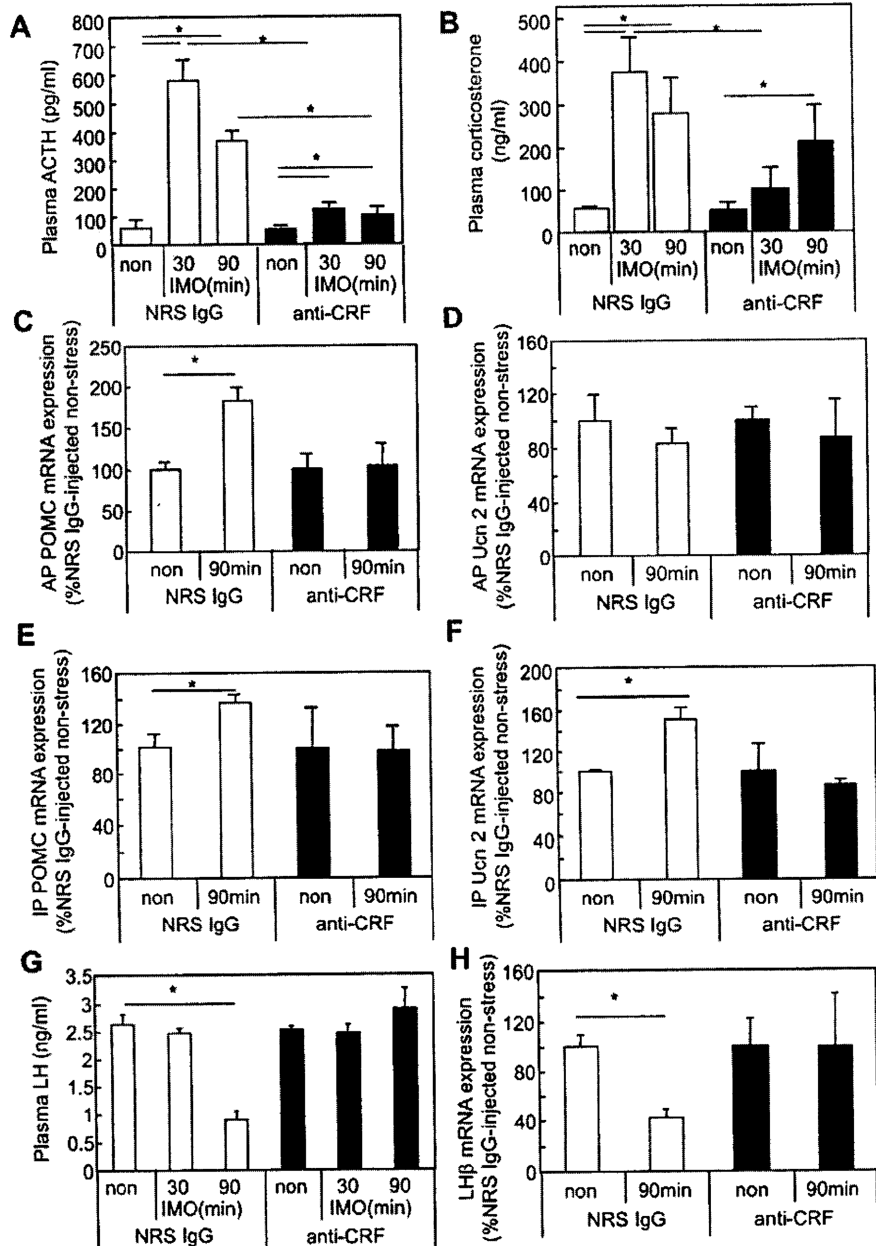

Fig. 4. Effects of anti-CRF IgG pretreatment on plasma ACTH, corticosterone, and LH levels and on POMC, Ucn 2, and LH β-subunit mRNA expression in the anterior and intermediate pituitary of immobilization stress-exposed rats. Male rats (7 wk old) pretreated with anti-CRF IgG (1 mg/kg body wt ip) were exposed to immobilization stress for 30 or 90 min (30 or 90). Their trunk blood was assayed for ACTH (*A*), corticosterone (*B*), and LH (*G*), and their anterior and intermediate pituitary RNA was extracted and assayed for mRNA expression of POMC (*C* for anterior pituitary and *E* for intermediate pituitary), Ucn 2 (*D* for anterior pituitary and *F* for intermediate pituitary), and LH β-subunit (*H*). Passive immunization using the same experimental protocol was performed two times, and data were combined and analyzed. Each mRNA expression level is shown as %normal rabbit serum (NRS) IgG-injected nonstressed controls. The no. of rats in each experimental group were 10 for *A* and *B* and 5 for *C–H*. $^*P < 0.05$ compared with the nonstressed controls. NRS IgG, normal rabbit serum IgG-injected rats; anti-CRF, anti-CRF IgG-injected rats; IMO, immobilization.

into the various peripheral tissues after entering the peripheral circulation because of the low concentrations of CRF in the pituitary portal vessels (31). Therefore, the POMC cells of the pituitary are the only likely targets of CRF released from the hypothalamus during stress, and they are the probable origin of the Ucn 2 that inhibits LH secretion during stress.

During stress, CRF and other ACTH secretagogues, such as vasopressin and norepinephrine, are released into the pituitary portal vessels, and the secretion of ACTH is subsequently stimulated by these secretagogues (1, 2, 7, 14, 17). The secretion of glucocorticoids is then increased by ACTH. Although CRF stimulates both the secretion and mRNA expression of Ucn 2 in both the anterior and intermediate lobes, the secretion of Ucn 2 is blocked by glucocorticoids in the anterior lobe but not in the intermediate lobe, as shown in our previous in vitro study (31). Therefore, the elevated secretion of Ucn 2 by POMC cells of the anterior lobe in response to CRF during immobilization stress would return to the basal level when the secretion of corticosterone is increased by ACTH and the corticosterone then counteracts the stimulatory effect of CRF on Ucn 2. The elevated secretion of Ucn 2 appears to last longer in the intermediate lobe than in the anterior lobe. Because local circulation from the intermediate lobe of the pituitary to the anterior lobe has been reported (30), Ucn 2 secreted by POMC cells of the intermediate lobe could act on gonadotrophs through the local circulation. On the other hand, because the amount of secreted Ucn 2 is extremely low compared with ACTH (1/40) in the culture media of anterior

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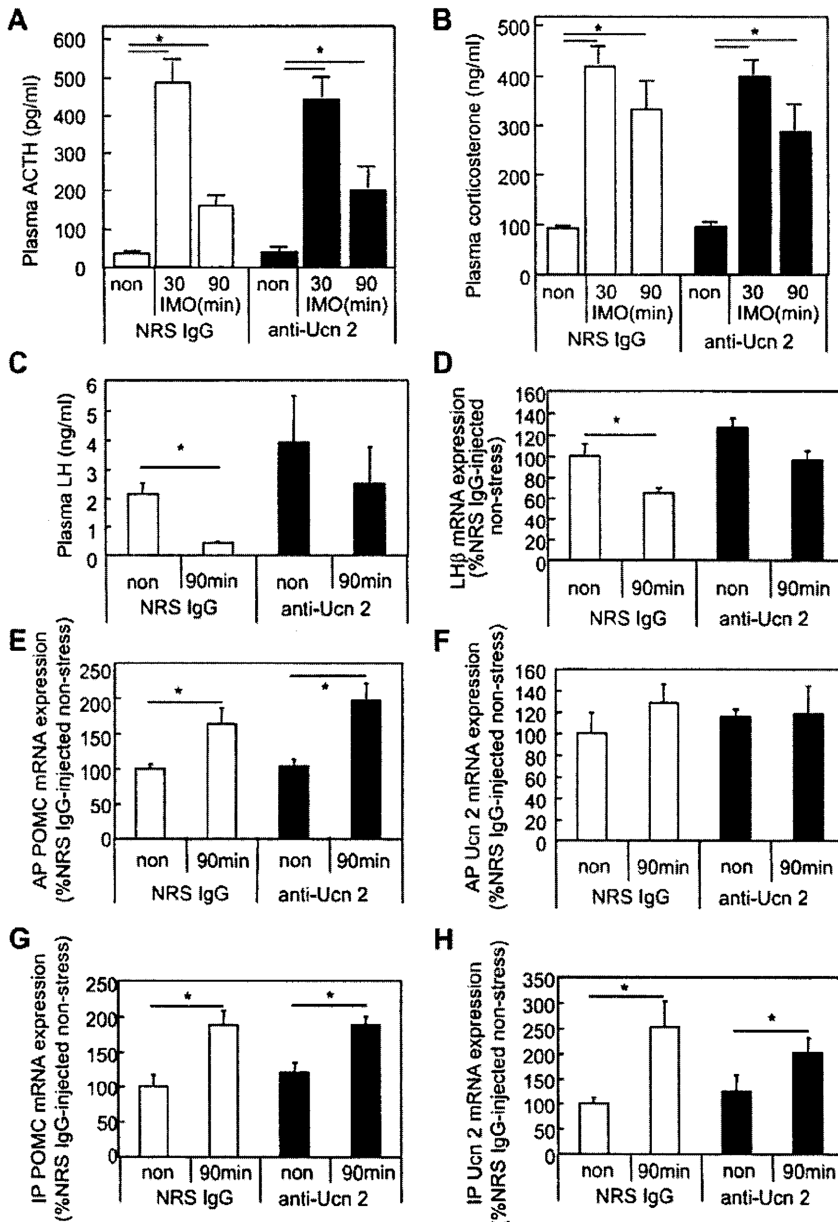

Fig. 5. Effects of anti-Ucn 2 IgG pretreatment on plasma ACTH, corticosterone, and LH levels and on POMC, Ucn 2, and LH β-subunit mRNA expression in the anterior and intermediate pituitary of immobilization stress-exposed rats. Male rats (7 wk old) pretreated with anti-Ucn 2 IgG (1 mg/kg body wt ip) were exposed to immobilization stress for 90 min (90). Their trunk blood was assayed for ACTH (*A*), corticosterone (*B*), and LH (*C*), and their anterior and intermediate pituitary RNA were extracted and assayed for LH β-subunit mRNA (*D*), POMC mRNA (*E* for anterior pituitary and *G* for intermediate pituitary), and Ucn 2 mRNA (*F* for anterior pituitary and *H* for intermediate pituitary) expression. Passive immunization using the same experimental protocol was performed two times, and data were combined and analyzed. Each mRNA expression level is shown as %NRS IgG-injected nonstressed controls. The no. of rats in each experimental group were 10 for *A* and *B* and 5 for *C-H*. $^*P < 0.05$ compared with the nonstressed controls. anti-Ucn 2, anti-Ucn 2 IgG-injected rats.

lobe cells of rat pituitary (31), the Ucn 2 secreted by POMC cells of anterior pituitary lobe may act on gonadotrophs in a paracrine manner.

Intracerebroventricular infusion of CRF reduces immunoreactive GnRH stores in the median eminence (39). Intracerebroventricular injection of CRF attenuates LH secretion by inhibiting immunoreactive GnRH release in the hypophysial-portal circulation in ovariectomized and estradiol-administered rats (37). Intravenous injection of CRF antagonist reverses the inhibitory effect of immobilization stress on LH secretion in rats (27). These findings suggest that hypothalamic CRF modifies reproductive function at the hypothalamic level during stress. In the present study, passive immunization with anti-CRF IgG also blocked immobilization stress-induced suppression of secretion and expression of LH mRNA. In conjunction with the results of our study using anti-Ucn 2 IgG, these findings indicate that immobilization stress-induced suppression of secretion and expression of LH is mediated probably by the hypothalamic CRF-pituitary Ucn 2 axis as well as the hypothalamic CRF-GnRH axis.

A recent study has shown that 13% of gonadotrophs express CRF-R1 (49). However, in the present in vitro study, no significant change in LH secretion or mRNA expression was induced by CRF, which mainly binds to CRF-R1. Therefore, it seems unlikely that CRF released from the hypothalamus directly suppresses the secretion and expression of LH in the pituitary. A study has shown that gonadotropin inhibitory hormone (GnIH) is expressed in the dorsomedial hypothalamus and that GnIH neurons express CRF-R1 (24), and thus CRF

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may also modify the reproductive function by acting on GnIH neurons during stress.

In addition to CRF, ACTH suppresses the LH surge. It has been reported that LH secretion in response to GnRH is inhibited by ACTH in adrenalectomized rams, suggesting that ACTH suppresses LH secretion without involving corticosteroid pathways, although the precise mechanism of ACTH action is not clear (15). We found no significant effect of Ucn 2 on ACTH secretion in the cultured rat anterior pituitary cells (32). Similarly, neither stresscopin-related peptide, a human homolog of Ucn 2, nor Ucn 2 affect ACTH secretion of cultured rat anterior pituitary cells in vitro (20) and in vivo (36). Therefore, ACTH does not seem to be involved in the mechanism by which Ucn 2 suppresses the secretion and expression of LH although ACTH released by CRF may inhibit LH secretion during stress. Glucocorticoids are reported to inhibit the LH response to GnRH in vivo and in vitro (5, 6, 26, 34). However, because anti-Ucn 2 IgG blocked the stress-induced suppression of plasma LH and LH β-subunit mRNA expression without affecting POMC mRNA expression in the anterior and intermediate lobes, it would appear that the increase in corticosterone secretion induced by ACTH during immobilization stress does not play a major role in stress-induced suppression of LH secretion.

Intracerebroventricular injection of Ucn 2 reportedly suppresses LH pulsatile secretion, and intracerebroventricular injection of CRF-R2 antagonist blocks stress-induced suppression of LH secretion in ovariectomized and estrogen-replaced rats (28). There have been no reports demonstrating the expression of CRF-R2 on GnRH neurons, although CRF-R1, which has low affinity for Ucn 2 (inhibitory constant >100 nM) (40), is reportedly expressed on GnRH neurons (21, 29). Therefore, the sites of action in the central nervous system of Ucn 2 and CRF-R2 antagonist injected intracerebroventricularly are unclear. The peptide and antagonist injected intracerebroventricularly might act on the pituitary by leaking into the peripheral circulation.

In summary, the present study suggest that Ucn 2 secreted by POMC cells of the pituitary in response to CRF acts on gonadotrophs and suppresses the expression and secretion of LH in stress. This is the first report outlining a novel pathway, namely a hypothalamic CRF-pituitary Ucn 2-pituitary LH axis, through which CRF suppresses the reproductive system in addition to the hypothalamic CRF-GnRH pathway in stress.

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DISCLOSURES

Nothing to disclose.

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Neuronal Histamine and its Receptors: Implication of the Pharmacological Treatment of Obesity

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Abstract: Obesity is the effect of imbalance between energy intake and expenditure and forms a fundamental basis of the metabolic syndrome. A number of substances implicated in the regulation of energy metabolism represent opportunities for anti-obesity drug development. Neuronal histamine and its receptors have been shown to regulate energy metabolism and are considered as anti-obesity targets. Several histamine receptor subtypes have been identified; of these, histamine H_1 and H_3 receptors (H_1-R and H_3-R) have been specifically recognized as mediators of energy intake and expenditure. In addition, several histamine drugs related to H_1-R and H_3-R, have been shown to attenuate body weight gain both in rodent and human. These results provide the reagents for histamine receptors biology and may find applications in the treatment of obesity and related metabolic disorders. In this review, the development of agonists and antagonists of histamine receptors are provided.

Keywords: Histamine, food intake, obesity, histamine H_1 receptors, histamine H_3 receptors, histidine decarboxylase, brain.

INTRODUCTION

Obesity is an important health problem because it is associated with a number of pathophysiologic conditions including hypertension, coronary heart disease, diabetes, and stroke [1, 2]. The detailed molecular mechanisms that underlie obesity have not been fully elucidated. There is increasing evidence that obesity is under control of several factors [3, 4] and a number of studies have revealed that the brain functions that regulate energy balance play a central role in the development of obesity [5, 6].

Histamine has been known to play a critical role in homeostatic regulatory functions such as control of food intake and obesity in animals [6, 7]. The histamine-containing neurons and its receptors are distributed throughout the brain [8, 9]. Neuronal histamine is involved in brain functions such as locomotor activity, circadian rhythm, feeding behavior, inflammation and obesity [5,6] through the histamine H_1, H_2, and H_3 receptors (H_1-R, H_2-R, and H_3-R) [9-12].

Several pharmacological and physiological studies have investigated the function of histamine receptors, using an agonist/antagonist of H_1-R and H_3-R [13, 14]. In addition, other studies have shown that histidine decarboxylase (HDC) is also target of food intake and obesity [15, 16].

NEURONAL HISTAMINE AND ITS RECEPTORS IN THE BRAIN

Histamine (**1**) is synthesized in the brain from l-histidine (**2**) by the enzyme HDC [3]. The termination of histamine's action in the brain may require its catabolism to telemethylhistamine (**3**) by the enzyme histamine N-methyltransferase (Fig. 1). Histamine H_1-R, H_2-R, and H_3-R have been demonstrated in the brain [10, 11]. Anatomically, histamine H_1-Rs are located postsynaptically; high densities of these receptors are seen in the hypothalamus [10]. Histamine H_2-Rs are also mainly located postsynaptically and high densities H_2-Rs are found in hippocampus, amygdala, and basal ganglia [10]. H_3-Rs are located on the somata and axon terminals of HA neurons where they serve as autoreceptors to modulate HA synthesis and release, and are also located pre- and postsynaptically in other brain regions [11].

NEURONAL HISTAMINE, H_1-R, AND ON FOOD INTAKE AND OBESITY

Extensive pharmacological experiments have demonstrated that the neuronal histamine system plays a critical role in the regulation of obesity and diabetes [17-20]. Centrally injected histamine suppresses food intake and body weight in rats [21]. Histidine has the same effect on food intake in rodents [22], suggesting that peripheral histidine may penetrate the blood brain barrier and be converted into histamine in the brain by HDC [23]. In fact, the histidine-induced suppression of food intake results from elevated histamine levels in the brain [23]. In contrast, the HDC inhibitor alpha-FMH (**4**) increases food intake in rodents. Pharmacological experiments using histamine H_1-R-agonists and antagonists have shown that H_1-Rs also help control food intake; central injection of the H_1-R-agonist 2-(3-trifluoromethylphenyl) histamine (betahistine) (**5**) decreases feeding in rodents [12]. Histamine H_1-R-agonist betahistine injected centrally activated the *c-fos* like immunoreactivity in paraventricullar nucleus (PVN) [24]. In contrast, central administration of the H_1-R-antagonists chlorpheniramine (**6**) and/or pyrilamine (**7**) elicit food intake in rats [14] (Fig. **2**). In addition, neuronal histamine and H_1-R regulate the uncoupling proteins expression in peripheral tissues [25-28]. Uncoupling proteins especially in brown adipose tissue are crucial roles in energy expenditure [29-32]. From these observations, neuronal histamine and histamine H_1-Rs may be important for neural regulation of energy intake and expenditure. In human study, betahistine is a centrally acting H_1-R agonist with partial H_3-R antagonistic activity and no H_2-R-binding effects [33]. Betahistine has been studied mainly as a vasodilator for conditions such as cluster headaches, vascular

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