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Growth hormone regulates the expression of UCP2 in myocytes

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ABSTRACT

Objective: To determine if and how growth hormone (GH) signaling is involved in energy metabolism.
Design: We used human embryonic kidney TSA201 cells, human H-EMC-SS chondrosarcoma cells, rat L6 skeletal muscle cells, and murine C2C12 skeletal muscle myoblasts to investigate GH-induced expression of uncoupling protein2 (UCP2) to the GHR/JAK/STAT5 pathway by a combination of a reporter assay, electrophoretic mobility shift assay (EMSA), real-time quantitative PCR, Western blotting.
Results: We demonstrated that the regulation energy metabolism, which was hypothesized to be directly acted on by GH, involves UCP2 via activated STAT5B, a signal transducer downstream of GH. We also showed that the sequence at the −586 'TTCnGA' may function as a novel putative consensus sequence of STAT5s.
Conclusion: The results suggest that GH regulates energy metabolism directly in myocytes and that UCP2 participates in the signal transduction pathway that functions downstream of the GHR/JAK/STAT.

1. Introduction

Growth hormone (GH) is a hormone secreted by the somatotrophs of the anterior pituitary gland. The binding of GH to GH receptors (GHR) on the hepatocyte membrane activates the Janus kinase (JAK)/signal transduction and activator of transcription (STAT) pathway and promotes the production of insulin-like growth factor-I (IGF-I) [1]. Most IGF-I is produced in the liver, and IGF-I plays an important role in bone development as a liver-derived growth factor [1]. Consequently, it was thought that much of the action of GH occurs indirectly, via IGF-I; however, in recent years, it has become clear that GH can also act directly. The direct actions of GH are mediated via GHRs, which are expressed throughout the body and are known to function not only in development but also in the promotion of energy metabolism through their action on cells such as myocytes and adipocytes [2,3]. However, the underlying signaling pathways that are activated during the direct action of GH on energy metabolism and the transcription factors and target genes acted upon have not been identified.

Upon the activation of GHR by the binding of GH, JAK2 is activated. Subsequently, pathways such as the STAT5 and mitogen-activated protein kinase (MAPK) pathways are activated, as is the phosphatidylinositol-3 kinase (PI3K) pathway via the insulin receptor substrate [4]. In adults, GHR is ubiquitously expressed in every tissue, and the action of GH via the aforementioned pathways is very diverse. For example, the GHR/JAK/STAT pathway promotes lipogenesis in preadipocytes [5] while inhibiting lipid synthesis in mature adipocytes; the pathway also promotes lipid degradation through the activation of hormone-sensitive lipase [6]. Clinically, patients with adult GH deficiency (AGHD) exhibit increased visceral fat, and frequently, dyslipidemia [7,8].

STAT5, one of the main transcription factors activated by GH has two homologs called STAT5A and STAT5B. These homologs share >90% homology at the amino acid level; however, STAT5A and STAT5B differ with respect to the number of amino acids present at their C-termini (20 and 8, respectively) [9,10]. The STAT5 proteins bind to a common consensus sequence (TTCnnnGA). However, despite this functional redundancy, knockout mice for each protein exhibit entirely different phenotypes. Female STAT5A knockout mice exhibit incomplete mammary gland development due to loss of prolactin sensitivity [11,12], while STAT5B knockout mice exhibit delayed growth due to loss of sensitivity to GH [12,13]. These findings suggest that the main transcription factor activated by GH is STAT5B.

A previous study demonstrated that GH administration in KK-Ay diabetic mice increased the mRNA expression of genes encoding

Abbreviations: AGHD, adult growth hormone deficiency; GH, growth hormone; GHR, growth hormone receptor; IGF-I, insulin-like growth factor-I; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide-3 kinase; STAT, signal transduction and activator of transcription; UCP, uncoupling protein.

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uncoupling proteins (UCPs) in several tissues, including muscle tissue [14]. UCPs uncouple the phosphorylation reactions occurring on the inner mitochondrial membrane, converting chemical energy such as that from fatty acids and glucose directly into heat. Consequently, activation of UCPs enhances thermogenesis and energy consumption. UCPs participate in energy metabolism and are of three types: UCP1, which is specifically expressed in brown adipocytes; UCP2, which is expressed widely throughout the body in tissues such as the skeletal muscle and white adipose tissue; and UCP3, which is mainly expressed in the skeletal muscle [15]. The above findings suggest that GH increases UCP expression via a specific signal transduction pathway. However, whether these effects are brought on by GH directly or indirectly via IGF-I remains to be clarified.

In the present study, we used myocytes to elucidate the signal transduction pathway that functions downstream of the GHR/JAK/STAT pathway and to clarify the mechanism by which GH participates in energy metabolism, paying special attention to both STAT5B and UCP2. Our findings suggest that GH regulates energy metabolism directly and that UCP2 participates in this process.

2. Materials and methods

2.1. Cell culture

TSA201 cells, which are clones of human embryonic kidney 293 cells [16], rat L6 skeletal muscle cells, and human H-EMC-SS chondrosarcoma cells were purchased from the JCRB-cell bank (Osaka, Japan); murine C2C12 skeletal muscle myoblasts were purchased from the Riken Cell Bank (Tsukuba, Japan). TSA201, L6, and C2C12 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Nissui Pharmaceutical, Tokyo, Japan), and H-EMC-SS cells were grown in minimal essential medium α (Wako Pure Chemical Industries, Osaka, Japan); both mediums contained 10% fetal bovine serum (HyClone laboratories Inc., UT), penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (2 mM). TSA201 cells were established from human embryos. H-EMC-SS cells were established from the quadriceps femoris muscle of a Japanese woman. L6 and C2C12 cells were induced to differentiate to 80% confluence in DMEM containing 2% horse serum for 10 days. Differentiated L6 cells were starved for 16 h in the presence of 25 mM HEPES, and 0.2% bovine serum albumin before GH (1.0 μg/mL) stimulating. The appropriate GH dose was determined in a previous report [17]. All cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Plasmid construction

To analyze the function of the promoter region of human (h) UCP2, we constructed various deletion mutants of the hUCP2 promoter by PCR with human genomic DNA as the template. PCR primers were constructed such that the *Xho*I and *Hin*dIII sites overhang the oligonucleotides used as sense and antisense primers, respectively. PCR products were subcloned between the *Xho*I and *Hin*dIII sites of the pGL4.10 vector (Promega, Madison, WI). The constructed plasmids were verified by sequencing. The inactive STAT5B (pCMX-iSTAT5B) (iSTAT5B) and constitutive active STAT5B (pMX-STAT5B1*6) (aSTAT5B) expression plasmids were kindly provided by Dr. Toshio Kitamura (Tokyo University) [18].

2.3. Transient expression assays

TSA201 cells were transfected with the constructed hUCP2 promoter-luciferase (Luc) plasmids by using the calcium phosphate method [19]. The total amount of expression plasmids was kept constant in different experimental groups by adding corresponding amounts of the empty vehicle plasmids. After exposure to the calcium phosphate-DNA precipitate for 6 h, phenol red-free DMEM (Wako Pure Chemical Industries, Osaka, Japan) with 10% charcoal-stripped fetal bovine serum was added. Cells were harvested after 20 h for the measurement of luciferase activity, according to the manufacturer's instructions (Dual-Luciferase® Reporter Assay System; Promega, Madison, WI). Transfection efficiency was monitored by using an internal control.

2.4. Electrophoretic mobility shift assay (EMSA)

Whole cell extracts from TSA201 cells transfected with pMX-STAT5B1*6 were prepared in cell lysis solution (50 mM Tris-HCl, 0.15 M NaCl, 0.1% sodium dodecyl sulfate, 1% Triton™ X-100, 10% sodium deoxycholate, and Halt Protease Inhibitor Cocktail [Thermo Fisher Scientific, Waltham, MA]). The extracts were pre-incubated at room temperature (20–25 °C) in 20 μL reaction solutions using a LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, Waltham, MA) with 1 μg/μL poly(dI-dC) for 15 min. Estimated biotin-labeled STAT5B oligonucleotides for consensus sequence were synthesized by Life Technologies Japan Ltd. (Tokyo, Japan). The estimated biotin-labeled STAT5B oligonucleotides were added, and the mixture was incubated for an additional 20 min at room temperature. The protein-DNA complexes were analyzed by electrophoresis on a 6% polyacrylamide gel in 0.5× Tris/borate/EDTA (TBE) buffer. The proteins were electroblotted onto nylon membrane and were detected with the streptavidin-horseradish peroxidase conjugate and chemiluminescence substrate. Non-labeled oligonucleotides were used as competitors, and unrelated non-labeled oligonucleotides of Epstein-Barr nuclear antigen were used as non-competitors of the biotin-labeled STAT5B oligonucleotides.

2.5. Expression analysis by quantitative reverse-transcription PCR (qRT-PCR)

Messenger RNA from TSA201 cells was isolated by using the RiboZol™ kit (AMRESCO, Solon, OH), and cDNA was synthesized from 1 μg RNA by reverse transcription, using iScript™ RT Supermix for RT-qPCR (BioRad, Hercules, CA). qPCR was performed in a CFX Connect™ Real-Time PCR Detection System (BioRad, Hercules, CA) using the iTaq™ Universal SYBR® Green Supermix (BioRad, Hercules, CA) and the following gene-specific primer pairs are listed in Table 1. PCR product quality was monitored by post-PCR melting curve analysis.

2.6. Western blotting

Whole cell extract from transfected L6 cells were prepared in cell lysis solution (described above) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% acrylamide gels. The proteins were then transferred onto nylon membranes and reacted with a polyclonal antibody against UCP2 (Santa Cruz Biotechnology, Dallas, TX).

2.7. Statistical analysis

The data are presented as the mean ± SD. Statistical analyses of the data were performed by unpaired *t*-test or one-way analysis of variance (ANOVA) followed by Bonferroni correction. Differences were considered statistically significant at $P < 0.05$.

Table 1
Primer sequences used in qRT-PCR.

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)
Rat UCP2	GCAGTTCTACACCAAGGGCT	GGAAGCGGACCTTTACCACA
Rat β-actin	CCCGCGAGTACAACCTTCTT	CCCACGATGGAGGGGAAGAC
Mouse UCP2	GAGAGTCAAGGGCTAGCGC	GCTTCGACAGTGCTCTGGTA
Mouse β-actin	CTTTGCAGCTCCTTCGTTGC	ACGATGGAGGGGAATACAGC
Human UCP2	CGGTTACAGATCCAAGGA	ACCAGCCCATTGTAGAGG
Human β-actin	CACTCTTCCAGCCTTCCTTCC	CGGACTCGTCATACTCCTGCTT