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Effect of the Orally Active Growth Hormone Secretagogue MK-677 on Somatic Growth in Rats

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Purpose: Growth hormone secretagogues (GHSs) possess the ability to release growth hormone (GH) in the body. This study aimed to investigate the effects of MK-677, an orally active GHS, on somatic growth in rats.

Materials and Methods: The serum levels of GH were measured after oral administration of MK-677 to confirm GH stimulatory effects. Body weight, body length, tibia length, epiphyseal plate width, and serum levels of insulin-like growth factor (IGF)-I were measured after oral administration of 4 mg/kg of MK-677 for 6 weeks to investigate growth-promoting effects.

Results: Oral administration of MK-677 at 4 mg/kg increased peak GH concentrations by 1.8-fold, compared to baseline. However, oral administration of MK-677 for 6 weeks did not increase body growth or serum levels of IGF-I. At 6 weeks after treatment, the GH response to MK-677 was abolished. Pituitary GH mRNA and hypothalamic GH-releasing hormone mRNA, and GH secretagogue receptor (GHSR) mRNA expression in the pituitary and hypothalamus did not differ between the control and treatment group. Somatostatin (SST) mRNA expression in the hypothalamus was markedly increased in the treatment group, whereas SST receptor (SSTR)-2 mRNA expression in the pituitary gland was decreased. Protein expression of hypothalamic GHSR, SST, and pituitary SSTR-2 showed patterns similar to those for mRNA expression.

Conclusion: Our results suggest that prolonged administration of MK-677 in rats does not promote growth despite the GH stimulatory effect of MK-677, which may be related to increased expression of SST in the hypothalamus. Further studies are needed to overcome the observed desensitization to GHS.

Key Words: Growth hormone-releasing peptide, oral administration, growth, rats, somatostatin

INTRODUCTION

Growth hormone secretagogues (GHSs) have been considered as alternatives in the treatment of diseases related to growth hormone (GH) deficiency because of their ability to release GH in the body.[1-3] GHSs enhance the pulsatile release of GH in the anterior pituitary gland, resulting in sustained elevation of insulin-like growth factor (IGF)-I levels. As GH is a large peptide molecule, it must be injected into subcutaneous tissue or muscle to get it into the blood. However, MK-677, an orally active non-peptide mimic of GHSs, can stimulate the release of GH effectively by intravenous, subcutaneous, intraperitoneal, and oral administration.[4,5] Growth hormone-releasing peptide (GHRP)-6, which is a synthetic hexapeptide, also shows potent GH-releasing activity after intravenous, subcutaneous, intranasal, and oral administration in humans. MK-677 is a non-peptide spiropiperidine previously shown to be functionally and mechanistically indistinguishable *in vitro* and *in vivo* from the potent peptide GHS GHRP-6.[1] MK-677 has been found to elevate GH levels, as well as IGF-I and cortisol levels, in dogs after oral administration:[6] this stimulatory effect appears to depend on the presence of an intact pituitary.[7] Previous studies in humans demonstrated that daily oral administration of MK-677 in healthy older adults,[8] GH-deficient adults[9] for 4 weeks, and GH-deficient children[10] for 7 days increased serum GH, IGF-I, and IGF binding protein (IGFBP)-3 concentrations. Further, clinical trial studies of MK-

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677 have been conducted to improve body composition and metabolism in older adults[11] or to increase bone mass in obese young males and older adults.[12,13] However, the beneficial effect of MK-677 on growth promotion in children is controversial. The aims of this study were to investigate whether oral administration of MK-677 1) stimulates the secretion of serum GH and increases the serum levels of IGF-I, 2) enhances the body length and width of growth plates in rats, and 3) influences mRNA expression of GH in the pituitary gland and mRNA and protein expression of GH-releasing hormone (GHRH), GHS receptor (GHSR), somatostatin (SST), and somatostatin receptors (SSTRs) in the hypothalamus.

MATERIALS AND METHODS

Animals and experimental design

Animals were provided access to regular chow and water *ad libitum* and were maintained at a temperature of 21±2°C and humidity of 60±10% on 12-h light/dark cycles. Female Sprague-Dawley (SD) rats, approximately 4 weeks of age, in each group were used for the experiments. The rats were fasted for 8 h before treatment and provided water *ad libitum*. MK-677 was administered via a stomach tube, and blood samples were collected from the tail vein during the experiment or from the heart after decapitation. Formulations of MK-677 were prepared at 1 mg/mL in distilled water. Distilled water was administered at 4 mL/kg as a placebo. All animal studies were approved by the Animal Care and Use Committee of the Yonsei University College of Medicine (No. 2013-0095).

To determine whether oral administration of MK-677 can stimulate GH secretion in rats, MK-677 was administered at 2 or 4 mg/kg via the stomach tube. Distilled water was administered at the same volume as a control. Blood samples were collected from the tail vein at 0, 30, 60, 90, and 120 min after treatment. Plasma was harvested by centrifugation and stored in -70˚C for determination of GH. The same experiment was conducted after treatment with MK-677 for 6 weeks to determine whether the GH stimulatory effect was sustained after long-term use of MK-677.

MK-677 was administered at 4 mg/kg via the stomach tube between 08:00 and 10:00 for 6 weeks. Distilled water was administered at 4 mL/kg as a control. To evaluate the growth-promoting effect of MK-677, body weight and body length were measured daily. The body length of rats was measured as the length from the nose to the anus. Blood samples were collected from the tail vein every 2 weeks for determination of IGF-I. The width of the tibia growth palate was measured after decapitation at 6 weeks after treatment. Right tibia tissues were fixed in 4% paraformaldehyde for 24 h. Decalcification was performed with an EDTA-G solution (14.5 g EDTA, 1.25 g NaOH, and 15 mL glycerol dissolved in distilled water, pH 7.3). The solution was then brought to 100 mL and stored for 10–14 days at 4°C. The fixed and decalcified tibia was embedded in paraffin and sectioned at 5 μm. Paraffin bone sections were stained with hematoxylin and eosin (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions.

To determine whether MK-677 can alter GH, GHRH, GHSR, SST, and SSTR mRNA and protein expression, rats were sacrificed by decapitation at 6 weeks after treatment. The pituitary gland and hypothalamus were collected and frozen for analysis of mRNA expression of GH, GHRH, GHSR, SST, and SSTR by real-time polymerase chain reaction (RT-PCR) and protein expression by Western immunoblotting.

Hormone analysis

Blood samples were collected in serum-separating tubes and centrifuged for 15 min at 3000 rpm at 4°C. Separated sera were stored at -20°C until ready for analysis. All hormone analyses were performed by Enzyme-linked Immunosorbent Assay (ELISA). GH was determined by ELISA (E-EL-R0029, Elabscience Biotech, Wuhan, China) with a sensitivity of 0.188 ng/mL, intra-assay coefficient of variation of 3.87–5.15%, and inter-assay coefficient of variation of 7.04–8.45%. Sera were diluted 10-fold with reference standard and sample diluent. 100 μL of sample was added to micro ELISA plate wells and incubated for 90 min at 37°C. After the solution was removed, 100 μL of biotinylated detection Ab working solution was added. The plates were incubated for 1 hour at 37°C and washed with 350 μL of wash buffer three times. Then, horseradish peroxidase conjugate working solution was added. After washing the wells five times with buffer, substrate reagent was added to each well and reacted by blocking light for about 15 min at 37°C. Finally, stop solution was added to the ELISA plates, and absorbance was measured with a micro-plate reader set to 450 nm. IGF-I was determined by ELISA (E-EL-R0010, Elabscience Biotech) with a sensitivity of 18.75 pg/mL, intra-assay coefficient of variation of 3.4–5.7%, and inter-assay coefficient of variation of 5.7–7.9%. The IGF-I ELISA experiment was performed according to the manufacturer's instructions. Sera were diluted 100-fold with reference standard and sample diluent. Absorbance was measured on a spectrophotometer at 450 nm wavelength.

Western immunoblotting

Antibodies against GHSR (E-AB-33767), SST (E-AB-32939), and SSTR-2 (E-AB-36114) were purchased from Elabscience Biotech. SSTR-5 antibody was purchased from Abcam (ab156864, Cambridge, MA, USA), and GAPDH was purchased from Cell Signaling Technology (#2118, Danvers, MA, USA), respectively. Protein from tissue was homogenized using RIPA buffer (Thermo Scientific, Waltham, MA, USA) with protease inhibitor. The samples were incubated on ice for 30 min and centrifuged at 13000 rpm for 15 min. After centrifugation, the supernatant was collected in a new tube. Protein concentrations were determined by a BCA assay (Pierce, Rockford, IL, USA). Equal

amounts of protein (50 μg) were electrophoresed on a 15% SDS-PAGE gel and transferred to a PVDF membrane. Subsequently, the blots were blocked with 5% nonfat dry milk for 1 hour and incubated with first antibody (dilution, 1:1000) overnight at 4°C. Membranes were washed three times for 5 min with PBST (0.2% Tween 20) and incubated with secondary antibody (dilution, 1:3000) for 1 hour. Blots were washed with PBST three times for 10 min. Protein expression was detected using an ECL (Amersham Biosciences, Piscataway, NJ, USA) solution according to the manufacturer's instructions. GAPDH was used as a sample loading control.

RT-PCR analysis

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Five micrograms of total RNA was subjected to reverse transcription using the Superscript™ III first-strand synthesis system (Invitrogen) according to the manufacturer's instructions. RT-PCR was conducted in 20 μL of reaction mixture containing cDNA, TaqMan primer pairs, and TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA). The primer pairs were obtained from Applied Biosystems (Rn01495894 for GH, Rn00580832 for GHRH, Rn00821417 for GHSR, Rn00561967 for SST, Rn01464950 for SSTR-2, Rn02535169 for SSTR-5, and Rn01775763 for GAPDH). Amplification was performed in duplicate with the ABI 7300 system (Applied Biosystems) with the following profile: 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s, and 60°C for 1 min. The gene expression in each sample was expressed in terms of the threshold cycle (C_t) normalized to GAPDH (ΔC_t). The ΔC_t values were compared between samples from MK-677 treated tissues (hypothalamus and pituitary gland) and control samples to calculate $\Delta\Delta C_t$. The final comparison of the transcript ratios between samples is given as $2^{-\Delta\Delta Ct}$.[14]

Statistical analysis

All statistical calculations were performed using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Two-tailed t test was used to test for differences between the treatment group and controls. *p* values <0.05 were considered statistically significant.

RESULTS

GH response to MK-677 at baseline and at 6 weeks after treatment

Serum GH concentrations after oral administration of 2 and 4 mg/kg of MK-677 or control at baseline are shown in Fig. 1A. Oral administration of MK-677 at 4 mg/kg significantly increased GH concentrations with a 1.8-fold increase in peak GH concentration (45.7 ng/mL), whereas administration of distilled water did not increase GH concentrations. The GH area under the curve (AUC) showed a similar significant increase after administration of 4 mg/kg of MK-677, compared to the distilled water control group (1090 vs. 206 ng/min/mL, $p<0.05$). The peak GH concentration was observed at 60 min after treatment and returned to near-pretreatment levels by 120 min. The increased response of GH after administration of MK-677 was abolished in animals treated with MK-677 for 6 weeks. Oral administration of MK-677 at 4 mg/kg in rats treated with MK-677 for 6 weeks did not increase GH concentrations (Fig. 1B).

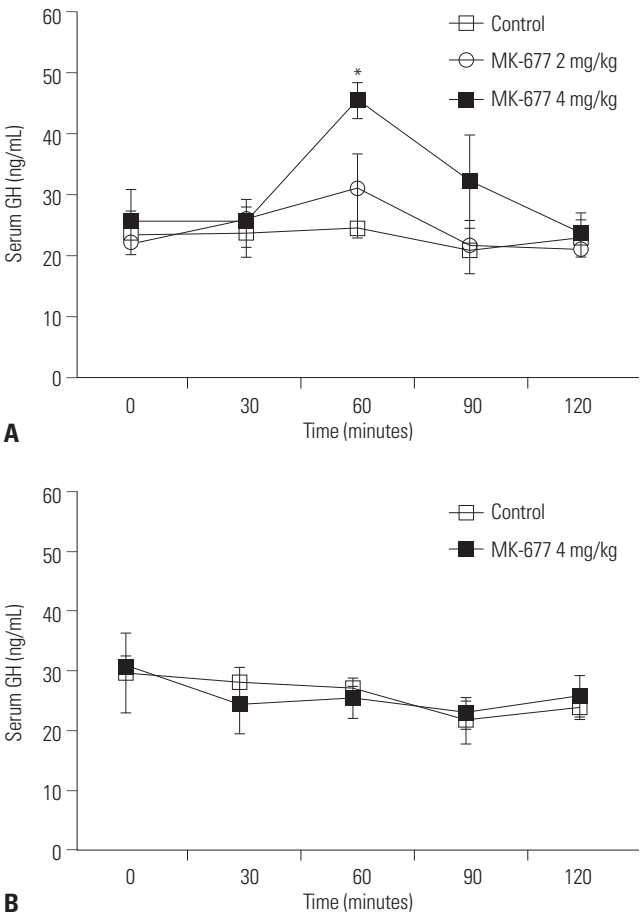

Fig. 1. Serum growth hormone (GH) levels after oral administration of MK-677. (A) Dose range study at baseline. MK-677 at 0, 2, and 4 mg/kg were administered. (B) MK-677 at 4 mg/kg was administered in rats at 6 weeks after treatment of MK-677. n=4/treatment. *$p<0.001$.

Efficacy of oral administration of MK-677 for 6 weeks

The treatment group showed no increase in body weight, body length, and width of the tibia growth plate after 6 weeks of MK-677 treatment, compared to the control group (Fig. 2). Serum IGF-I levels at 2, 4, and 6 weeks were not changed, compared to those at baseline, and did not differ between the treatment group and control group at each time point (Fig. 3).

Effect of MK-677 on mRNA and protein expression of GH, GHRH, GHSR, SST, and SSTRs in the pituitary gland and hypothalamus

Pituitary GH and hypothalamic GHRH mRNA levels did not

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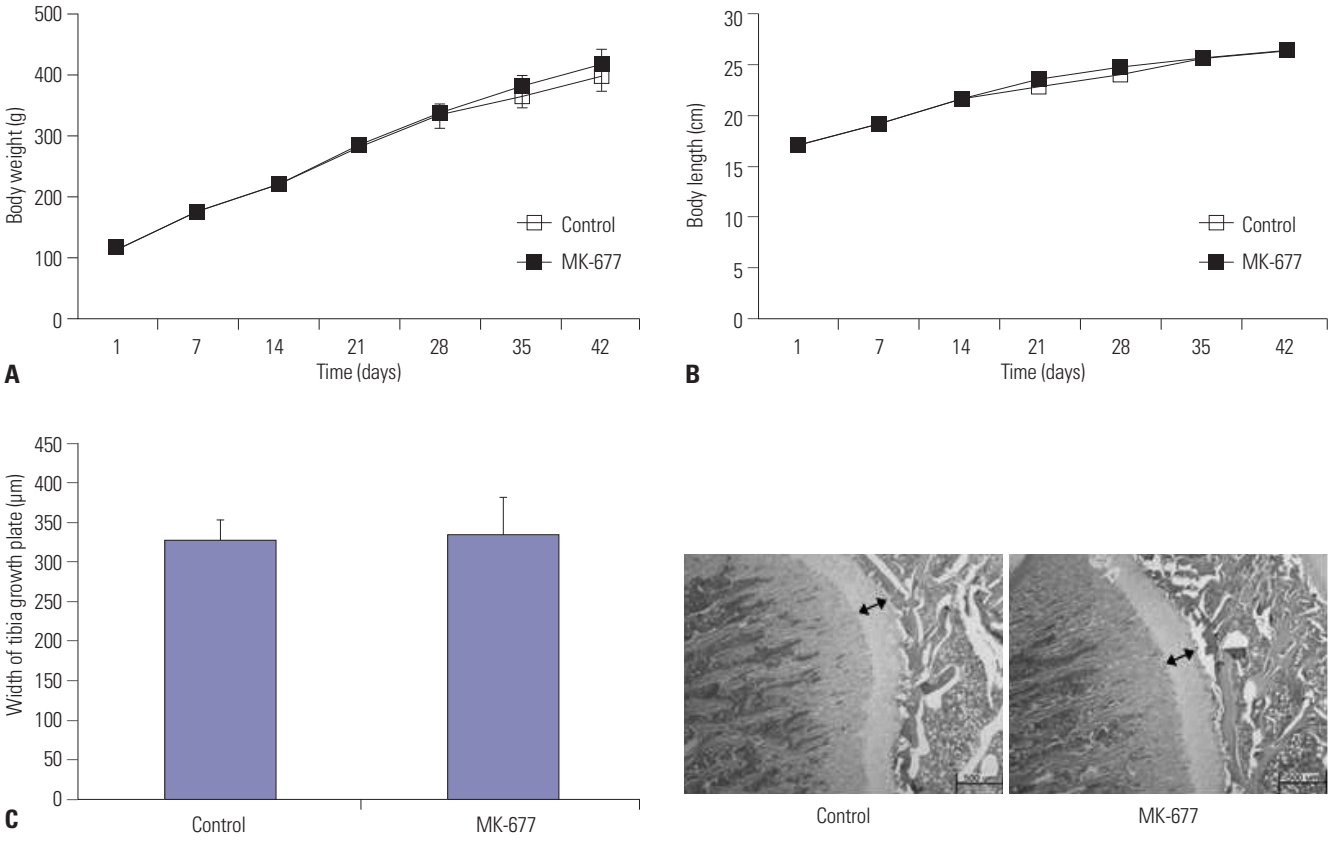

Fig. 2. Efficacy of oral administration of MK-677 for 6 weeks. (A) Body weight, (B) body length, and (C) width of tibia growth plate (arrows) detected by hematoxylin-eosin staining (×40). n=4/treatment.

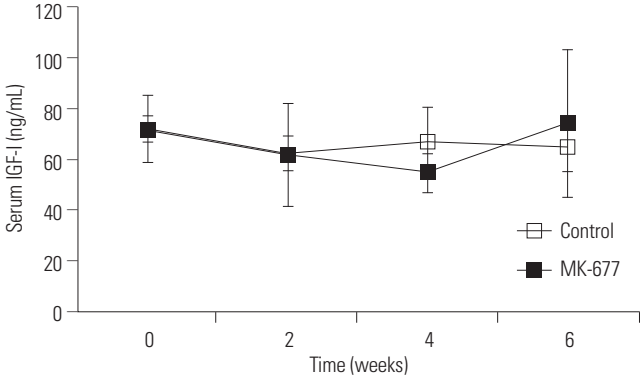

Fig. 3. Serum insulin-like growth factor (IGF)-I levels during oral administration of MK-677 for 6 weeks. n=4/treatment.

differ between the control and treatment groups (Fig. 4A and B). Pituitary and hypothalamic GHSR mRNA expression did not differ between the control and treatment groups (Fig. 4C). SST mRNA expression in the hypothalamus was markedly increased in the treatment group, compared to the control group (Fig. 4D). In addition, SSTR-2 mRNA expression in the pituitary gland was decreased in the treatment group, compared to the control group (Fig. 4E), whereas SSTR-5 mRNA expression in the pituitary gland was not different between two groups (Fig. 4F). Protein expression of hypothalamic GHSR, SST, and pituitary SSTR-2 and -5 showed the same patterns as those for mRNA expression (Fig. 4G).

DISCUSSION

Secretion of GH in the pituitary gland is regulated by GHRH and SST in the hypothalamus. GHRH stimulates the release of GH through GHRH receptor, whereas SST represses the release of GH through SSTR.[15] In 1977, new synthetic peptides with GH-releasing ability were discovered.[16] These new compounds were enkephalin analogs, with weak GH-releasing activity *in vitro* and inactivity *in vivo*. Subsequently, more potent GHRPs were developed by chemical modifications.[17] GHRPs have no sequential homology with GHRH and are more effective for inducing GH release even at the same dose of GHRH.[18] Many studies have found that GHRPs have GH releasing activity in several species via their own receptors known as GHS receptor, not GHRH receptor.[19] Recently, to overcome the limit of low oral bioavailability, peptide-mimicking and non-peptidyl GHS, which can be administered orally, were developed.[4,5,20] SM-130686, an orally active non-peptidyl GHS, was developed and was confirmed to have GH-releasing activity. In rats, SM-130686 was found to enhance GH secretion and body weight gain.[20] Another non-peptidyl GHS, ibutamoren

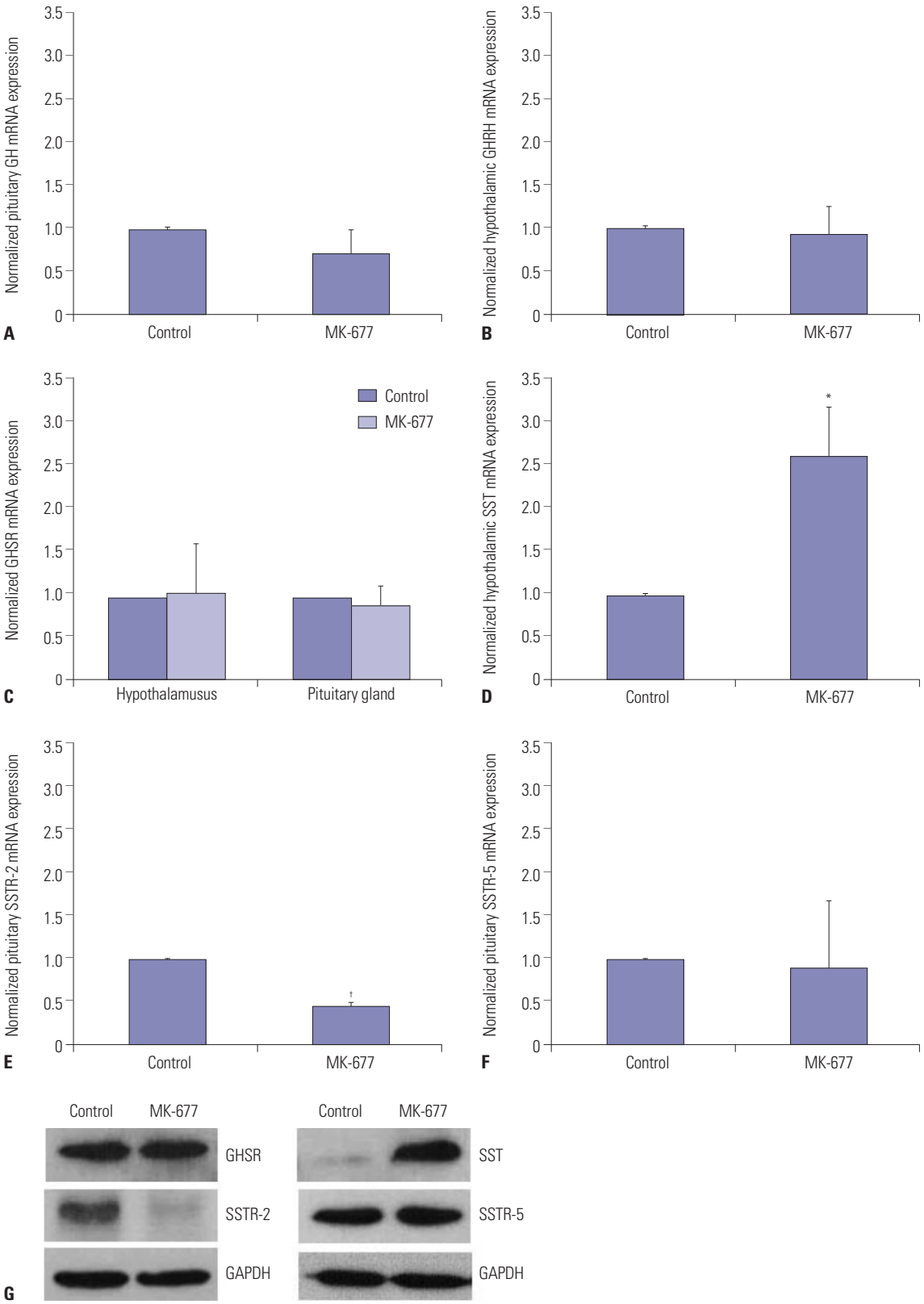

Fig. 4. Effect of MK-677 on mRNA and protein expression of growth hormone (GH), GH-releasing hormone (GHRH), GH secretagogue receptor (GHSR), somatostatin (SST), somatostatin receptor (SSTR)-2, and SSTR-5 in the hypothalamus or pituitary gland. (A) Pituitary GH mRNA level, (B) hypothalamic GHRH mRNA level, (C) hypothalamic and pituitary GHSR mRNA level, (D) hypothalamic SST mRNA level, (E) pituitary SSTR-2 mRNA level, (F) pituitary SSTR-5 mRNA level, and (G) protein levels of GHSR and SST in the hypothalamus and SSTR-2 and SSTR-5 in the pituitary gland. n=4/treatment. *p<0.01 or †p<0.001.

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mesylate (MK-677), was reported to stimulate the release of GH and to show an oral bioavailability of more than 60%.[4,18] Oral administration of MK-677 was also shown to elevate GH levels, as well as IGF-I and cortisol levels, in dogs[6] and that this stimulatory effect is dependent on the presence of an intact pituitary.[7] In humans, daily oral administration of MK-677 in healthy older adults,[8] GH-deficient adults,[9] or GH-deficient children[10] increases serum GH, IGF-I, and IGFBP-3 concentrations, suggesting that these GHSs are potential treatments for growth disorders. However, repeated administration of GHRP has been reported to desensitize its stimulatory effect on GH secretion, and there have been no studies of the growth promoting effect after its long-term use, thereby limiting clinical application.[21]

In our study, oral administration of 4 mg/kg of MK-677 increased the peak concentrations of serum GH by 1.8-fold, compared to basal levels, and GH AUC was significantly increased, suggesting that oral administration of MK-677 has GH-releasing activity. However, the growth promoting effect assessed by measuring the body weight, body length, width of tibia growth plate, and serum level of IGF-I was not observed after oral administration of 4 mg/kg of MK-677 for 6 weeks. The desensitization phenomenon of GHRP has been reported previously.[22-26] In an animal study using transgenic growth-retarded rats, infusion of GHRP-6 for 7 days produced a dose-dependent increase in body weight gain and accelerated skeletal growth.[26] However, this growth promoting effect was observed only in the group infused with GHRP-6 at 3-h pulses. Continuous infusion of GHRP-6 was only effective in accelerating growth for the first 2 days of infusion, suggesting that the growth response with continuous infusion is transient because of desensitization. In normal female rats, continuous infusion of GHRP has been reported to induce tachyphylaxis after an initial increase in growth velocity, suggesting that continuous infusion of GHRP induces desensitization.[27] Repeated administration of hexarelin at 120-min intervals decreased the magnitude of the GH response in healthy adult males.[25] In our study, oral administration of MK-677 did not stimulate GH secretion after treatment for 6 weeks, suggesting that the initial GH stimulatory effect of MK-677 was abolished in animals treated with MK-677 for 6 weeks.

The mechanisms of GHRP desensitization after continuous infusion or long-term use have not been fully clarified. Many studies have demonstrated that continuous infusion of GHRP did not alter GH stores and GHRH receptor expression in the pituitary gland or GHRH expression in the hypothalamus.[22,23,27] The most direct and probable mechanism is the down regulation of GHSR in the pituitary gland and hypothalamus after treatment with GHRP. However, previous studies demonstrated that continuous infusion of GHRP-6 did not alter hypothalamic GHSR expression in arcuate and ventromedial nuclei[28] or even increase arcuate GHSR expression.[26] The results of our study also showed that oral administration of MK-677 for 6 weeks did not alter mRNA expressions of pituitary GH and hypothalamic GHRH and GHSR in the pituitary gland and hypothalamus or protein expression of GHSR in the hypothalamus. Another possible mechanism of desensitization induction is increased expression of SST in the periventricular nucleus following continuous infusion of GHRP.[26,29] In our study, mRNA and protein expression of SST in the hypothalamus was markedly increased after treatment with MK-677 for 6 weeks, which inhibits GHRH- and GHS-induced GH secretion. In contrast, mRNA and protein expression of SSTR-2 in the pituitary gland was decreased, which may have resulted from elevated expression of SST in the hypothalamus.

In conclusion, oral administration of the GHS MK-677 stimulates GH secretion. However, prolonged oral administration for 6 weeks does not promote growth and abolishes the GH stimulatory effect of MK-677, potentially resulting from increased expression of SST in the hypothalamus. Further studies are needed to develop a strategy for overcoming the increased expression of SST, thereby leading to growth promotion.

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