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Induction of Growth Hormone Release by Glycyrrhizae Radix on Rat

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Induction of growth hormone (GH) by Glycyrrhizae Radix (GR), one of the most popular herbal medicine, and its major ingredients were studied in rat pituitary cells *in vitro* and *in vivo* assay. The MeOH extract and the *n*-hexane (HX) fraction of GR induced rat GH (rGH) release up to 1.89 times (0.34 ± 0.04 nM) and 4.59 times (0.83 ± 0.03 nM), compared to the basal level ($p < 0.05$). Among many ingredients isolated and purified from GR both glycyrrhetinic acid and glycyrrhizin induced significantly rGH release compared to the control ($p < 0.05$). After an intravenous injection of rat growth hormone releasing hormone (rGHRH) (10 μg/kg) as positive control, in SD rats, T_{max} of plasma rGH level was 10 min, C_{max} was 3.84 ± 0.01 nM (n = 3), and enhanced plasma rGH level returned to the baseline in 90 min. Both $AUC_{0\text{-}90}$ (area under the curve) of plasma rGH level after HX fraction and that after rGHRH administration were increased significantly from the basal level, respectively ($p < 0.01$). In conclusions, HX fraction is the most active fraction of MeOH extract of GR in rGH induction.

Keywords: Glycyrrhizin, Glycyrrhizae Radix, Growth hormone (GH), Growth hormone releasing hormone (GHRH), Growth hormone secretagogue (GHS), Pituitary cells

Introduction

Glycyrrhizae Radix (GR), a perennial herb, is the dried roots and rhizomes of *Glycyrrhiza glabra* L. (Leguminosae) or *Glycyrrhiza uralensis* FISCH, and their variances. GR has been used as a tonics and one of the most popular herbal medicine in Northeast Asia for several thousand years. GR contains, as major ingredients, the triterpenes of the oleanane type, mainly glycyrrhizin (GZ), glycyrrhetinic acid (GA), liquiritic acid (LA) and glabrolide (GL); liquiritigenin (LG), isoliquiritigenin (IL) of the flavones; formononetin (FO) of the isoflavone (Kitagawa *et al*., 1993; Kitagawa *et al*., 1993; Fukai *et al*., 2002). It further contains coumarins, chalcones and polysaccharides (Zeng *et al*., 1990). It has been reported that GR and its ingredients have been used antiulcer activity (Yano *et al*., 1989), liver protection, anti-inflammatory and antiallergic actions, antiviral (Shibata, 2000) and antitoxic activities, antitussive (Kamei *et al*., 2005) and expectorant properties (Shaikh *et al*., 1999), demulcent action, and anti-fatty liver activity (Shin, 1988).

It has been reported that GR and its ingredients have glucocorticoid-like action and release adrenocorticotropic hormone (ACTH) (Eun *et al*., 1989; Hanafusa *et al*., 2002; Kageyama *et al*., 2004). The secretion of ACTH from the anterior pituitary corticotrophs is controlled by the hypothalamic release of corticotropin-releasing hormone and vasopressin. They can also stimulate steroidogenesis directly in rat adrenal glands, stimulates the secretion of glucocorticoids, minernalocorticoids, and anterior pituitary corticotroph-releasing hormone and vasopressin from the adrenal cortex (Wang *et al*., 2000). Glucocorticoid in excess inhibits growth in children, and this adverse effect is a major complication of therapy. This may be a direct effect on bone cells, although decreased growth hormone (GH) secretion and somatomedin generation also contribute (Greenspan *et al*., 2001). However the endocrine activity of both ghrelin and synthetic growth hormone secretagogue (GHS) is not specific for GH but also includes a stimulatory effect on corticotroph (Smith *et al*., 1997; Emanuela *et al*., 2002). The ACTH-releasing activity of GHS is independent of gender but shows peculiar age-related variation.

GH, as an anabolic hormone essential for the regulation of normal growth rate, accelerates both protein synthesis and fat decomposition. Pulsatile GH release from anterior pituitary is regulated by GH-releasing hormone (GHRH) and somatostatin-releasing inhibition factor (SRIF) (Shimokawa *et al*., 1997), as well as the synthetic peptide or non-peptide molecule of

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GHSs (Muller *et al*., 1999). Many kinds of GHS including GH-releasing peptides (GHRPs) (Bowers *et al*., 1991; McDowell *et al*., 1995; Hansen *et al*., 1999) have been developed, which have improved bioavailability and pharmacokinetic properties (Smith RG *et al*., 1993; Smith *et al*., 1997). GHS elevated low concentration of insulin-like growth factor I (IGF-I) in blood of healthy elderly people aged 60-80 as high as young people and improved the quality of life (Lanfranco *et al*., 2003); it further increased lean body mass, spinal bone mineral density and the thickness of skin, while reducing the quantity of fat tissue (Rudman *et al*., 1990).

In this study, the induction of rGH and rLH release by GR and its ingredients was studied in order to develop substitution for GHRH or GHS that made up defects of hGH administration and showed stability to prolonged administration, as well as was capable of oral administration.

Materials and Methods

Materials. The GR (specimen number in our herbarium: KIOM 01-3-32) was purchased from Korea Oriental Medicinal Herb Association (Seoul, Korea) and evaluated by Je-Hyun Lee, O.M.D., Ph.D. (Dongguk University, Gyeongju, Korea), prior to its use. This specimen was stored in the herbarium of Korea Institute of Oriental Medicine (Daejeon, Korea). The major ingredients of GR, GZ and GA, were purchased (Wako Pure Chemical industries, Ltd.) as reference standards and all other chemicals used reagent grade.

Preparation of Samples. The MeOH extract of GR and its fractions were obtained as follows: dried GR was extracted by 70% methanol (MeOH) at room temperature for 7 days. The 70% MeOH extraction repeated 3 times. The extract was concentrated using a rotary evaporator and lyophilized. The MeOH extract was dissolved in distilled water, followed by extraction with *n*-hexane (HX), ethyl acetate (EA), BuOH, and aqueous (H_2O) residue, sequentially.

GR (1 kg) was pulverized and extracted with HX. The HX extract was dried following the method described above (yield: 14 g). Residues of GR were extracted with 10 L of 70% MeOH, followed by concentration under reduced pressure to give MeOH extract (yield: 150 g) which was described above. The mixed solution of 5% HCl-80% dioxane (1 L) was added to the MeOH extract and hydrolyzed in a boiling water bath for 5 h. The hydrolysate was concentrated to a half volume at room temperature using a rotary evaporator. Iced water was added to the obtained concentrate to give precipitates and the precipitates were collected and washed with distilled water, followed by drying to give powder (yield: 60 g).

The hydrolyzed product was poured onto water and then defatted with *n*-hexane. The aqueous layer was partitioned with chloroform and EtOAc successively. The chloroform extract (10 g) was separated into 8 subfractions on a silica gel column (5 cm ID) chromatography using a *n*-hexane-EtOAc-MeOH gradient system (2 : 1 : 0.1 → 2 : 1 : 10, 500 ml each). Combined subfractions (0.9 g) of 6-8 was further separated by silica gel column (2 cm ID) chromatography (*n*-hexane-EtOAc = 8 : 5, 650 ml) to give GL (30 mg), LA (20 mg) and GA (50 mg). The EtOAc extract (5 g) was purified by chromatography on a silica gel column (3 cm ID), with a gradient of MeOH in $CHCl_3$ (100 : 0 → 0 : 100, 200 ml each), to afford 10 subfractions. Subfractions 5-7 (500 mg) were further purified by a reversed phase chromatography on a RP-18 column (1 cm ID), with MeOH-H_2O (4 : 6, 300 ml) as eluent, to give FO (5 mg), LG (10 mg) and IL (2 mg), respectively. The known compounds, GL, LA, GA (Kang *et al*., 2004), FO (Kang *et al*., 2000), LG (Nakanishi *et al*., 1985; Kang *et al*., 2000) and IL (Kang *et al*., 2000) were identified by comparison of their physical and spectral data with those of reported values. GZ and GA were purchased from Wako Pure Chemical industries. Each of the isolated compounds was identified by comparison between spectroscopic data and reference (Fig. 1). The MeOH extract, its sequential fractions and ingredients were dissolved in the culture medium to contain 0.1% DMSO (Sigma Chem. Co., St. Louis, USA) prior to their use.

Rat pituitary cell culture. The Sprague-Dawley (SD) rats (3-4 weeks old; Daehan Biolink) were adapted for 1 week. Preparation of pituitary cells were followed the method of Kim *et al*. (Kim *et al*., 2003; Jung *et al*., 2004). In a brief, the rat pituitary was isolated rapidly, washed with cold Hank's balanced salt solution (HBSS, pH 7.4, Sigma Chem. Co.), and cut finely. The chopped tissues were treated with 0.2% hyaluronidase type II (Sigma Chem. Co.) and 0.2% collagenase type IV (Gibco BRL) to isolate cells, wherein their viability was 90% or more. The isolated pituitary cells were washed with Dulbecco's Modified Eagle's medium (DMEM) containing 2.5% fetal bovine serum (FBS) and 10% horse serum (Gibco BRL), followed by addition to the same medium and incubation in a humidified 5% CO_2-95% air atmosphere at 37°C for 4-5 days.

Treatment of samples on rat pituitary cells. The cultured rat pituitary cells were collected, washed with HBSS, suspended in HBSS and adjusted to 7.5×10^4 cells/mL, then followed by addition of various concentrations of rGHRH (Bachem, Budendorf, Switzerland) anged 0-1.0 μM, extract, fractions and ingredients and then incubation at 37°C for 15 min. rGHRH was used a positive control. The supernatant was collected and measured concentration of hormones.

Each samples were added to the pituitary cells: the MeOH extract (1 mg/mL corresponding to 1 mg of dried herb/mL); HX, EA, BuOH, and aqueous fractions were added at 20 μg/mL, respectively; sub-fractions and ingredients were added at 10 μg/mL, respectively; and the pituitary cells were incubated at 37°C for 15 min and then the supernatant was collected after centrifugation at 13,000 rpm, 4°C for 10 min and stored at −20°C to quantify concentration of rGH, rACTH, and rLH.

***In vivo* assay; rGH induction in rats.** Male SD rats (250-300 g; Daehan Biolink, Eum-seong, Chung-buk, Korea) were anesthetized with 50 mg/kg of pentobarbital (Hanlim Pharm. Co.) intraperitoneal injection. rGHRH (10 μg/kg), MeOH extract (corresponding to 1 mg of dried herb/kg), each of sequential fractions (20 μg/kg), and GA (20 μg/kg) were given through jugular vein, respectively. Blood (0.5 mL) was taken at time (0, 10, 20, 30, 45, 60, 90, and 120 min) from tail vein, and followed by centrifugation to gather

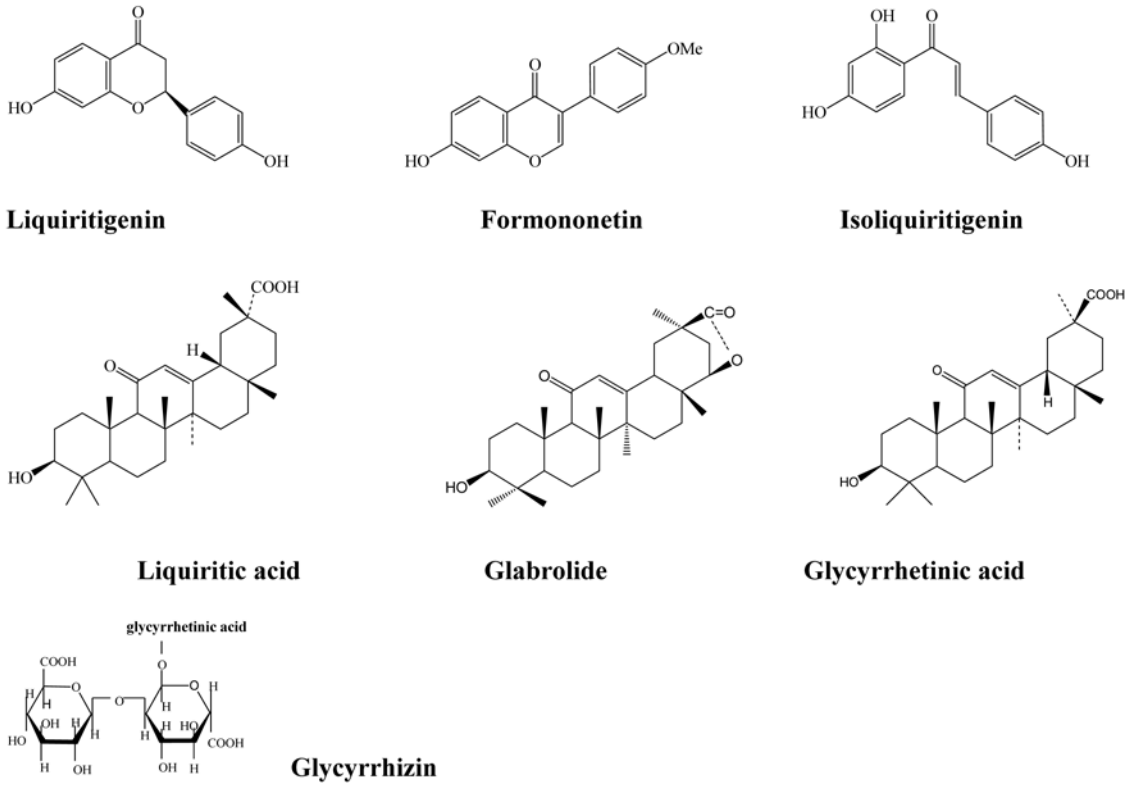

Fig. 1. Structures of ingredients which were isolated and purified from Glycyrrhizae.

plasma. The obtained plasma was stored at −20°C until the quantitative analysis of rGH. All samples were prepared in saline and the control group was given only saline intravenously.

Quantitation of rGH. The concentrations of rGH release on the pituitary cells and plasma rGH were analyzed with a radioimmuno assay kit (RIA; Amersham Pharmacia Botech, UK). In a brief, the concentrations of standard rGH ranged from 0.07 nM to 4.55 nM. rGH-antibody (Ab) complexes were obtained by the competitive reaction for anti-rGH between a regular amount of [^{125}I]- labeled rGH and the standard GH by concentration gradient. The rGH-Ab complexes were precipitated by addition of second antibodies and measured the radioactivity with a γ-counter (Wallac 1470, Wallac EG&G comp, Turku, Finland) after non-reacted antibodies were then removed. The concentrations of rGH in the culture media and plasma rGH were calculated using the standard calibration curve plotted by various concentrations of standard rGH.

Quantitation of rACTH. The concentrations of rACTH release on the pituitary cells and plasma rACTH were analyzed with a radioimmuno assay kit (RIA; Phoenix Pharmaceuticals, Inc.) as like rGH assay. The concentration of standard rACTH used ranged from 0.22 pM to 28.2 pM.

Quantitation of rLH. The concentrations of rLH release on the pituitary cells and plasma rLH were analyzed with a radioimmuno assay kit (RIA; Amersham Pharmacia Botech, UK) as like rGH assay. The concentration of standard rLH used ranged from 0.02 nM to 0.14 nM.

Statistical analysis. Data was represented by mean ± SEM and defined that $p < 0.05$ had statistical significance by ANOVA or Bonferroni Multiple comparison method (SYSTAT 10.0, SPSS Inc., USA).

Results

Induction of rGH on rat pituitary cells. As the result of addition of rGHRH (0.1, 0.3, 0.5, and 1.0 μM, respectively) on rat pituitary cells, rGH release was increased 1.12, 1.55, 2.97 ($p < 0.01$) and 7.41 ($p < 0.01$) times as high as that of the control (0.19 ± 0.02 nM, n = 9), respectively. rGHRH increased rGH release concentration-dependently (Fig. 2).

The concentrations of rGH released by the MeOH extract of GR (1 mg/mL), HX and EA (20 μg/mL) were increased approximately 1.89 times ($p < 0.05$), 4.60 times ($p < 0.01$), and 1.86 times ($p < 0.01$) as high as the control (0.19 ± 0.03 nM, n = 12). BuOH and H_2O had no significance in rGH release, compared to the control. GA and GZ increased significantly rGH release approximately 1.42 (0.25 ± 0.02 nM, n = 5) and 1.43 times (0.26 ± 0.02 nM, n = 5) as high as the control, respectively ($p < 0.05$) (Fig. 3). HX consequently had the most effect on rGH release.

Induction of rACTH and rLH on rat pituitary cells. The concentrations of rACTH released by the MeOH extract of GR (1 mg/mL), HX, EA, BuOH, and H_2O fraction (20 μg/mL)

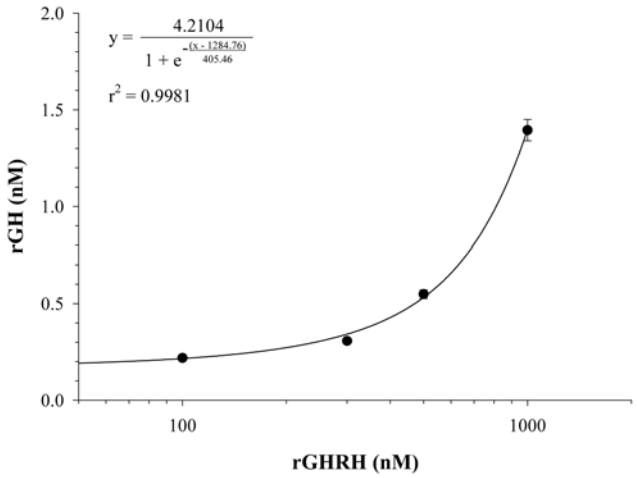

Fig. 2. Induction of rGH release by various concentrations of rGHRH. Each of data is represented by mean ± SEM (n = 9). Bar indicates standard error of mean (SEM). ** means $p < 0.01$, compared to 0 nM of rGHRH based on Bonferroni multiple comparisons.

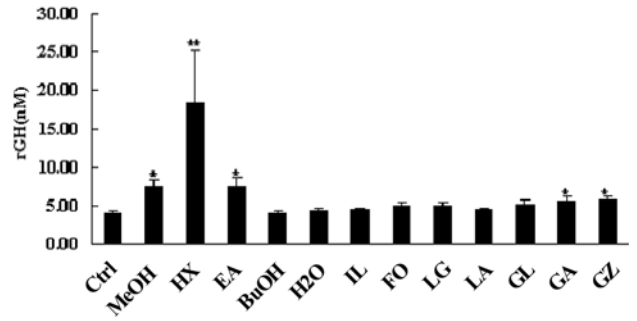

Fig. 3. Induction of rGH release by MeOH extract of Glycyrrhizae Radix (1 mg/mL), its sequential fractions (20 μg/mL), and its ingredients (10 μg/mL) on rat pituitary cells. Bar indicates standard error of mean (SEM). Ctrl; Control (n = 15), MeOH; MeOH extract (n = 15), HX; hexane fraction (n = 8), EA; ethyl acetate fraction (n = 7), BuOH; BuOH fraction (n = 9), H_2O; aqueous fraction (n = 8), IL; isoliquiritigenin (n = 6), FO; formononetin (n = 6), LG; liquiritigenin (n = 6), LA; liquiritic acid (n = 6), GL; glabrolide (n = 6), GA; glycyrrhetinic acid (n = 5), GZ; glycyrrhizin (n=5). * and ** mean $p < 0.05$ and $p < 0.01$, respectively, compared to the control based on Bonferroni multiple comparisons.

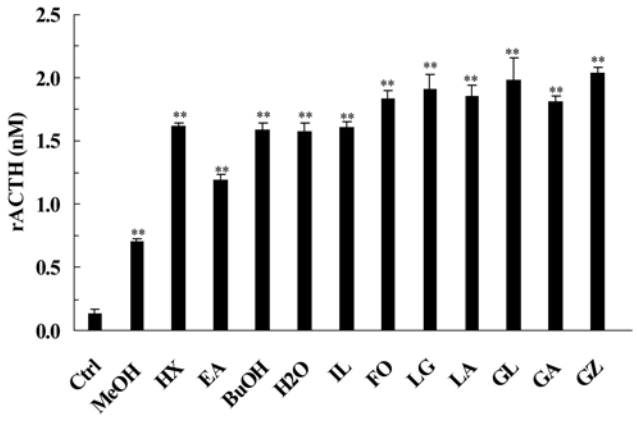

Fig. 4. Induction of rACTH release by MeOH extract of Glycyrrhizae Radix (1 mg/mL), its sequential fractions (20 μg/mL), and its ingredients (10 μg/mL) on rat pituitary cells. Bar indicates standard error of mean (SEM). Ctrl; Control (n = 15), MeOH; MeOH extract (n = 15), HX; hexane fraction (n = 8), EA; ethyl acetate fraction (n = 7), BuOH; BuOH fraction (n = 9), H_2O; aqueous fraction (n = 8). * and ** mean $p < 0.05$ and $p < 0.01$, respectively, compared to the control based on Bonferroni multiple comparisons.

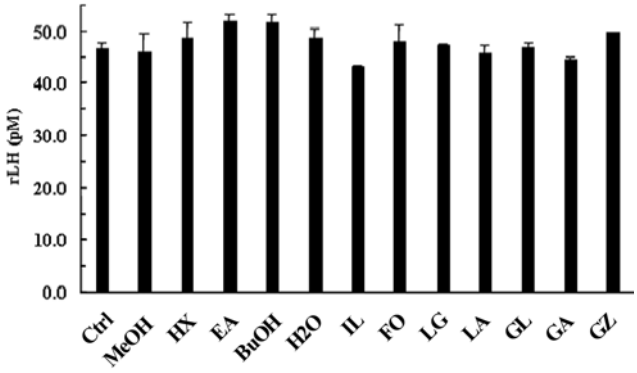

Fig. 5. Induction of rLH release by MeOH extract of Glycyrrhizae Radix (1 mg/mL), its sequential fractions (20 μg/mL), and its ingredients (10 μg/mL) on rat pituitary cells. Bar indicates standard error of mean (SEM). Ctrl; Control (n = 15), MeOH; MeOH extract (n = 15), HX; hexane fraction (n = 8), EA; ethyl acetate fraction (n = 7), BuOH; BuOH fraction (n = 9), H_2O; aqueous fraction (n = 8). * and ** mean $p < 0.05$ and $p < 0.01$.

were increased approximately 5.13, 11.66, 8.53, 11.48, and 11.35 times as high as the control (0.14 ± 0.03 nM, n = 12) ($p < 0.01$) (Fig. 4). IL, FO, LG, LA, GL, GA and GZ increased rACTH approximately 11.6, 13.2, 13.8, 13.4, 14.3, 13.0 and 14.7 times as high as the control, respectively ($p < 0.01$). All of GR and its ingredients released rACTH on rat pituitary cells. The concentration rLH release by MeOH extracts, fraction, and ingredients had no significant compared to the control (Fig. 5).

***In vivo* assay; rGH induction in rats.** The effective fractions *in vitro* assay were studied *in vivo* assay. A time course of rat plasma rGH level after i.v. injection of rGHRH (10 μg/kg) is shown in Fig. 6, wherein T_{max} of rat plasma rGH level was 10 min, C_{max} was 4.02 ± 0.52 nM (n = 7) and plasma rGH level returned rapidly to basal level within 90 min after the injection. In the MeOH extract group (1 mg/kg), T_{max} of rGH level was 30 min and C_{max} was 0.75 ± 0.02 nM (n = 11). In the HX group (20 μg/kg), T_{max} was 10 min and C_{max} was 7.17 times (1.42 ± 0.53 nM, n = 4) as high as the control ($p < 0.01$). There was no significant difference in the EA group (20 μg/kg) compared to the control. A time course of rat plasma rGH after administration of GA, one of the active ingredient *in*

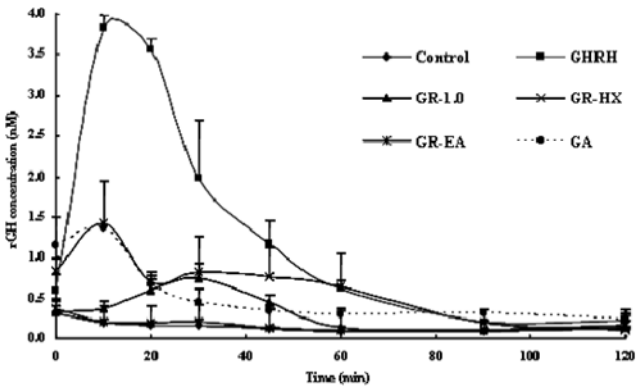

Fig. 6. Time courses of rat plasma rGH levels after i.v. of samples in rats. GHRH; rGHRH (10 μg/kg, n = 7), GR 1.0; MeOH extract of Glycyrrhizae Radix (1.0 mg of dried herb/kg, n = 11), GR-HX; hexane fraction (20 μg/kg, n = 4), GR-EA; EA fraction (20 μg/kg, n = 3), and GA; glycyrrhetinic acid (20 μg/kg, n = 3). The control group was administered with saline (1.0 mg/kg, n = 19). Each point is expressed by mean ± SEM.

vitro assay (Fig. 3), follows the similar pattern to that of rGHRH. $AUC_{0\text{-}90}$ (area under the curve 0-90 min) was increased significantly in the groups treated with rGHRH (144.6 ± 12.6 nM · min, n = 7, $p < 0.01$) and HX fraction (58.8 ± 15.2 nM · min, n = 4, $p < 0.01$), compared to the control (17.1 ± 1.8 nM · min, n = 9). $AUC_{0\text{-}90}$ in the HX group was significantly lower than that of the rGHRH group ($p < 0.01$). MeOH extract of GR and EA fraction had no effect on rGH induction *in vivo*, differently from *in vitro* test. $AUC_{0\text{-}90}$ in the GA group (47.1 ± 10.43 nM · min, n = 3 $p < 0.01$) was increased significantly compared to the control. T_{max} of rat plasma rGH level was 10 min which is the same as those of rGHRH and HX groups. C_{max} of the GA group was 30.00 ± 1.36 nM (n = 3) which is similar to that of HX group.

Discussion

Recently, GH has been the treatment for obesity, anti-aging and osteoporosis as well as dwarfism. However, recombinant human GH (rhGH) is not widely supplied because of price and method of dosage; injection etc. We are studying the induction of GH by medicinal herbs. It has reported that the Glycyrrhizae radix (GR) of medicinal herbs released ACTH and GHS. And ACTH is released by GR of medicinal herbs and GHS. Also, it has many reported that GHS release the GH. We studied whether GR which released ACTH in duce the GH.

GR was well known as a medicinal herb that regulated drug properties well, when used with other medicinal herbs. GR was imported over 2,000 tons annually and its consumption was the greatest in the domestic market in Korea. Further, it was reported that both blood corticosterone level and blood histamine level were increased when GR extract and GZ were injected into an ICR mouse (Eun *et al*., 1989). Glucocorticoid and catecholamine act on homeostasis maintenance in the body. It has been reported that GR and its ingredients release ACTH so that they have glucocorticoid-like action (Fig 4) (Eun *et al*., 1989; Kageyama *et al*., 2004). The endocrine activity of both ghrelin and synthetic GHS is not specific for GH but also includes a stimulatory effect on both lactotroph and corticotroph secretion (Smith *et al*., 1997). Hypothlamus-pituitary receptors explain the stimulatory effect of GHS on GH, prolactin, and ACTH secretion (Bowers *et al*., 1991; Takara *et al*., 2000).

Induction of GH *in vivo* was not similar to that *in vitro*. When rGHRH was added (especially 1 μM), it increased rGH release 7.41 times as high as the control on the pituitary cells (Fig. 2). This represents GHRH acts on a pituitary somatotroph cell and enhances the synthesis and release of rGH (Mayo *et al*., 1995), and a GHRH receptor gene is expressed predominantly in anterior pituitary (Mayo 1992). The administration of GHRH increased rGH release concentration-dependently *in vivo* (Fig. 6). $AUC_{0\text{-}90}$ of plasma rGH as shown in Fig. 6 represents the accumulation of GH concentration in plasma within 90 min after administration and it showed that rGHRH enhanced rGH release very strongly. T_{max} of HX fraction and GA was 10 min and plasma GH level returned to the basal level within 90 min. $AUC_{0\text{-}90}$ of the HX fraction and GA group was much lower than that of the rGHRH group ($p < 0.01$), significantly higher than the control. In the changes of plasma rGH level by time, T_{max} corresponded to the rGHRH group, but it showed another release pattern differently from pulsatile release occurred by the peptide of GH enhancer, such as GHRP or ghrelin as endogenous GHS (Jacks *et al*., 1996). The peptide of GH enhancer was disposed to show short-lasting blood GH level, while a part of the non-peptide of GH enhancer showed long-lasting blood GH level (Pettersson *et al*., 2002). According to Frohman *et al*. (2001) as short-term effect (within 4 h), GHS and GHRH inhibited the expression of mRNAs of GHS-receptor and GHRH-receptor, respectively. However, as long-term effect (over 12 h), therefore, it is considered that mechanistic relationship between GHS and GHRH should be examined prior to long-term administration of GR.

In results, GR and GA released ACTH as like many reported and those were induced rGH on rat pituitary cells. However it didn't release LH. We thought that GR and GA reacted on the ACTH and GH in pituitary hormones, especially we are stying on pathway which GR and GA belongs to GHS or GHRH.

In summary, the HX fraction and GZ increase GH as well as ACTH in pituitary hormones. On the other hand, those were not released rLH. The HX fraction and GZ may either increases endogenous GHRH concentration directly in pituitary level or acts on GHS-receptor or GHRH-receptor as a direct partial agonist or antagonist (Tannenbaum *et al*., 2003). It is consequently considered that the isolation, verification and mechanism of effective ingredients contained in GR such as GA need to be studied in future.

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