Journal of Biochemistry and Molecular Biology, Vol. 40, No. 6, November 2007, pp. 1016-1020

JOURNAL OF Biochemistry and Molecular Biology

Induction of Growth Hormone Release by Dioscin from *Dioscorea batatas* DECNE

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Received 22 February 2007, Accepted 8 August 2007

In this study, dioscin was isolated from Dioscoreae Rhizoma (DR), which is the rhizome of *Dioscorea batatas* DECNE. that inhabits broad areas of Korea and Japan. To determine whether dioscin induced growth hormone (GH) release, we evaluated its induction effects on GH release both *in vitro* and *in vivo*. The 70% methanol extract of DR, and its *n*-hexane and *n*-BuOH fractions, induced rat GH (rGH) release in rat pituitary cells 10-fold, 8-fold, and 5-fold higher than the control (0.36 ± 0.02 nM), respectively ($p < 0.05$ each). The dioscin-induced rGH release of the cells was concentration-dependent and its ED_{50} was 1.14×10^{-5} M. Within 90 minutes after intravenous administration of 10 μg/kg ($p < 0.05$ at t_{max}), dioscin caused the greatest increase in rGH concentration (C_{max}) in the rat plasma (34.16 ± 14.10 ng/ml) (n = 4), which was twice as high as the control group (12.88 ± 3.29 ng/ml) (n = 27).

Keywords: Dioscin, Dioscoreae rhizoma, Growth hormone (GH), Rat pituitary cells

Introduction

Growth hormone (GH) is a peptide hormone secreted from the pituitary of animals, and consists of 191 amino acids with a molecular weight of 22 kD. GH release is regulated by GH-releasing hormones (GHRH or GRF) and somatotropin-release inhibiting factor (SRIF). In particular, it is regulated by neuro-transmitters or neuropeptides such as serotonin, dopamine, calcitonin, neuropeptide Y (NPY), etc. (Argente *et al*., 1996).

Additionally, ghrelin (Kojima *et al*., 1999), which is secreted from the stomach, is identified as a natural ligand of the GH secretagogue (GHS) receptor. GH is used to treat dwarfism and various diseases such as obesity and osteoporosis, and is also used for anti-aging (Horber *et al*., 1990) because it was found to inhibit protein loss and delay the aging process during middle- and old-age. GHSs (Bowers *et al*., 1981) are widely classified into sub-groups of peptides (Bowers *et al*., 1984), including partial peptides (Momany and Bowers, 1996), nonpeptide, and peptidemimetics (Cheng *et al*., 1993). Also, there are the orally administered L-629, 429, and L-163, 191 (Smith *et al*., 1993; Patchett *et al*., 1995) that are under clinical trials.

Dioscorea batatas DECNE. (Dioscoreaceae) grows extensively on mountains as well as in fields within Korea and Japan. Its rhizomes (Dioscoreae Rhizoma; DR) are traditionally used to treat coronary artery disorder or disease caused by blood clotting (Au *et al*., 2004), and are also known to improve immunity (Choi *et al*., 2003), have antioxidant properties (Farombi *et al*., 1999; Choi *et al*., 2002), and decrease blood glucose levels (Brakohiapa *et al*., 1997; Morrison *et al*., 2006).

Dioscin, a common component of *Dioscorea batatas* DECNE. and *Polygonatum odoratum* D., was isolated and purified from the BuOH fraction of the DR extract, and its structure was identified, as described before (Kim *et al*., 1989). Dioscin is known to inhibit the growth of leukemia cells (Wang *et al*., 2001), has antifungal activity (Sata *et al*., 1998), and has efficacy as an antineoplastic agent, an immunomodulator, and an anticarcinomic agent (Chiang *et al*., 1992; Hu *et al*., 1996). Yet, no one has reported on the use of dioscin in the treatment of various diseases caused by GH deficiency.

In this study, the herbal medicine Dioscoreae Rhizoma, which is capable of inducing GH, was selected from natural products, and its fractions and components abilities for inducing GH release were examined.

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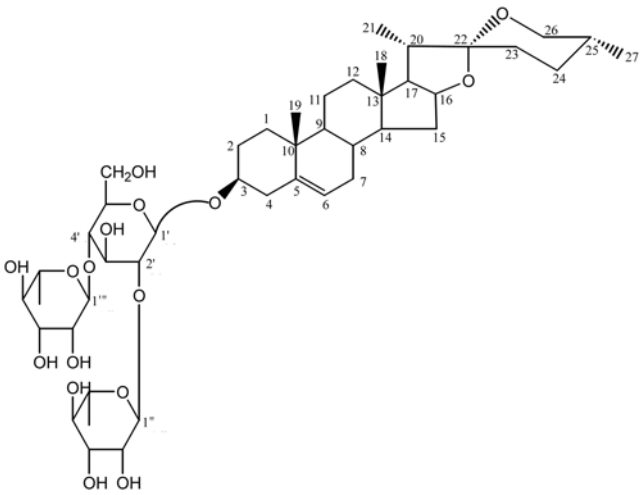

Fig. 1. The structure of dioscin.

Materials and Methods

Isolation of dioscin. Dioscoreae Rhizoma (DR) was collected from Kyeng-buk province (Korea) in August, 2000, and it was evaluated by Je-Hyun Lee, O.M.D., Ph.D. (Dongguk University, Gyeongju, Korea) prior to use. A voucher specimen (KIOM-02-3-0027) is stored in the Herbarium of the Korea Institute of Oriental Medicine (Daejeon, Korea). The dried DR (5 kg) were cut finely and percolated in methanol (8 L) at room temperature for 7 days and then filtered. The filtrate was concentrated under reduced pressure to give the methanol extract of DR (200 g). The methanol extract was solvent-extracted with n-hexane (1.5 L), EtOAc (1.5 L), and *n*-butanol (1.5 L) sequentially, 3 times each, and the solvent extracts were then filtered, evaporated, and lyophilized.

The dried n-butanol fraction (78 g) was sub-fractionated into 6 sub-fractions by silica gel column chromatography (80 cm × 10 cm) with a $CHCl_3$-methanol-water (7 : 3 : 1, lower part 6 L) gradient system. Fraction 3 was further purified by column (90 cm × 3 cm) chromatography with unsaturated EtOAc-MeOH (98 : 2, 2 L) followed by EtOAc-MeOH (95 : 5, 2 L) to isolate dioscin (550 mg). The compound was further re-crystallized using methanol, to give a colorless, needle-shaped crystalline dioscin (Fig. 1) of the following structure (Wang *et al*., 2001):

Melting point: 289~292°C;

$[\alpha]_D^{25}$: -110.5° (*c* 0.3 in MeOH)

IR (KBr, cm^{-1}) 3420, 1645, 1100~1000, 920, 900, 863, 835, (900>920, 25(R)-spiroketal), 810

FAB MS *m/z* (rel. int.): 891 $[M + Na]^+$ (2.5), 415 $[genin + H]^+$ (3.4)

^1H NMR (300 MHz, pyridine-d_5) d: 0.68 (3H, d, J = 5.2 Hz, 27-CH_3); 0.82 (3H, s, 18-CH_3); 1.05 (3H, s, 19-CH_3); 1.12 (3H, d, J = 6.9 Hz, 21-CH_3); 1.60 (3H, d, J = 6.9 Hz, Rha-CH_3); 1.74 (3H, d, J = 6.2 Hz, Rha-CH_3); 4.92 (1H, d, J = 6.7 Hz, anomeric H of glucose); 5.32 (1H, br d, J = 4.6 Hz, H-6); 5.80 (1H, br s, anomeric H of rhamnose); 6.34 (1H, br s, anomeric H of rhamnose).

^{13}C NMR (75.5 MHz, pyridine-d_5) d: 37.5 (C-1), 30.2 (C-2), 78.1 (C-3), 39.0 (C-4), 140.8 (C-5), 121.8 (C-6), 32.2 (C-7), 31.7 (C-8), 50.3 (C-9), 37.1 (C-10), 21.1 (C-11), 39.9 (C-12), 40.5 (C-13), 56.7 (C-14), 32.3 (C-15), 81.1 (C-16), 62.9 (C-17), 16.3 (C-18), 19.4 (C-19), 42.0 (C-20), 15.0 (C-21), 109.3 (C-22), 31.8 (C-23), 29.3 (C-24), 30.6 (C-25), 66.9 (C-26), 17.3 (C-27), 100.3 (C-1), 78.6 (C-2), 77.0 (C-3), 78.1 (C-4), 77.8 (C-5), 61.3 (C-6), 102.0 (C-1"), 72.6 (C-2"), 72.6 (C-3"), 74.1 (C-4"), 70.4 (C-5"), 18.5 (C-6"), 102.9 (C-1), 72.8 (C-2), 72.9 (C-3), 73.9 (C-4), 69.5(C-5), 18.7 (C-6).

Rat pituitary cell culture. The pituitaries of Sprague-Dawley (SD) rats (3 to 4 weeks old; Daehan Biolink Co., Ltd.) were washed with a cold Hank's balanced salt solution (HBSS, pH 7.4, Gibco BRL.). They were then combined with 0.2% collagenase type IV (Gibco BRL.) and 0.2% hyaluronidase type III (Sigma Co.), and cultured at 37°C to isolate the pituitary cells (Kim *et al*., 2003; Jung *et al*., 2004). The isolated cells were centrifuged at 2,000 rpm and the precipitates were washed with Dulbecco's Modified Eagle's medium (DMEM, Gibco BRL.) containing 10% horse serum (Gibco BRL.), 2.5% fetal bovine serum (FBS, Gibco BRL.), and antibiotics (gentamicin and nystatin, Gibco BRL.). The cells were incubated under humidified conditions and a 5% CO_2-95% air atmosphere at 37°C for 4~5 days. The cultured cells were collected, washed with HBSS (pH 7.4) twice, and suspended at 7.5×10^4 cells/ml. The suspended pituitary cells were combined with various concentrations (0~1000 nM) of rGRF (Bachem Co.), as a positive control, and with the extracts (corresponding to 1 mg of dried herb/ml), fractions (20 μg/ml), and dioscin (5 μg/ml) from DR. The cells were then incubated at 37°C for 15 min. To determine the concentration dependency of dioscin, various dioscin concentrations (0~10 μg/ml) were added to the pituitary cells, which were then incubated as described above. After incubation, the supernatant was collected and stored at −70°C to determine the concentration of GH; GH concentration was determined using an rGH RIA kit (Amersham).

Identification of the dioscin binding receptor. To identify whether the dioscin binding receptor is GHRH receptor (GRRH-R) or GHS-R, the pituitary cells were incubated with dioscin (1.15×10^{-5} M) and human GHRH antagonist (1×10^{-6} M or 1×10^{-7} M). The supernatants were collected to determine the concentrations of rGH. At the same time, the pituitary cells were incubated with GHRP-6 antagonist (1×10^{-5} M or 1×10^{-6} M), somatostatin-14 (1×10^{-5} M~1×10^{-7} M), and cyclo-somatostatin (1×10^{-5} M~1×10^{-7} M) as described above. The concentration of rGH released was determined using an rGH RIA kit (Amersham).

Induction of GH in SD rats. The SD rats (8 weeks old) were anesthetized with 50 mg/kg of pentobarbital (Hanlim Pharm. Co.) intraperitoneally, and then intravenously administered a saline solution containing 0.1% DMSO and 10 μg/kg of dioscin through the jugular vein. The blood samples were collected from the tail vein at regular time intervals, centrifuged to isolate the plasma, and stored at −70°C. As a control group, select SD rats were intravenously administered 0.1% DMSO-containing saline only. Their plasma was collected after centrifugation and stored by the same procedure. The rGH levels in the plasma were determined using an rGH RIA kit.

Statistical analysis. The data are presented as mean ± SEM, with statistical significance defined as $p < 0.05$ by ANOVA or the

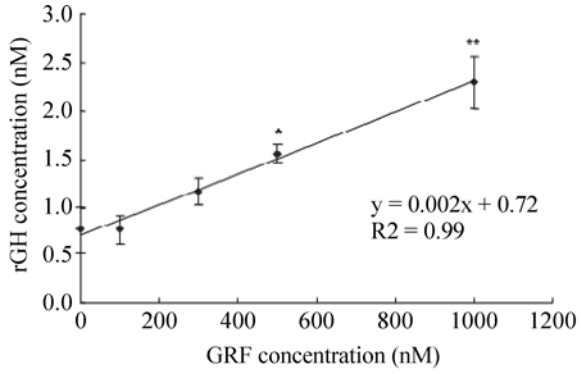

Fig. 2. rGH release by various concentrations of rGRF. Bars indicate standard errors of means (SEM) (n = 9). The fitted standard curve of GRF on rGH release is Y = 0.002 X + 0.72 (R^2 = 0.99). Y represents rGH concentration (nM) and X represents rGRF concentration (nM).

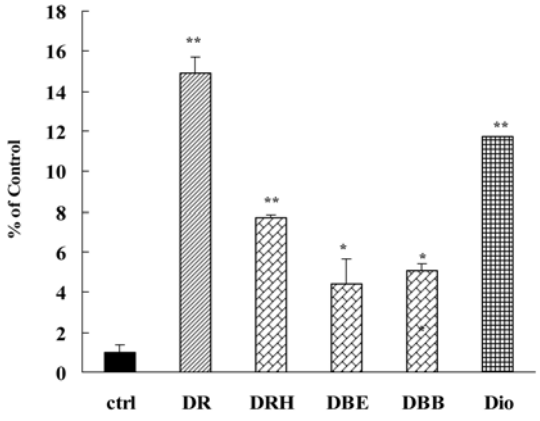

Fig. 3. Induction of rGH release by the medicinal herb (1 mg/ml), the fractions (20 μg/ml), and dioscin (5 μg/ml) on rat pituitary cells. Bars indicate standard errors of means (SEM) (n = 3). Ctrl: control, DR: Dioscoreae Rhizoma, DRH: n-hexane fraction of DR, DBE: EtOAc fraction of DR, DBB: BuOH fraction of DR, Dio: dioscin. *$p < 0.05$ and **$p < 0.01$ as compared to the control by Bonferroni multiple comparisons.

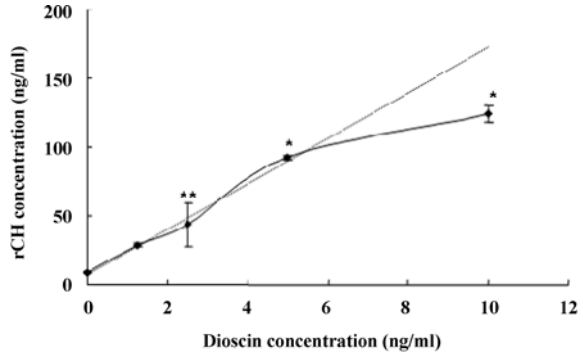

Fig. 4. rGH concentration (ng/ml) induced by various concentrations of dioscin (ng/ml). Bars indicate standard errors of means (SEM) (n = 3). *$p < 0.05$ and **$p < 0.01$ as compared to the control by Bonferroni multiple comparisons.

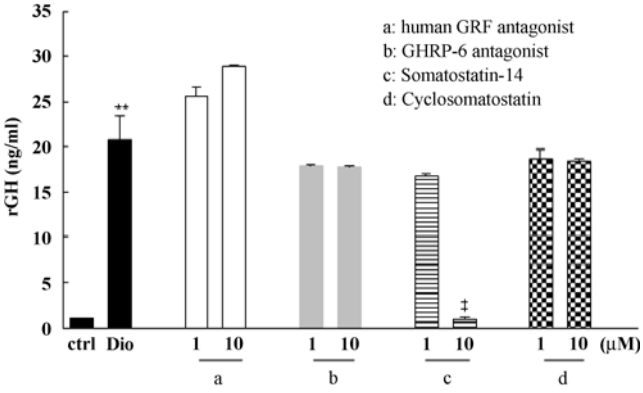

Fig. 5. Competitive inhibition of rGH release in the presence of dioscin (1.15×10^{-5} M) by each addition of various concentrations of human GHRH antagonist, GHRP-6 antagonist, somatostatin-14, and cyclo-somatostatin. Bars indicate standard errors of means (SEM). The control group (ctrl) represents rGH release in the absence of dioscin, and Dio indicates that in the presence of dioscin. **$p < 0.01$ as compared to the control and $p < 0.001$ as compared to the dioscin-only group by Bonferroni multiple comparisons.

Bonferroni Multiple comparison method (SYSTAT 10.0, SPSS Inc., USA).

Results

The correlation between rat GRF (rGRF) concentration and rGH level was analyzed by additions of various concentrations of rGRF (1-40) to the rat pituitary cells, in order to minimize experimental errors. The results show that GH release was GRF concentration-dependent (Fig. 2). To estimate the rGH releasing effect of DR, 1 mg of dried herb/ml of DR methanol extract was added to the pituitary cells, and the concentration of released GH was determined (Fig. 3). The DR extract induced rGH release that was 15-fold ($p < 0.01$) higher than that of the control (0.36 ± 0.02 nM). When the DR fractions were added to the pituitary cells (20 μg/ml), the *n*-hexane, EtOAc, and BuOH fractions induced rGH release that was 8-fold (2.76 ± 0.29 nM), 4-fold (1.59 ± 0.05 nM), and 5-fold (1.81 ± 0.43 nM) higher than the control (0.36 ± 0.02 nM), respectively ($p < 0.01$, Fig. 2). Dioscin (5 μg/ml), a component of DR, induced rGH release at a level that was approximately 11-fold (2.40 ± 0.11 nM) higher than the control ($p < 0.01$) (Fig. 3). Additionally, we analyzed the induction on rGH release by using various concentrations of dioscin, and the results indicated that dioscin increased rGH release in a concentration-dependent manner. The ED_{50} value of dioscin calculated using the Michaelis-Menten's equation was 1.14×10^{-5} M (Fig. 4).

The effects of the antagonists on dioscin-induced rGH

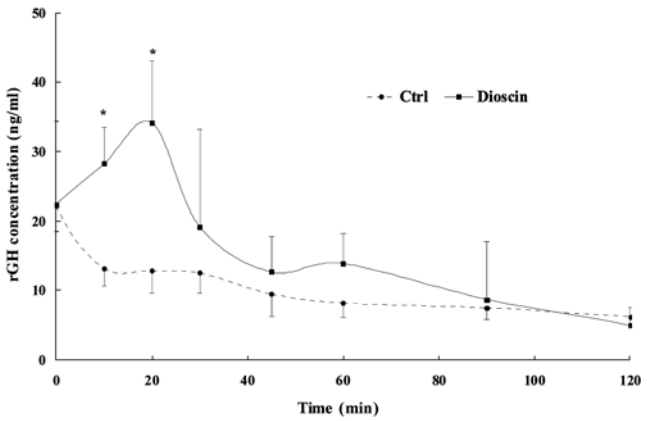

Fig. 6. Time course of released rGH concentration after intravenous administration of dioscin (10 μg/kg) in SD rats. Bars indicate standard errors of means (SEM). Ctrl (n = 27) represents the control group and dioscin (n = 4) indicates the group with dioscin administraion. t_{max} is 20 min after the dose. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to the control by ANOVA.

release are shown in Fig. 5. Incubating the pituitary cells with human GHRH antagonist or GHRH-6 antagonist (1×10^{-5} M and 1×10^{-6} M) did not affect rGH release, but somatostatin-14 (1×10^{-5} M) inhibited rGH induction to the basal level.

The induction effects of dioscin on GH release were evaluated *in vivo,* as described below. When dioscin was administered at 10 mg/kg intravenously, the C_{max} of the rGH level in rat plasma (34.16 ± 14.10 ng/ml) (n = 4) was approximately twice as high as in the control group (12.88 ± 3.29 ng/ml) (n = 27) ($p < 0.05$ at 20 min after the dose) within 60 min, and then the rGH level returned to the basal level within 90 min (Fig. 6).

Discussion

The concentration range of rGRF was used as a positive control to analyze the results (Fig. 2) and it showed that rGRF directly participates in GH release. In this report, the DR extract and its fractions resulted in 10-fold and 8- to 4-fold increases in the *in vitro* rGH concentration, respectively (Fig. 3). In general, there are many active compounds within the MeOH extract of herbs. Even though the MeOH extract showed the highest activity when compared to the negative control, the DBH, DBE, and DBB fractions showed lower activity than the MeOH extract. This may be attributed to the active compounds of the MeOH extract being divided into each fraction during fractionation, causing the fractions to show lower activity than the MeOH extract.

Consequently, the results demonstrate that the MeOH extract and its fractions have rGH release-inducing activity. Medicinal herbs are regarded as having excellent pharmaceutical efficacy and less adverse effects on humans, and their fractions and components like dioscin are easily obtained from the natural product.

It is known that GH prevents and cures various degenerative diseases, and GH release is reduced with aging. The GHS receptor (GHS-R) is expressed in the hypothalamus and pituitary gland, and it induces GH release by a mechanism that is different from that of GHRH. In this study, GH induction by dioscin may have been caused by direct action on the GHRH receptor (GHRH-R), because GH release was not inhibited by the human GHRH antagonist and GHRP-6 antagonist (Fig. 5). Further, GH induction by dioscin was inhibited by the addition of somatostatin-14 (1×10^{-5} M), while cyclosomatostatin had no effect.

GHRP-6 has a lower induction of GH release than GRF, yet it is regarded as a useful synthetic peptide because it has a small molecular weight (873.0 g/mol). The receptors, with which GH release inducing agents bind, are divided into the sub-groups of GHRH-R and GHS-R, and may have a synergistic effect on GH release according to their reaction mechanism. GHRH-R belongs to the G-protein receptor family and binds with G-proteins by a stimulus to activate cAMP. This increases cAMP and free Ca^{2+}, which results in GH release. However, a synergistic effect on GH release by GHRH, GHS, and GHRP is recognized, yet they react independently (Bowers *et al*., 1990). This means that GHRH and GHRP have their own receptors. Previously, it was shown that the concentration of GH release increased 4.5-fold when GHRP-2 was administered to GH deficient (GHD) rats with mutated GHRH-Rs, while a normal group showed a 79-fold increase in its GH level. This demonstrates that GHRP-2 has a GHRH-independent effect on pituitary somatotroph cells, and an intact GHRH is necessary for the proliferation of normal somatotroph cells (Gondo *et al*., 2001).

To identify the dioscin binding receptor between GHRH-R and GHS-R, we added ED_{50} (1.14×10^{-5} M) of dioscin and a 1×10^{-6} M concentration each of human GHRH antagonist [{Ac-Tyr^1, D-Arg^2}-GHRH{1-29}] amide, GHRP-6 antagonist [{D-lys^3}-GHRP-6], cyclosomatostatin [cyclo-7-aminoheptanoyl-Phe-D-Trp-Lys-Thr (BZL) acetate salt], and somatostatin-14 to the rat pituitary cells. The concentrations of rGH release by the treated cells were similar to that when only dioscin was added (Fig. 5). The rGH concentration, when 1×10^{-5} M GHRP-6 antagonist or cyclosomatostatin was added to the pituitary cells, was similar to that when dioscin alone was added. On the other hand, with the addition of 1×10^{-5} M somatostatin-14, rGH concentration was nearly 2.3% of that when only dioscin was added (Fig. 5). Consequently, it is assumed that the mechanism for dioscin is different from that of the human GHRH antagonist, GHRP-6 antagonist, and cyclosomatostatin, and that somatostatin-14 acts on the active site of dioscin as a competitive inhibitor. Therefore, dioscin is considered a clinically useful GH alternative that induces GH release by pituitary stimulation. Further study on its reaction mechanism is currently in progress.

We are studying the isolation and efficacy of compounds from all fractions. We initially found the efficacious component

of the BuOH fraction, and presently, the Hx fraction is being studied for its effective compound.

Acknowledgments This research was supported by a grant (# PF0321102-00) from the Plant Diversity Research Center of the 21st Century Frontier Research Program, which is funded by the Korean government's Ministry of Science and Technology.

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