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Effects of Steaming Time and Frequency for Manufactured Red *Liriope platyphylla* on the Insulin Secretion Ability and Insulin Receptor Signaling Pathway

Sun Il Choi[1], Hye Ryun Lee[1], Jun Seo Goo[1], Ji Eun Kim[1], So Hee Nam[1], In Sik Hwang[1], Young Ju Lee[1], So Hae Prak[2], Hee Seob Lee[2], Jong Sup Lee[3], In Surk Jang[4], Hong Ju Son[1] and Dae Youn Hwang[1]*

[1]*College of Natural Resources and Life Science, Pusan National University, Miryang, Republic of Korea*
[2]*College of Human Ecology, Pusan National University, Pusan, Republic of Korea*
[3]*Noghyup Sandong Processing Plant, Miryang, Republic of Korea*
[4]*Department of Animal Science & Technology, Gyeongnam National University of Science and Technology, Republic of Korea*

In oriental medicine, *Liriope platyphylla* (LP) has long been regarded as a curative herb useful for the treatment of diabetes, asthma, and neurodegenerative disorders. The principal objective of this study was to assess the effects of steaming time and frequency for manufactured Red LP (RLP) on insulin secretion ability and insulin receptor signaling pathway. To achieve our goal, several types of LPs manufactured under different conditions were applied to INS cells and streptozotocin (STZ)-induced diabetic ICR mice, after which alterations in insulin concentrations were detected in the culture supernatants and sera. The optimal concentration for the investigation of insulin secretion ability was found to be 50 ug/mL of LP. At this concentration, maximum insulin secretion was observed in the INS cells treated with LP extract steamed for 3 h (3-SLP) with two repeated steps (3 h steaming and 24 h air-dried) carried out 9 times (9-SALP); no significant changes in viability were detected in any of the treated cells. Additionally, the expression and phosphorylation levels of most components in the insulin receptor signaling pathway were increased significantly in the majority of cells treated with steaming-processed LP as compared to the cells treated with LP prepared without steaming. With regard to glucose transporter (GLUT) expression, alterations of steaming time induced similar responses on the expression levels of GLUT-2 and GLUT-3. However, differences in steaming frequency were also shown to induce dose-dependent responses in the expression level of GLUT-2 only; no significant differences in GLUT-3 expression were detected under these conditions. Furthermore, these responses observed *in vitro* were similarly detected in STZ-induced diabetic mice. 24-SLP and 9-SALP treatment applied for 14 days induced the down-regulation of glucose concentration and upregulation of insulin concentration. Therefore, these results indicated that the steaming processed LP may contribute to the relief of diabetes symptoms and should be regarded as an excellent candidate for a diabetes treatment.

Key words: *Liriope platyphylla*, diabetes, steaming process, insulin, glucose

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Liriope platyphylla (LP) is an herb which has been used for a very long time in oriental medical protocols for the treatment of asthma and bronchial and lung inflammation [1]. LP is a perennial seed-reproducing plant, and is distributed broadly throughout the temperate climate region of the northern hemisphere. In Korea, these plants grow principally in the low mountain areas, at altitudes less than 500 m above sea level, and their leaves remain green throughout the year [2]. The effects of extracts of LP roots on preventing obesity, diabetes, inflammation and neurodegenerative disease have recently been demonstrated in several studies [3-7]. Among these therapeutic effects, the effects of LP against obesity

*Corresponding author: Dae Youn Hwang, Department of Biomaterials Science, College of Natural Resources & Life Science, Pusan National University, 50 Cheonghak-ri, Samnangjin-eup, Miryang, Gyeongnam 627-706, Republic of Korea
Tel: +82-55-350-5388; Fax: +82-55-350-5389; E-mail: dyhwang@pusan.ac.kr

and diabetes are quite well-known in Korea. Gyeongshing-angjeehwan (GGEx), the principal component of which is LP, appears to effectively prevent obesity and hyper-triglyceridemia by inhibiting appetite and activating hepatic PPARg in OLETF male rats [4]. A homoisoflavone-enriched fraction in a methanol extract was also shown to increase insulin-stimulated glucose uptake in 3T3-L1 adipocytes via induced increases in GLUT-4 content in the plasma membranes [3]. Additionally, a novel compound of LP (LPMH80-H) has been shown to regulate GLUT-1 and GLUT-3 biosynthesis via the Akt and p38 MAPK signaling pathways of the insulin signaling pathway in the livers and brains of mice [8].

Steaming is often applied to medicinal plants to increase the levels or efficacy of their functional components and to induce chemical transformations of specific components [9]. This process has been most successfully applied in the ginseng plant, derivations of which are taken orally as adaptogens, aphrodisiacs, nourishing stimulants, and in the treatment of type II diabetes, as well as for sexual dysfunction in men [10-12]. There are two kinds of Korean ginseng; Korea white ginseng (KWG) (*Panax ginseng* C.A. Meyer) is air-dried ginseng, and Korea red ginseng (KRG) (*Ginseng Radix Rubra*) is steamed ginseng [9]. During the steaming-process of ginseng, several important components, including ginsenosides, acidic polysaccharides, and phenolics, are transformed into different components, and several new compounds, including non-saponinpolyaceylene, maltol, and amino acid, are formed [13,14]. However, the steaming process has never previously been applied to the roots of LP in an effort to improve its functionality in therapy for diabetes.

In this study, we have assessed the effects of LP steaming time and frequency on insulin secretion ability and the insulin receptor signaling pathways as a part of a broader effort to develop a novel diabetes drug. The results of this study can provide scientific evidence that will be useful in determining the optimal conditions for the LP steaming process.

Materials and Methods

Preparation of LP sample

The roots of LP were collected from plantations in the Chungbuk area (Korea) and dried with a hot-air drying machine at 60°C. In order to prepare five extracts at different steaming times (0-SLP, 3-SLP, 9-SLP, 15-SLP and 24-SLP), 200 g of dry roots were steamed at 99°C for a variety of durations (0, 3, 9, 15 and 24 h) and air-dried for 24 h at 70°C. These steamed roots were reduced to powder using an electric blender and the water extracts were purified for 2 h at 100°C using circulating extraction equipment (IKA Labortechnik, Staufen, Germany) after adding 200 mL of distilled water. Additionally, the solution of extracts was concentrated into dry pellets with rotary evaporator (EYELA, Tokyo, Japan) and stored at −80°C until use.

In order to prepare six extracts with different steaming frequencies (0-SALP, 1-SALP, 3-SALP, 5-SALP, 7-SALP and 9-SALP), the specific process comprising two steps (200 g of dry roots were steamed at 99°C for 3 h and air-dried at 70°C for 24 h) was carried out with different numbers of repetitions (0, 1, 3, 5, 7 and 9 times). The roots obtained via these processes were treated with the same procedures used in the above protocol in order to prepare dry pellets.

Cell culture and treatment

The INS pancreatic beta cell line producing insulin was obtained from the Korean Food and Drug Administration (Seoul, Korea). This cell line was maintained for 24 to 36 h in RPMI-1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum, 1% non-essential amino acids, 2 mL L-glutamine, 100 IU/mL of penicillin, and 100 μg/mL of streptomycin. In order to determine the insulin concentration and cell viability, the wells in 96-well plates were divided into as many groups as were required for each experiment. The INS cells were seeded at a density of 4×10^4 cells/200 μL in 96-well plates and grown for 24 h in a 37°C incubator. When the cells attained 70-80% confluence, vehicle and each extract of LP dissolved in dH_2O was added to each well and incubated further for another 24 h. After 24 h, the culture supernatants were collected from these wells to determine the insulin concentration, and the remaining cells were used for the analysis of cell viability.

3–[4,5–Dimethylthiazol–2–yl]–2,5–diphenyltetrazolium bromide (MTT) assay

INS cells were seeded at a density of 4×10^4 cells/200 μL in the wells of 96-well plates and then were grown for 24 h in a 37°C incubator. When the cells attained 70-80% confluence, they remained untreated (vehicle) or were exposed to various types of LP extracts dissolved in dH_2O for another 24 h. Cell proliferation was determined using the tetrazolium compound MTT (Sigma-Aldrich, St. Louis, MO, USA). After the supernatants in the vehicle or LP-treated wells were discarded, 200 μL of fresh MEM and 50 μL of MTT solution [2 mg/mL in phosphate buffered saline (PBS)] were added to each well. The cells were then incubated in a 37°C incubator. The reduction of MTT to insoluble purple formazan dye crystals by viable cells was evaluated in 220 μL, recovered after 4 h. The formazan precipitate was dissolved in dimethyl

sulfoxide and the absorbance was read directly at 570 nm in the wells using a SoftMax Pro5 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Additionally, the data was analyzed in terms of cell number versus absorbance, allowing for changes in cell proliferation to be quantified.

Enzyme-linked immunosorbent assay (ELISA) for insulin detection

The levels of insulin in the culture supernatant and mice sera were detected using the ultra-sensitive assay procedure and reagents in the insulin ELISA kit (Mercodia, Uppsala, Sweden). In brief, the sample and standards were incubated in a plate shaker at 100-150 rpm at room temperature for 2 h on antibody-coated plates. The wells were then washed six times with an automatic plate washer (Hoefer, San Francisco, CA, USA), after which horseradish peroxidase (HRP) conjugate was added to each of the wells. The plates were subsequently incubated in a shaker for 30 min at room temperature. The reaction was terminated via the addition of 50 mL of stop solution (0.5 M H_2SO_4), after which the plates were analyzed by evaluating absorbance at 450 nm using a Molecular Devices V_{max} Plate reader (MD, Sunnyvale, CA, USA).

Western blot analyses

INS cells harvested from 100 mm-diameter culture dishes were solubilized with 1% Nonidet P-40 in 150 mM NaCl, 10 mM Tris HCl (pH 7.5), and 1 mM EDTA, and supplemented with a protein inhibitor mixture (Roche, Basel, Switzerland). They were then centrifuged for 10 min at 10,000 *g* at 4°C. The homogenized proteins were separated for 3 h via 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes over 2 h at 40 V. The membranes were then incubated with anti-insulin receptor β (Cell Signaling Technology, Danvers, MA, USA), anti-Akt (Cell signaling Technology), anti-p-Akt (Cell signaling Technology), anti-GLUT-2 (Abcam, Cambridge, UK), anti-GLUT-3 (Abcam) and anti-β-actin antibodies (Sigma-Aldrich) to detect the levels of each protein. Each antigen-antibody complex was visualized using a biotinylated secondary antibody (goat anti-rabbit)-conjugated HRP streptavidin Histostain-Plus Kit (Zymed, South San Francisco, CA, USA) diluted to 1:1,500 in PBS.

Care and use of animals

All animal experimental procedures employed herein were approved by the Institutional Animal Care and Use Committee (IACUC) of the Pusan National University. The animals were handled in the Pusan National University-Laboratory Animal Resources Center accredited by Korea FDA in accordance with the USA NIH guidelines (Accredited Unit Number-000996). All mice were housed under specific pathogen-free (SPF) conditions with a strict light cycle (lights on at 06:00 and lights off at 18:00), and provided with a standard irradiated chow diet (Purina Mills, St. Louis, MO, USA) *ad libitum*. Adult ICR mice were purchased from SamTako (Osan, Korea).

Treatment of LP and detection of glucose level

Two types of LP powders (24-SLP and 9-SALP) prepared under the different conditions specified above were dissolved in distilled water to adjust a final concentration of 50 mg/mL. ICR mice were divided randomly into two groups. The first group of ICR mice was not treated with any compounds, and used as a control group. The second group received 70 mg/kg body weight of streptozotocin (STZ) via intraperitoneal injections for 7 days in order to induce diabetic conditions. After 7 days, the second group was further divided into two subgroups [vehicle-treated group, Red LP (RLP)-treated group]. The first subgroups of mice received a comparable volume of daily water (vehicle-treated group), whereas the second group received 50 mg/kg body weight/day of 24-SLP or 9-SALP via gavage for 7 days (RLP-treated group) [8]. Also, KWG and KRG were treated with same concentration as a control. After 1, 7 and 14 days, the glucose concentrations were measured after 24 h of fasting using the sensitive strip of the Blood Glucose Monitoring System (I-Sens, Seoul, Korea).

Statistical analysis

Tests for significance between LP-treated and vehicle-treated groups in INS cells were carried out using a one-way ANOVA test of variance (SPSS for Windows, Release 10.10, Standard Version, Chicago, IL, USA). Additionally, the tests for significance between 1-day-treated mice and 7- or 14-day-treated mice were carried out via *post hoc* tests (SPSS for Windows) of variance, and significance levels are provided in the text. All the values are reported as the mean±SD. A P value of <0.05 was considered as significantly different.

Results

Determination of optimum concentration

Prior to the test of LP effects on insulin secretion ability, the optimal LP concentration was determined by assessing the viability and insulin concentrations of INS cells at different LP concentrations. In the MTT assay, cell viability was not significantly changed in INS cells treated with all tested concentrations (from 500 to 3.9 mg/mL) relative to the cells treated with vehicle alone (Figure 1A). However, insulin

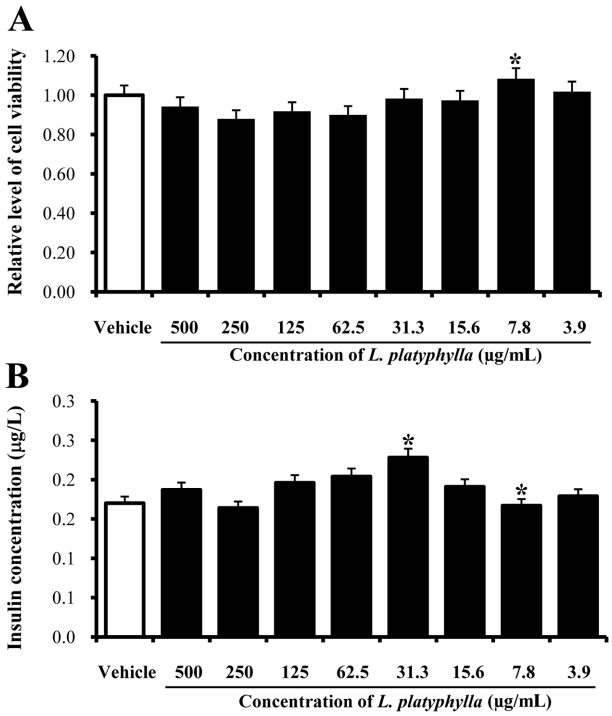

Figure 1. Optimal concentration of steamed *Liriope platyphylla* on the toxicity and insulin secretion of INS cells. Cells were cultured with one of 8 different concentrations in dH_2O for 24 hrs. Activity of cell viability was measured using an MTT assay (A). The culture supernatants were collected from each of the cells. Insulin concentration in the supernatant was measured with an anti-insulin ELISA kit (B). The values of data represented the means±SD of three experiments. **P*<0.05 is the significance level relative to the vehicle-treated group.

concentrations were increased significantly in INS cells treated with both 62.5 and 31.3 mg/mL of LP (Figure 1B). Therefore, 50 mg/mL of RLP was determined as the optimal concentration to investigate insulin secretion ability in the INS cells.

Effects of RLP manufactured by different steaming time on the insulin secretion ability of INS cells

In order to evaluate the effects of steaming time on the viability and insulin secretion of INS cells, the cell viability and insulin concentration were determined in INS cells treated with five types of LPs manufactured with different steaming times (0, 3, 9, 15 and 24 h). In the case of the ginseng treatment group, cell viability was slightly higher in the KRG-treated than KWG-treated cells as compared to the cells treated with vehicle alone. Additionally, the cell viability of the KWG-treated cells was quite similar to that of the LP-treated cells. However, in the groups of cells treated with other LP extracts, the highest viability was observed in INS cells treated with 3-SLP, followed by 9-SLP, 24-SLP and 15-SLP (Figure 2A). In insulin ELISA analysis, the INS cells treated with ginseng evidenced slightly higher insulin concentrations than the group of cells treated with vehicle alone. However, the increase in insulin concentration ratio was greater in the KRG-treated INS cells than in the KWG-treated cells. Additionally, in the LP-treated group, the insulin concentrations were dramatically increased in the INS cells treated with 0-SLP and 3-SLP, whereas they were reduced in the INS cells treated with 9-SLP and 15-SLP. Furthermore, the cells treated with 24-SLP maintained the levels observed in the KWG-treated cells (Figure 2B). Therefore, these results indicated that 3 h of steaming time could be considered as the optimal conditions for increasing insulin secretion ability in INS cells, as 3-SLP induced the maximum increase in insulin secretion.

Effects of RLP manufactured by different steaming frequencies on the insulin secretion ability of INS cells

Next, in an effort to evaluate the effects of LP steaming frequency on insulin secretion ability, the cell viability and insulin concentration of INS cells treated with six types of LPs manufactured under different steaming frequencies (0, 1, 3, 5, 7 and 9 times) were evaluated. First, in cell viability analysis, the cells treated with KWG and KRG evidenced slightly higher levels of viability than vehicle-treated cells. Among the LP-treated cells, the highest (albeit slightly so) viability was noted in the INS cells treated with 1-SALP, followed by 3-SALP, 5-SALP and 9-SALP (Figure 2C). However, drastic changes in insulin concentration were noted. The insulin concentrations were increased dramatically in INS cells treated with 9-SALP, whereas they were reduced in INS cells treated with 3- and 5-SALP. The 1- and 7-SALP-treated cells maintained middle levels of insulin concentration (Figure 2D). Therefore, the above results demonstrated that the 9-SALP could induce the maximum increase in insulin secretion ability on INS cells.

Effect of RLP manufactured by different steaming time on the insulin receptor signaling pathway

On the insulin receptor signaling pathway, the insulin receptor transduces the insulin signal into cytoplasm via the activation of various pathways including the Ras-Raf-MEK-ERK, the PI3K-PDK-AKT, the c-Cbl-GLUT-4, the PI3K-Rab4-GLUT-4 and the PI3K-Rac-MEKK1-MKK4-JNK pathways [15,16]. In order to assess the effect of LPs manufactured under different steaming times on the insulin receptor signaling pathway, the expression levels of components were involved in this pathway were measured using Western blot. In the control group, the expression level of insulin receptor was higher in the KRG-treated cells than in vehicle or KWG-

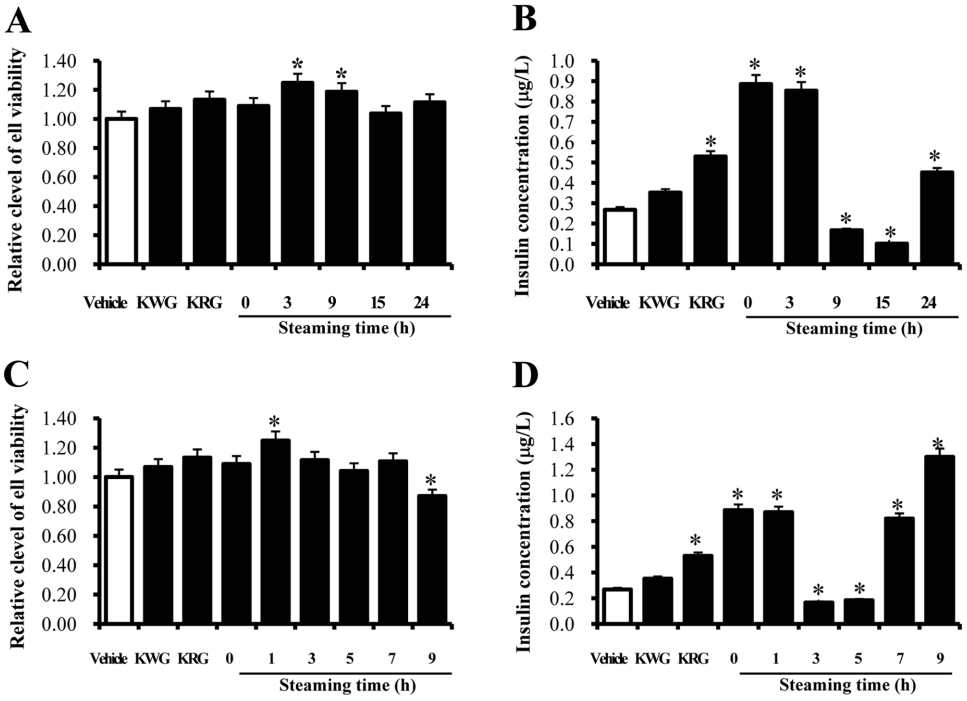

Figure 2. Effects of the different steaming time and frequency of *Liriope platyphylla* on toxicity and insulin secretion in INS cells. Cells were cultured with one of the 5 different extracts manufactured under the different concentrations and two ginseng types in dH_2O for 24 hrs. Activity of cell viability was measured via MTT assays (A). The culture supernatants were collected from each of the cells. Insulin concentration in the supernatant was measured using an anti-insulin ELISA kit (B). The values of data represented the means±SD of three experiments. **P*<0.05 is the significance level relative to the vehicle-treated group.

treated cells. Additionally, the expression level of insulin receptor was increased in groups treated with 4 different types of steamed LP compared to the 0-SLP treated cells, although the increase in their ratios differed among groups. Additionally, in the case of the downstream signaling pathway for insulin receptors, the level of Akt phosphorylation was decreased in the KRG-treated cells compared with the KWG-treated cells. However, in PL-treated cells, Akt phosphorylation was higher in cells treated with all steamed LPs than in cells treated with 0-SLP except for the cells treated with 24-SLP. Among these LP-treated cells, the cells treated with 3-SLP evidenced the highest levels of Akt phosphorylation. But, this level gradually declined from the cells treated with 3-SLP to the cells treated with 24-SLP. Furthermore, the expression level of GLUT-2 was increased in the cells treated with 3-SLP and 9-SLP, although this level was only decreased in the 0-SLP treated cells (Figure 3D). Also, the similar response was observed in Glut-3 expression. The expression level of Glut-3 was significantly increased in the 0-SLP and 3-SLP treated cells, while other groups maintained steady expression, regardless of steaming time (Figure 3E). Therefore, these results indicated that the steaming process could induce the capability of LP on the increased levels of insulin receptor expression, Akt phosphorylation and Glut-2 expression. In particular, the LP steamed for 3-SLP was found to be the most appropriate formulation for activating the insulin receptor signaling pathway.

Effect of RLP manufactured by different steaming frequencies on insulin receptor signaling pathway

Additionally, we investigated the effect of LP manufactured by different steaming frequencies on the insulin receptor signaling pathway. After the steaming process, insulin receptor expression was significantly increased in the cells treated with 1-SALP to 7-SALP, while they were maintained in the 9-SALP-treated cells. Additionally, the levels of Akt phosphorylation were higher in the cells treated with a repeatedly steamed LP than those treated with 0-SALP. Of these repeatedly steamed LP, the cells treated with 5-SALP evidenced the highest levels of Akt phosphorylation. The expression level of GLUT-2 was increased dramatically in the cells treated with 1-SALP to 7-SALP, whereas the expression level of GLUT-3 remained at a constant level in all groups (Figure 4). Therefore, these results showed that the increased levels of insulin receptor expression, Akt phosphorylation and GLUT-2 expression depended roughly on the steaming frequency of LP.

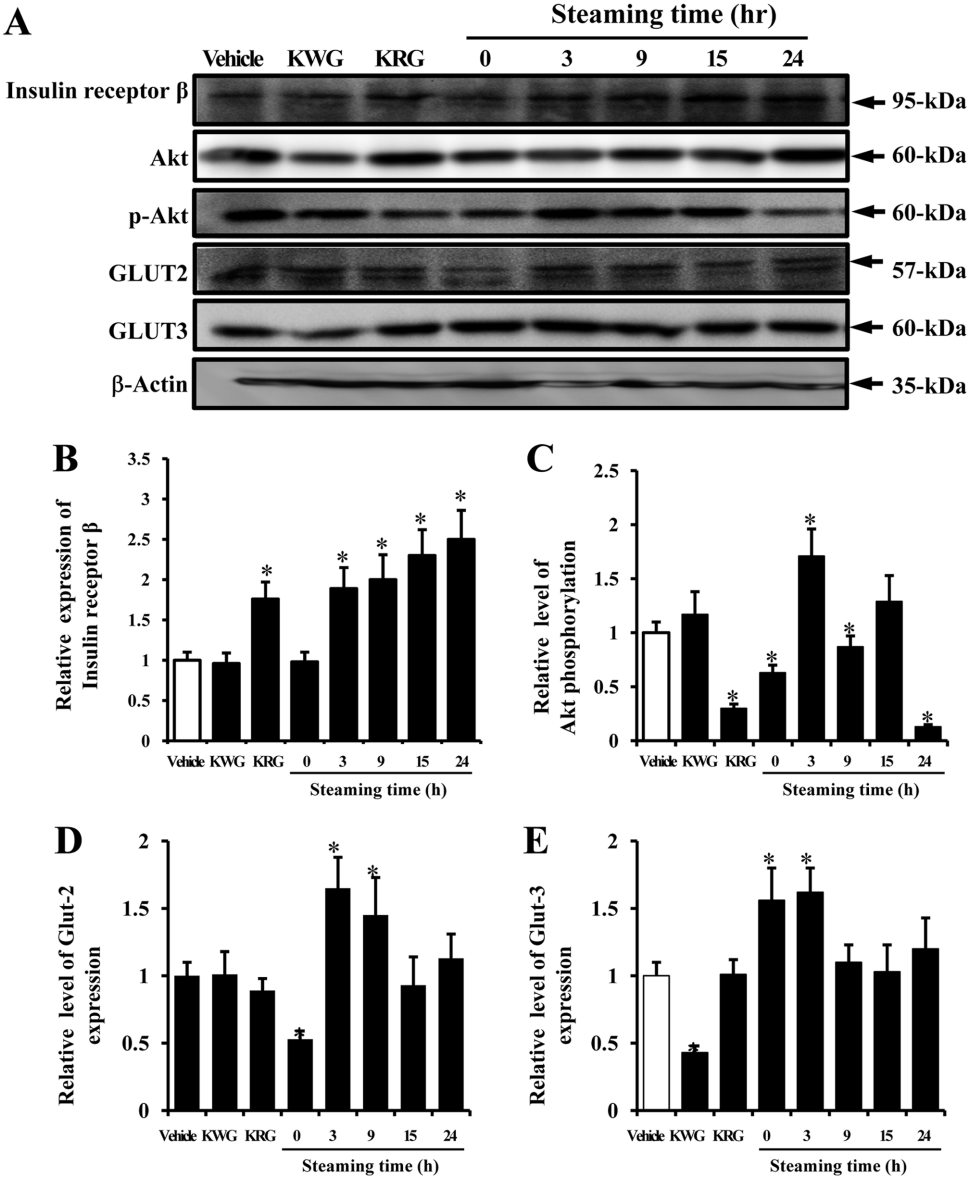

Figure 3. Effects of the different steaming times of *Liriope platyphylla* on the down-stream signaling pathway of the insulin receptor signaling pathway via Western blotting. Total cell lysates were prepared from INS cells treated with vehicle, KWG, KRG and five different LPs prepared under different steaming conditions, as described in the Materials and Methods section. Fifty micrograms of protein per sample were immunoblotted with antibodies for each protein. Three samples were assayed in triplicate via Western blotting. The values are expressed as means±SD. **P*<0.05 is the significance level compared to the vehicle-treated group.

Improving effect of LP on the insulin secretion of STZ-induced diabetic mice

Finally, in an effort to determine whether the 24-SLP and 9-SALP treatments utilized herein could influence the regulation of glucose metabolism in diabetic model animals, the glucose and insulin concentrations in serum were measured in STZ-induced mice after 24-SLP or 9-SALP treatment for 14 days. As shown in Figure 5, STZ treatment for 7 days successfully induced diabetic conditions in ICR mice. After two types of steamed LP treatment, the glucose concentrations were altered significantly as time passed. In the 24-SLP treatment group, the glucose concentration declined dramatically at 7 days, although this level was slightly increased at 14 days. In the case of 9-SALP treatment, the glucose concentration was maintained at a higher level until 7 days. However, this level was reduced significantly, by 20%, at 14 days. Furthermore, at 14 days, the insulin concentration was increased in diabetic mice treated with 24-SLP and 9-SALP as compared to the vehicle-treated mice. Therefore, these results showed that 24-SLP and 9-SALP could induce a reduction in glucose

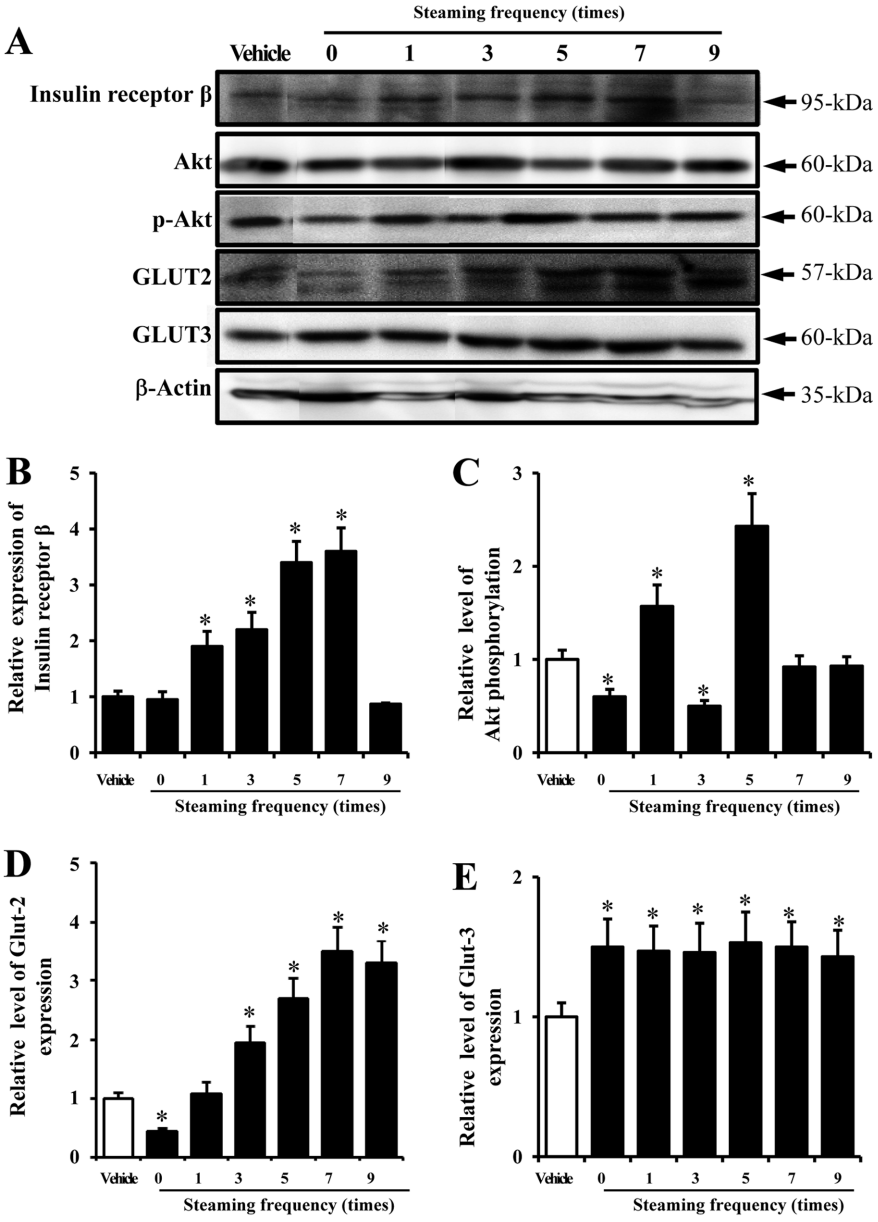

Figure 4. Effects of the different steaming frequencies of *Liriope platyphylla* on down-stream signaling pathway of insulin receptor signaling pathway via Western blotting. Total cell lysates were prepared from INS cells treated with vehicle, KWG, KRG and six different LPs prepared under different steaming conditions, as described in the Materials and Methods sections. Fifty micrograms of protein per sample were immunoblotted with antibodies for each protein. Three samples were assayed in triplicate via Western blotting. The values are expressed as the means±SD. **P*<0.05 is the significance level compared to the vehicle-treated group.

concentration and an increase in insulin concentration in STZ-induced diabetic model mice.

Discussion

Among all of the medicinal properties of LP, its anti-inflammation and anti-microbial effects have been studied for a very long time. Kim *et al*. [5] screened the effects of antibacterial drugs for the extracts of many plants by evaluating the inhibitory activity of soratase. Among 80 medicinal plants tested, LP, *Cocculustrilobus*, *Fritillariaverticillata*, and *Rhusverniciflua* evidenced strong bacteriocidal activity. Also, in a murine model of asthma, LP was shown to have profound inhibitory effects on airway inflammation and hyper-responsiveness by modulating the relationship between the Th1/Th2 cytokine imbalances [1]. Additionally, LP has also been considered to be an effective therapeutic drug for human subjects suffering from Alzheimer's disease (AD). AD is the

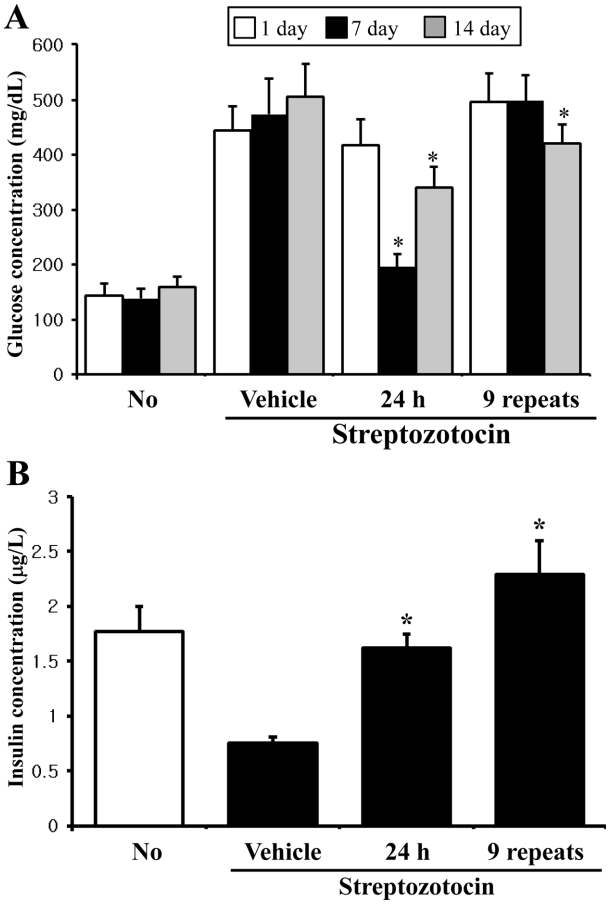

Figure 5. Effects of steamed *Liriope platyphylla* on glucose metabolism of streptozotocin-induced diabetic model animals. The blood was collected from the abdominal veins of streptozotocin-induced diabetic model mice after steaming *Liriope platyphylla* for 14 days. The glucose concentration was measured at three different times using the sensitive strip of the Blood Glucose Monitoring System (A). The insulin concentration was detected in the serum of streptozotocin-induced diabetic model animals on the final day (B). The values of data were expressed as the means±SD of three experiments. **P*<0.05 is the significance level compared to the vehicle-treated group.

most common form of dementia, characterized by the progressive deterioration of cognitive functions, with loss of memory concurrent with pathogenic alterations. In particular, the spicatoside A isolated from LP induced neuritic outgrowth, similar to the effects of NGF in its activation of ERK1/2 and PI3-kinase/Akt on PC12 cells [7]. Additionally, the butanol fraction of LP induced the expression and secretion of nerve growth factor (NGF) via a PKC-dependent pathway, increasing the neuritic outgrowth of PC12 cells [6]. Furthermore, the sub-chronic administration of ethanolic (70%) extracts improved learning and memory by the enhancement of brain-derived neurotrophic factor (BDNF) or NGF expression [17]. Recently, the novel function of LP on atopic dermatitis was reported by our previous study. The aqueous extracts of LP could reduce the symptoms of atopic dermatitis induced by phthalic anhydride in NC/Nga mice. Therefore, considering these results, LP was considered as a new drug with potential value as a therapy for diabetes.

Generally, the steaming process induced the chemical transformation or novel formation of compounds according to their condition. The steamed ginseng was manufactured by steaming raw ginseng at 98-100°C for 2-3 h, although the steaming process was repeated under several conditions [9]. In this study, we wished to determine the optimal conditions for the LP steaming process. Of four different steaming times, the maximum insulin secretion was noted in INS cells treated with 3 h-steamed LP (Figure 2). Therefore, these results demonstrated that the steamed LP could greatly induce insulin secretion compared to KRG, although the applied condition for steaming process was very similar in two medicinal plants. Furthermore, our study determined the optimal frequency of the steaming process at 3 h of steaming time. The highest insulin concentrations were detected in INS cells treated with 9 repeat-steamed LP (Figure 2). Therefore, we suggested that the increase in insulin secretion induced by treatment with steamed LP may be induced by the component changes caused by the steaming process.

KWG and LP are very homogenous in terms of their saponin content. There is a long history of ethnopharmacological evidence suggesting that both KWG and KRG exert therapeutic effects against diabetes. KWG was previously shown to exert antidiabetic functions in type I diabetic model animals [18,19] and type II diabetic model animals [20,21]. However, in the case of KRG, their therapeutic effects have been reported only in type II diabetic model animals [22,23] and diabetic patients [24]. In this study, we evaluated the therapeutic effects of steamed LP on STZ-induced type I diabetes model animals. As shown in Figure 5, steamed LP evidenced therapeutic effects by increasing insulin secretion, although further studies will require actual treatment of diabetic patients.

Additionally, several studies have evaluated the mode of insulinotrophic action of KWG and KRG. KWG performs as a potentiator which stimulates the glucose-induced insulin release from pancreatic islets [18,25]. However, KRG does not depend on glucose concentration, and functions as an initiator for insulin release. In this study, our results suggested no clues as to the mode of insulinotrophic action, because the INS cells used in this analysis were cultured in high glucose concentration media. Therefore, further studies were required to determine in detail the mechanisms underlying the insulinotrophic action of steamed LP.

Insulin is crucial to metabolism related to critical energy functions, such as glucose and lipid metabolisms [26]. This

regulation process is known to involve the insulin receptor signaling pathway, which is composed of insulin receptors, IRS and Akt. After binding to the appropriate receptor, insulin activates the insulin receptor tyrosine kinase which phosphorylates and recruits the IRS family of proteins. Activated IRS subsequently displays binding sites for numerous signaling partners such as PI3K. PI3K plays a major role in insulin function via the activation of Akt/PKB. Furthermore, activated Akt could induce glycogen synthesis, protein synthesis, and cell survival via a variety of mechanisms. Additionally, the insulin receptor signaling pathway also induced growth and mitogenic effects in cells through the Akt cascade and the Ras/MAPK pathway [27,28]. In particular, our study results also demonstrated the effects of steamed LP on the insulin receptor signaling pathway. The steamed LP at different steaming times and frequencies induced the differential upregulation of expression and phosphorylation for specific proteins in the insulin signaling receptor pathway (Figures 3 and 4). However, no evidence has, until now, been collected regarding the effects of KRG on the insulin receptor signaling pathway.

In conclusion, the steam-processed LP evidences more profound insulin secretion ability than unsteamed LP, under both *in vitro* and *in vivo*. Additionally, alterations of the insulin receptor signaling pathway were noted in the INS cells treated with steam-processed LP. Therefore, our overall results suggest the possibility that steam-processed LP will be developed into a therapeutic drug that exerts beneficial effects in the treatment of diabetes.

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