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ORIGINAL CONTRIBUTION

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Long-term consumption of fermented soybean-derived Chungkookjang enhances insulinotropic action unlike soybeans in 90% pancreatectomized diabetic rats

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Abstract *Background* We previously reported that Chungkookjang (CKJ), fermented unsalted soybeans, exhibited better anti-diabetic action than cooked soybeans (CSB) in vitro, but its effectiveness and mechanism have not been studied in vivo. *Aim of the study* We investigated whether CKJ modulated insulin resistance, insulin secretion, and pancreatic β-cell growth and survival in 90% pancreatectomized (Px) diabetic rats. *Methods* The Px rats weighing 201 ± 12 g were divided into four groups and fed for 8 weeks with a CSB diet, a CKJ diet, a casein diet, or a casein diet plus rosiglitazone (20 mg/kg body weight/day). With the exception of protein sources and contents of isoflavonoid aglycones and glycosides, the composition of the diets was made identical by adding soybean oil and cellulose to a casein diet. At the end of the experimental periods, hyperglycemic clamp was performed in conscious, unstressed and overnight fasted Px rats to measure insulin secretion capacity. Insulin/IGF-1 signaling was measured by immunoblotting in isolated islets from the treated rats, and β-cell mass, proliferation and apoptosis were also determined by immunohistochemistry. *Results* After 8-week administration, CSB did not modulate glucose-stimulated insulin secretion, but surprisingly, CKJ enhanced insulin secretion. In addition, CKJ potentiated insulin/IGF-1 signaling in islets via the induction of insulin receptor substrate-2 expression, leading to increasing pancreatic duodenal homeobox-1, insulin promoter transcription factor. In parallel with the enhancement of the signaling, CKJ elevated pancreatic β-cell hyperplasia by increasing its proliferation and decreasing apoptosis, whereas CSB did not. *Conclusion* Based on these results, the fermentation of soybeans predominantly with *Bacillus subtilis* generated isoflavonoid aglycones and small peptides, which improved insulinotropic action in islets of type 2 diabetic rats. Overall, the anti-diabetic action of CKJ was superior to CSB in type 2 diabetic rats.

Key words insulin secretion – β-cell proliferation – insulin/IGF-1 signaling – IRS2 – PDX-1

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Introduction

Type 2 diabetes is a heterogeneous metabolic disorder characterized by the impairment of insulin secretion and insulin resistance [1]. Many studies revealed that type 2 diabetes does not develop until insulin secretion compensates for insulin resistance [1, 2]. Thus, the enhancement of glucose-stimulated insulin secretion promotes the reduction of insulin resistance in preventing or delaying type 2 diabetes [1, 2].

In Asian countries, soybeans (*Glycine max* MERILL) have been consumed for a long time as an important protein source to complement grain protein. In addition to proteins, they contain various nutritious and functional components such as isoflavonoids, which are beneficial against metabolic diseases [3]. Soybeans are fermented to generate various products in Asian countries. For example, in Korea, there are several traditional fermented soybean products, such as Chungkookjang (CKJ), Doenjang, Kochujang, and soy sauce.

Chungkookjang has distinct characteristics when compared to other fermented soybean products. It is fermented predominantly with *Bacillus subtilis* for short-periods (2 days) without salts or other seasonings. During fermentation, isoflavonoids are converted from glycosides into the corresponding aglycones, and most proteins are degraded into small peptides and amino acids [4, 5].

Our previous in vitro study revealed that CKJ increased isoflavonoid aglycones and small peptides during fermentation with *Bacillus subtilis*. While both CSB and CKJ enhanced insulin-stimulated glucose uptake in 3T3-L1 adipocytes, only CKJ stimulated peroxisome proliferator-activated receptor (PPAR)-γ activity, such as rosiglitazone (RGZ), and induced glucose-stimulated insulin secretion in Min6 cells, insulinoma cell line [6]. Based on these results, we hypothesized that CKJ improved anti-diabetic activities better than CSB through insulin sensitizing and/or insulinotropic actions in diabetic rats due to compositional changes, such as increased isoflavonoid aglycones and small peptides with low polarity found in CKJ. The present study was designed to explore the insulin sensitizing and insulinotropic effects and mechanism of CSB and CKJ in vivo, as our previous results were obtained in vitro.

Materials and methods

Animals and diets

Male Sprague Dawley rats, weighing 201 ± 12 g, were housed individually in stainless steel cages in a controlled environment (23°C and a 12 h light and dark cycle). All surgical and experimental procedures were performed according to the guidelines of the Animal Care and Use Review Committee at Hoseo University, Korea. The rats had a 90% pancreatectomy (Px) using the Hosokawa technique [7] or received a sham pancreatectomy (Sham). Px rats included in the experiments showed characteristics of type 2 diabetes, while the Sham rats did not.

Table 1 Composition of experimental diets

	Casein diet	Cooked soybean (CSB) diet	Chungkookjang (CKJ) diet
Carbohydrates (En%)	39.6	39.8	40.1
Protein (En%)	19.9	18.3	18.1
Fat (En%)	40.5	41.9	41.8
Fiber (%)	8.9	9.0	8.7
Total isoflavonoids (%)	–	0.036	0.027
Isoflavonoid Aglycones (%)	–	0.001	0.015

Chungkookjang was generated by a traditional processing method at Moonokrae Foods (Soonchang, Korea). Soybeans were sorted, washed, and soaked in water for 12 h at 15°C and boiled for 4 h at 100°C. The cooked soybeans were made to 40°C and fermented predominately with *Bacillus subtilis* in a fermentation chamber at 30°C for 43 h. According to the results of our preliminary study that insulin-stimulated glucose uptake was highest in the 43-h fermentation of CSB, the 43-h fermented CKJ and unfermented CSB were used for dietary protein source for the present study.

The Px rats were randomly assigned to four different groups (CSB, CKJ, casein, and RGZ groups) of 20 animals, according to dietary protein source. The rats in the RGZ group were orally administered rosiglitazone (20 mg/kg body weight) with a casein diet on a daily basis. RGZ was selected as a positive control, since it is a commercial insulin sensitizer utilized to attenuate hyperglycemia by activating PPAR-γ, and recent studies revealed some impact on β-cell function [8, 9]. Moreover, in our previous cell-based experiments, CKJ had distinct activities in glucose-stimulated insulin secretion and PPAR-γ agonist, compared to CSB. The Sham rats were provided a casein diet as a normal control. All of the Px and Sham rats freely consumed water and corresponding diets for 8 weeks.

The diets were made semi-purified diets, by modifying a base AIN-93 formulation for experimental diets [10]. The diet compositions of each group are shown in Table 1. Protein sources of the control, CSB, and CKJ groups were casein, cooked soybeans, and Chungkookjang, respectively. Since cooked soybeans and Chungkookjang contained a mixture of carbohydrates, protein, and lipids, their compositions were analyzed. According to the results of analysis, the macronutrient composition was tailored to show

equal proportions in all cases by adding soybean oil and cellulose. All diets consisted of approximately 40 energy percent (En%) carbohydrates, 20 En% protein, and 40 En% fats (Table 1) in order to study the effect of CKJ and CSB on insulin sensitizing and insulinotropic actions under an aggravated diabetic condition. The differences among these diets were essentially the degree of hydrolysis of protein and the presence of isoflavone, mainly as glycosides (CSB) or aglycones (CKJ). The contents of isoflavonoids were measured in our previous study; total isoflavonoids were decreased during fermentation, but the ratio of isoflavonoid aglycones was greatly increased (Table 1).

Insulin secretion capacity

After 7 weeks of treatment, catheters were surgically implanted into the right carotid artery and left jugular vein of rats anesthetized with intraperitoneal injections of ketamine and xylazine (100 mg and 10 mg/kg body weight, respectively). After 5–6 days of implantation, 25% glucose was continuously infused for 120 min to raise blood glucose levels 5.5 mM above the baseline, and serum glucose and insulin levels were measured from arterial blood at designated periods. The hyperglycemic clamp was performed in conscious and overnight fasted rats to determine insulin secretion capacity, as described in previous studies [7, 11]. After completing the clamp, the rats were provided with food and water for 2 days and the islet isolation or pancreas fixation were performed, as described below.

Overnight fasted serum glucose levels, food and water intake and body weight were measured every Tuesday at 10 AM. Oral glucose tolerance test (OGTT) was performed every 3 weeks in overnight fasted animals by orally administering 3 g/kg of glucose. Serum glucose and insulin were measured at 0, 10, 20, 30, 45, 60, 90, and 120 min after glucose loading by tail bleeding, and the average of area under the curve of glucose and insulin was calculated. Serum glucose levels were analyzed with a Glucose Analyzer II (Beckman, Palo Alto, CA) and serum insulin levels were measured by commercial RIA kit (Linco Research, St. Charles, MO).

Immunohistochemistry and islet morphometry

At the end of the 8-week experimental period, nine to ten rats from each group were injected with BrdU (100 μg/kg body weight). Six hours post-injection, rats were anesthetized with intraperitoneal injections of ketamine and xylazine, and pancreas was immediately dissected. The pancreas was fixed with 4% paraformaldehyde and paraffin-embedded, as described in previous studies [12, 13]. Two serial 5-μm paraffin-embedded tissue sections were selected out of seventh or eighth section to avoid counting the same islets twice in measuring β-cell area, BrdU incorporation, and apoptosis. Endocrine β and α-cells were identified by applying guinea pig anti-insulin and rabbit anti-glucagon antibodies to the sections. BrdU incorporation in β-cells was determined by staining rehydrated paraffin sections with anti-insulin and anti-BrdU antibodies [12]. Apoptosis of β-cells was measured by TUNEL kit (Roche Molecular Biochemicals, Indianapolis, IN) and counterstained with hematoxylin and eosin to visualize islets [13].

Pancreatic β-cell area was measured by examining all of non-overlapping images in two insulin-stained sections of each rat at a magnification of 10× with a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Thornwood, New York). Results of β-cell quantification were expressed as the percentage of the total surveyed area containing insulin-positive cells, measured by IP Lab Spectrum software (Scanalytics Inc., Fairfax, VA). Pancreatic β-cell mass was calculated by multiplying the percentage of insulin-positive area by the weight of the corresponding pancreatic portion [12, 14]. The individual β-cell size was determined as the insulin-positive area divided by the number of nuclei counted in the corresponding insulin-positive structures in randomly immunofluoresence-stained sections. Enlarged individual β-cell size indicates the induction of β-cell hypertrophy [14]. Beta-cell proliferation was calculated as the total $BrdU^+$ nuclei in β-cell nuclei per pancreas section [12, 13]. Apoptosis of β-cells was measured by the total number of apoptotic bodies in β-cell nuclei per pancreas section [13].

Islet isolation and immunoblotting

Pancreatic islets from nine to ten rats of each group were isolated by collogenase digestion at the end of an 8-week treatment of casein, CSB or CKJ, after anesthetizing the rats with intraperitoneal injections of ketamine and xylazine [13]. Isolated islets were incubated for 6 h to recover from the isolation process and at the end of incubation, they were administered with 10 nM of insulin-like growth factor-1 (IGF-1) for 10 min to determine insulin/IGF-1 signaling cascade. The IGF-1 treated islets were lysed with a 20 mM Tris buffer (pH 7.4) containing 2 mM EDTA, 137 mM NaCl, 1% NP40, 10% glycerol, 12 mM α-glycerol phosphate, and protease inhibitors. Lysates with equal amounts of protein (30–50 μg) were used for immunoblotting with specific antibodies against insulin receptor substrate (IRS)-2, protein kinase A (PKB, Akt), cAMP responding element binding protein (CREB), phosphorylated PKB^{Ser473}, phosphory-

lated CREB^{Ser133} (Cell Signaling Technology, Beverly, MA), and pancreatic duodenal homeobox-1 (PDX-1), as previously described [15]. Quantification of the relative band intensity was performed by laser scanning densitometry. These experiments were repeated four times for each group.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from islets of the rats from each group using a monophasic solution of phenol and guanidine isothiocyanate (Trizol reagent, Gibco-BRL, Rockville, MD), followed by extraction and precipitation with isopropyl alcohol [16]. The cDNA was synthesized from equal amounts of total RNA with superscript III reverse transcriptase, and polymerase chain reaction (PCR) was performed with high fidelity Taq DNA polymerase. The primers used to detect rat IRS2, PDX-1, and 18S genes were the following: IRS2 forward 5′-gaggactgaggaagaggac-3′, reverse 5′- ggttactgctggaactcttg-3′; 18S forward 5′-agttgctgcagttaaaaagc-3′, reverse 5′-actcagctaagagcatcgag-3′. The primers were designed to sandwich at least one intron to discriminate the products derived from mRNA and genomic DNA. These experiments were repeated four times for each group.

Statistical analysis

All results are expressed as a mean ± SD. Statistical analysis was performed using the SAS statistical analysis program [17]. CSB and CKJ effects were determined by one-way ANOVA. Significant differences in the effects among groups were identified by Tukey tests. Differences with a $P < 0.05$ were considered statistically significant in Tukey tests.

Results

Body weight, fasting glucose, insulin, and non-esterified fatty acids

Sham rats consumed fewer calories daily than Px rats, but their body weight was higher (Table 2), possibly due to urinary glucose excretion. Px rats displayed comparable daily food intake and body weight gain, regardless of diet composition (Table 2). Px rats exhibited diabetic symptoms, polyphagia, and hyperglycemia (Table 2). Hyperglycemia in Px rats was accompanied by a concomitant decrease in serum insulin levels (Table 2). The improvement in glucose utilization in response to the administration of CSB, CKJ, or RGZ was represented by lowered serum glucose. However, overnight fasted serum insulin levels were not different among groups of Px rats (Table 2). Similar to serum glucose levels, fasted serum non-esterified fatty acid levels were lowered in the CSB, CKJ, or RGZ groups, compared to the control (Table 2).

Area under the curve of glucose and insulin in OGTT

After the oral glucose load, CSB and CKJ diets in Px rats resulted in significantly lower area under the curve of serum glucose by 19 and 23%, respectively, compared with the casein diet (Fig. 1). This decrease was comparable to that of Px rats administered RGZ. Thus, Px rats consuming the CSB and CKJ diets had improved glucose tolerance via attenuating insulin resistance compared to Px rats consuming the casein diets (Fig. 1). Unlike CSB and RGZ, CKJ displayed higher total incremental areas under the insulin curves to the glucose load during the OGTT (Fig. 1), nearly reaching that of Sham rats. The CSB diet and RGZ administration had no impact on serum insulin increment, compared to casein diets in OGTT (Fig. 1).

Table 2 Physiological characteristics

	Control (n = 20)	CSB (n = 20)	CKJ diet (n = 20)	RGZ diet (n = 20)	Sham rats (n = 20)
Body weight (g)	343 ± 28	335 ± 29	355 ± 18	337 ± 22	385 ± 25**
Food intake (g/day)	23.9 ± 1.8	21.9 ± 2.8	24.3 ± 3.5	21. 8 ± 2.9	18.2 ± 2.8**
Serum glucose (mM)	7.0 ± 0.6[a]	6.0 ± 0.7[b]	5.5 ± 0.4[b]	5.7 ± 0.6[b]*	4.8 ± 0.5**
Serum insulin (ng/mL)	0.51 ± 0.08	0.62 ± 0.1	0.57 ± 0.08	0.58 ± 0.09	0.73 ± 0.15**
Serum non-esterifield fatty acids (μM)	958 ± 122[a]	754 ± 87[b]	737 ± 94[b]	705 ± 95[b]*	635 ± 83***

Values are mean ± SD. The control group was fed a casein diet,CSB group a cooked soybean diet, CKJ group a chungkookjang diet, and the RGZ group a casein diet plus rosiglitazone (20 mg/kg body weight/day)
*Significantly different among groups at $P < 0.05$, **$P < 0.01$, ***$P < 0.001$
[a,b]Values on the same row with different superscripts (a,b) were significantly different at $P < 0.05$ by Tukey test

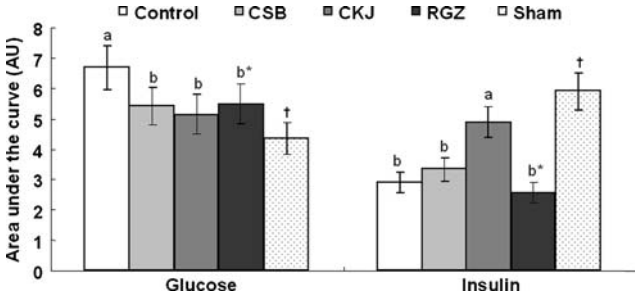

Fig. 1 The area under the curve of serum glucose and insulin during oral glucose tolerance test. Oral glucose tolerance tests were performed on Px rats fed with diets made of cooked soybeans (CSB), Chungkookjang (CKJ), casein or casein with rosiglitazone (RGZ; 20 mg/kg body weight/day) for 8 weeks following oral loading with 3 g glucose per kg body weight. Blood samples were taken at the time points indicated, glucose and serum were measured, and the area under the curve of glucose and insulin was calculated. The sample size in each group was the same as in Table 2. The bars represent mean ± SD. *Significantly different among the groups at $P < 0.05$. [a,b]Values of the bars with different superscripts (a, b, c) were significantly different in Tukey test at $P < 0.05$

First and second phase insulin secretion during hyperglycemic clamp

During hyperglycemic clamp, serum insulin levels peaked at 2–5 min and then declined to a nadir at 10 min, when glucose levels remained elevated and stable [11]. This is known as first phase insulin secretion. An ascending second phase of plasma insulin was observed at 60–90 min in all rats. Serum insulin levels in the first and second phases represent insulin secretion capacity [11, 13]. Serum insulin levels of Px rats were half of those in Sham rats during first and second phase insulin secretion under hyperglycemic clamp (Table 3). Consumption of CKJ, but not CSB, elevated first phase insulin levels of Px rats to those of Sham rats under hyperglycemic clamp (Table 3). In addition, CKJ consumption increased second phase insulin secretion in Px rats, but the increment did not reach that of Sham rats. In contrast, RGZ reduced second phase insulin secretion, but not first phase insulin secretion, compared to the control (Table 3). In fact, the area under the curve of serum insulin levels was paralleled with first and second phase insulin secretion (Table 3).

Glucose infusion rates in hyperglycemic clamp indicated β-cell function and insulin sensitivity at hyperglycemic state, calculated as the ratio of glucose infusion rate to steady-state plasma insulin levels [18]. The insulin sensitivity at hyperglycemic state was found to be higher in the RGZ-treated group than the other groups (Table 3). In order to maintain serum glucose levels 5.5 mM above baseline, glucose infusion rates were 50% higher in Sham rats than Px rats (Table 3). Comparable to Sham rats, the CKJ fed Px rats increased the rates more than Px rats fed the others, indicating CKJ improved β-cell function. Since Px rats impaired β-cell function, glucose infusion rates at hyperglycemic clamp state was related to the restoration of β-cell function rather than insulin sensitivity.

Pancreatic β-cell mass, proliferation, and apoptosis

Pancreatic β-cell mass, calculated by multiplying β-cell area by the pancreas weight was increased by CKJ in Px rats compared to the control. The percentage of β-cell area in total pancreas area of a section was not significantly different among the groups of Px rats. However, pancreas weight was significantly higher in CKJ fed Px rats, compared to other groups of Px rats (Table 4). As a result, total β-cell mass was significantly higher in CKJ, compared to the other groups. In addition to total β-cell mass, the characteristics of β-cells, such as individual cell size and numbers were different in the groups. CKJ decreased the individual β-cell size, suggesting the recuperation of hypertrophy resulted from increased insulin resistance (Table 4). In contrast, CKJ increased the number of β-cells (hyperplasia) by enhancing proliferation and reducing apoptosis in Px rats (Table 4). The ratio of β-cells and α-cells was higher in CKJ and CSB fed Px rats compared to the control (Table 4).

Table 3 Insulin secretion capacity during hyperglycemic clamp

	Control ($n = 9$)	CSB ($n = 10$)	CKJ ($n = 9$)	RGZ ($n = 9$)	Sham rats ($n = 9$)
Serum insulin at basal state (ng/mL)	0.49 ± 0.12	0.65 ± 0.11	0.62 ± 0.12*	0.57 ± 0.11	0.79 ± 0.12
Serum insulin at first phase (ng/mL)	4.4 ± 0.6[c]	5.6 ± 0.6[b]	8.8 ± 0.9[a]	5.1 ± 0.5[bc]*	9.5 ± 0.9**
Serum insulin at second phase (ng/mL)	5.2 ± 0.7[b]	6.1 ± 0.6[b]	8.3 ± 0.9[a]	3.3 ± 0.4[c]*	12.3 ± 1.3**
Area under the curve of insulin (AU)	4.2 ± 0.5[b]	4.8 ± 0.5[b]	6.8 ± 0.7[a]	3.1 ± 0.4[c]*	8.9 ± 1.0***
Glucose infusion rate (mg/kg body weight/min)	7.2 ± 0.8[b]	8.9 ± 0.9[ab]	10.6 ± 1.1[a]	8.3 ± 1.0[b]*	13.5 ± 1.3**
Insulin sensitivity (μmo1 glucose min 1.100 g^{-1} per μmol insulin/L)	22.1 ± 3.3[b]	23.9 ± 3.5[b]	20.3 ± 2.8[b]	35.2 ± 4.1[a]*	19.8 ± 2.7

Values are mean ± SD. The control group was fed a casein diet, CSB group a cooked soybean diet, CKJ group a chungkookjang diet, and the RGZ group a casein diet plus rosiglitazone (20 mg/kg body weight/day). First phase insulin secretion was defined as the average of serum insulin levels at 2 and 5 min, with second phase at 60, 90 and 120 min. Insulin sensitivity at hyperglycemic state was calculated as the ratio of glucose infusion rate to steady-state plasma insulin levels
*Significantly different among groups at $P < 0.05$, **$P < 0.01$, ***$P < 0.001$
[a,b,c]Values of the bars with different superscripts (a, b, c) were significantly different in Tukey test at $P < 0.05$

Table 4 The modulation of islet morphometry

	Control (n = 9)	CSB (n = 10)	CKJ (n = 9)	RGZ (n = 9)	Sham rats
β-cell area (%)	6.7 ± 0.8	7.4 ± 0.9	7.6 ± 0.9	7.1 ± 0.9	5.8 ± 0.7**
Individual β- Cell size (μm^2)	223.4 ± 29.1^{a}	205.6 ± 26.2^{ab}	181.4 ± 24.5^{b}	210.6 ± 25.8^{ab}*	178.5 ± 27.2**
Absolute β cell mass (mg)	20.2 ± 3.6^{b}	22.1 ± 3.5^{b}	30.9 ± 4.5^{a}	21.4 ± 3.1^{b}*	34.5 ± 4.7***
BrdU$^+$ cells (% BrdU$^+$ cells of islets)	0.85 + 0.10^{b}	0.95 + 0.11^{ab}	1.09 + 0.13^{a}	0.88 + 0.11^{b}*	0.71 + 0.10**
Apoptosis (% apoptotic bodies of islets)	0.68 ± 0.08^{a}	0.63 ± 0.07	0.60 ± 0.08	0.61 ± 0.07	0.61 ± 0.09
Ratio of β:α cells	4.8 ± 0.6^{b}	5.5 ± 0.6^{a}	5.8 ± 0.7^{a}	5.2 ± 0.7^{ab}*	5.2 ± 0.8

Values are mean ± SD. The control group was fed a casein diet, CSB group a cooked soybean diet, CKJ group a chungkookjang diet, and the RGZ group a casein diet plus rosiglitazone (20 mg/kg body weight/day)
*Significantly different among Px groups at $P < 0.05$, **$P < 0.01$, ***$P < 0.001$
^{a,b}Values on the same row with different superscripts (a, b) were significantly different at $P < 0.05$ by Tukey test

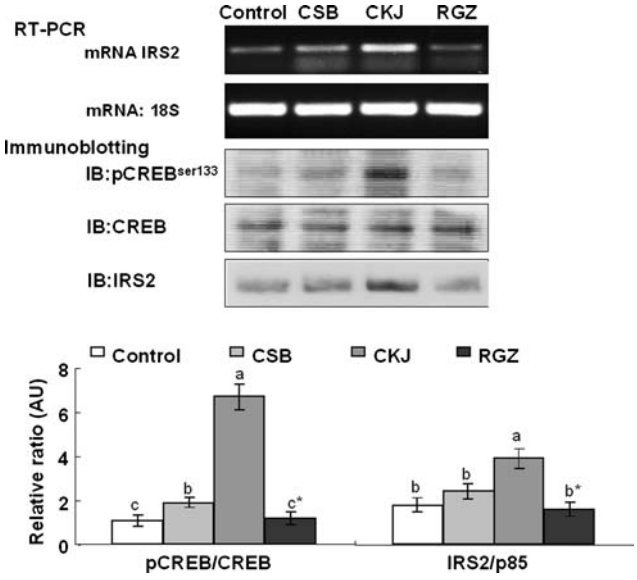

Fig. 2 The mRNA and protein levels of IRS2 and CREB phosphorylation in islets. These experiments were repeated four times from isolated islets, and the bars represent mean ± SD. *Significantly different among the groups at $P < 0.05$. ^{a,b,c}Values of the bars with different superscripts (a, b, c) were significantly different in Tukey test at $P < 0.05$

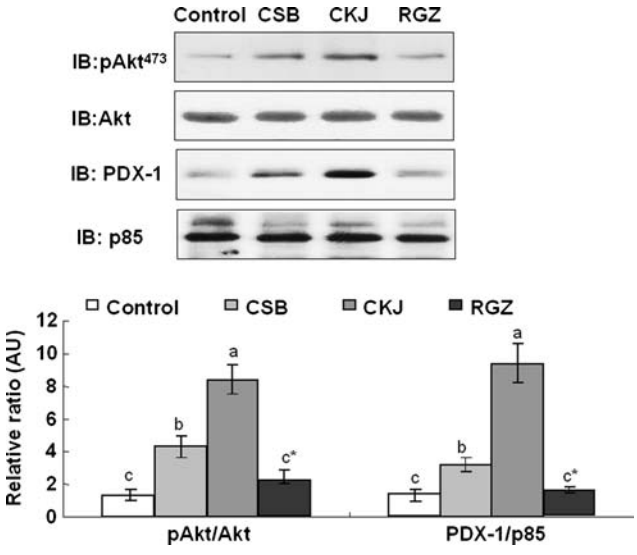

Fig. 3 Modulation of insulin/IGF-1 signaling in islets. These experiments were repeated four times for isolated islets, and the bars represent mean ± SD. *Significantly different among the groups at $P < 0.05$. ^{a,b,c}Values of the bars with different superscripts (a, b, c) were significantly different in Tukey test at $P < 0.05$

Since β-cell mass increased by hyperplasia sufficiently compensates for insulin resistance over a long period, CKJ may exhibit a better insulin secretion pattern in hyperglycemia.

Insulin/IGF-1 signaling in islets

Chungkookjang increased both IRS2 mRNA and protein levels in isolated islets in parallel with CREB phosphorylation, but CSB and RGZ minimally changed the levels (Fig. 2). Along with an absolute increment of IRS2 expression, serine^{473} phosphorylation of Akt was potentiated in islets isolated from CKJ fed rats, when compared to the control (Fig. 3). Even though CSB induced IRS2 expression in a small proportion, Akt phosphorylation in islets was increased more than the control, but not as much as CKJ. Another pathway was involved in Akt activation by CSB. In parallel with the intensity of Akt phosphorylation, expression of PDX-1, insulin promoter transcription factor, was increased in islets (Fig. 3). Increased expression of IRS2 and PDX-1 indirectly implied that an activated IGF-1/insulin signaling cascade resulted in enhancing insulin synthesis and hyperplasia of β-cells in the CKJ group.

Discussion

Soybeans exhibit various activities, such as anti-carcinogenic, anti-diabetic, anti-oxidant, and anti-lipidemic properties [19, 20]. Many researchers have suggested that coexisting isoflavonoids are associated

with various biological actions exerted by dietary soybean protein [19–21]. Thus, the compositional changes of isoflavonoids and proteins alter biological function. Since the fermentation of soybeans changes the contents and structures of isoflavonoids and protein, CSB and CKJ each have distinct biological functions. However, functional studies on CKJ have not been performed.

The present study showed that both CSB and CKJ attenuated insulin resistance, but unlike CSB, CKJ had insulinotropic agent through enhanced glucose-stimulated insulin secretion and pancreatic β-cell hyperplasia in Px rats exhibiting type 2 diabetic symptoms. This action increased proliferation of β-cells and decreased apoptosis by enhanced insulin/IGF-1 signaling cascade, suggesting that CKJ had better anti-diabetic action than CSB. These differences between CKJ and CSB resulted from increased isoflavone algycones and small peptides during fermentation.

A high fat diet accelerated the impairment of insulin secretion and exacerbation of insulin resistance, which worsened type 2 diabetic symptoms [12, 13]. We selected a high fat diet for all rats in order to determine the anti-diabetic effect of CSB and CKJ under an exacerbated diabetic condition. High fat diets have been known to elevate serum non-esterified fatty acids, which act as a key modulator of hypertrophy to increase insulin secretion, compensating for increased insulin resistance [22]. At first, insulin secretion capacity increased to compensate for insulin resistance with hypertrophy of β-cells. However, enlarging individual cell size, otherwise known as hypertrophy, is a temporary adjustment to compensate for increased insulin resistance, but it cannot be sustained for long periods [23]. The Px rats, our diabetic animal model, had induced type 2 diabetes through impaired insulin secretion capacity, followed by increased insulin resistance. Hyperplasia of β-cells is needed in order to reverse the impairment of insulin secretion capacity. Increased serum non-esterified fatty acids are known to accelerate hypertrophy, but attenuate hyperplasia [22, 23]. In addition, non-esterified fatty acids elevated apoptosis and reduced proliferation, leading to a decreased number of β-cells, similar to our results [23]. CKJ reduced individual β-cell size with decreased serum non-esterified fatty acids, but increased the number of β-cells by enhanced proliferation and decreased apoptosis in spite of the high fat contents in the diet. Due to the number and size of individual β-cells, the percentage of area β-cells was not different among the groups of Px rats, including the CKJ group.

Chungkookjang exhibited insulinotropic action as much as exendin-4, glucagons-like peptide-1 (GLP-1) agonist. In several studies, exendin-4 has been observed to improve glucose-stimulated insulin secretion, as well as to expand pancreatic β-cell mass via increased β-cell proliferation and neogenesis from precursor cells and decreased apoptosis [16, 24, 25]. The growth and survival of β-cells work through IRS2-PI_3 kinase pathway via the induction of IRS2 expression. Our previous study showed that mRNA levels of IRS2 increased in human islets treated with 2.5 nM exendin-4 for 4–8 h by approximately 5-fold and continuous 8-week administration of exendin-4 elevated IRS2 protein levels in islets of B6 mice by 3-folds [16]. This increase of IRS2 mRNA and protein levels was comparable to CKJ administration in Px rats in the present study. The induction of IRS2 was found to be associated with CREB activation, since the human and murine IRS2 gene contains several half-cre elements in the 5′-untranslated region that bind phosphorylated CREB. The activation of cAMP $\rightarrow$ CREB signaling increases the expression of IRS2 in Min6 cells and murine β-cells [12, 26]. Comparable to exendin-4, CKJ increased IRS2 induction in islets through phosphorylation of CREB, indicating the activation of cAMP signaling cascade. Lui et al. [27] reported that genistein elicited a significant glucose-stimulated insulin secretion at a concentration as low as 10 nM with a maximal effect at 5 μM. They explained that the improvement was associated with the activation of the cAMP/PKA signaling cascade in pancreatic β-cells. This suggested that increased genistein in CKJ was involved in enhanced glucose-stimulated insulin secretion through activating cAMP signaling.

Furthermore, Vedavanam et al. [28] suggested that isoflavonoids, including genistein and diadzein, might enhance anti-diabetic activity through activation of their estrogen-like action. Their ability to prevent glucose-induced lipid peroxidation and inhibit intestinal glucose uptake by decreasing sodium-dependent glucose transporter may also enhance anti-diabetic activity, especially by reducing insulin resistance. Estrogen improved glucose-stimulated insulin secretion, as well as β-cell proliferation and survival via increasing IRS2 expression in islets through CREB activation in our previous study [13]. In this study, their increased contents in CKJ activated CREB and led to the induction of IRS2 expression, since genistein and diadzein were found to increase intracellular cAMP, as mentioned previously. Thus, insulinotropic action of CKJ was closely related to estrogenic activity of isoflavonoid aglycones.

IRS2 induction improves β-cell growth and survival since IRS2 play a key modulator of insulin/IGF-1 signaling [29, 30]. Increased IRS2 expression in CKJ administration enhances insulin/IGF-1 signaling cascade by potentiating phosphorylation in β-cells, which improves the growth and survival by increasing β-cell proliferation and decreasing apoptosis [12, 13,

16]. Our previous studies showed that the condition in induced IRS2 expression improved the phosphorylation [13, 16]. Along with induction of IRS2, many other studies have shown that the expression levels of PDX-1 in islets were consistent with the proliferation of β-cells, resulting in increased mass, consistent with our results [12, 29, 30]. Thus, increased expression levels of IRS2 and PDX-1, as a result of CKJ, led us to the assumption that an IGF-1/insulin signaling cascade would be potentiated in the islets.

In conclusion, regardless of fermentation of soybeans mainly with *Bacillus subtilis*, insulin sensitivity was improved possibly through PPAR-γ activation in type 2 diabetic rats. Increased isoflavonoid aglycones and small peptides resulting from the fermentation improved glucose-stimulated insulin secretion and β-cell growth and survival by enhancing insulin/IGF-1 signaling cascade in islets of diabetic rats. Thus, CKJ is superior to CSB in ameliorating diabetic symptoms in diabetic Px rats with insulin deficiency.

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