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ScienceDirect
Metabolism Clinical and Experimental 56 (2007) 599–607
Metabolism
Clinical and Experimental
www.elsevier.com/locate/metabol

Long-term consumption of caffeine improves glucose homeostasis by enhancing insulinotropic action through islet insulin/insulin-like growth factor 1 signaling in diabetic rats

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Received 1 June 2006; accepted 20 December 2006

Abstract

Our previous study demonstrated that long-term cola consumption reduced body weight and improved insulin sensitivity in healthy male rats. In this study, we investigated the effect and mechanism of caffeine and sucrose, major components of cola, on glucose metabolism in 90% pancreatectomized diabetic rats. After a 12-week administration of 0.01% caffeine solution, the rats exhibited reduced body weight, fats, and insulin resistance, without a change in food intake, regardless of an 11% sucrose solution supplementation. In addition, caffeine enhanced glucose-stimulated first- and second-phase insulin secretion and beta-cell hyperplasia. This insulinotropic action was explained by potentiating an insulin/insulin-like growth factor 1 (IGF-1) signaling cascade via induction of insulin receptor substrate 2 in islets. In contrast, sucrose supplementation deteriorated insulin sensitivity and attenuated insulin/IGF-1 signaling in islets, which reduced the number of beta cells. Caffeine nullified the adverse effect of sucrose on glucose homeostasis. These findings indicate that long-term caffeine consumption can help alleviate diabetic symptoms by enhancing insulin sensitivity and beta-cell function through improved insulin/IGF-1 signaling via induction of insulin receptor substrate 2 in mildly diabetic rats.
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1. Introduction

The major characteristics of type 2 diabetes mellitus are insulin resistance and insulin deficiency [1]. People with insulin resistance do not develop diabetes if they have a sufficient insulin secretion capacity to compensate for insulin resistance. The prevalence of type 2 diabetes mellitus has been continuously and rapidly increasing in Asia, including Korea, because of the increased consumption of fat and simple sugars that accompanies the westernization of lifestyles and eating habits [2,3]. Recent statistics show that this prevalence now affects more than 8% of the entire population of Korea [4]. In contrast to Western diabetic patients, Asian type 2 diabetic patients, including Koreans, are not obese, and their serum insulin concentrations remain at or below the reference range [5]. This reflects that nonobese Koreans have insufficient insulin secretion capacity when insulin resistance is induced, making them susceptible to diabetes. Thus, reducing insulin resistance or increasing insulin secretion capacity can contribute to a decrease in susceptibility to type 2 diabetes mellitus.

Our previous study surprisingly revealed that long-term cola consumption with 33 energy percentage (En%) fat ameliorated insulin resistance in nondiabetic male rats from weaning [6]. This improvement was due to a reduction in body weight and body fat without a decrease in energy intake, which was associated with long-term caffeine consumption. However, most studies concerning acute caffeine response to glucose homeostasis revealed increased insulin resistance due to an immediate secretion of epinephrine [7-9]. In contrast, most long-term epidemiological studies revealed that long-term caffeine consumption via caffeinated drinks, such as coffee, had a rather beneficial action in glucose homeostasis to reduce the risk of type 2 diabetes mellitus [10,11]. However, some prospective studies on Pima Indians [12] and US participants in the Health Professionals Follow-Up Study and Nurses' Health Study [13] found no evidence of any relation between coffee consumption and type 2 diabetes mellitus. In the present study, we examined the effect and mechanism of 2 major

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0026-0495/$ – see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.metabol.2006.12.004

components of cola (caffeine and sucrose) on insulin secretion capacity and insulin resistance in nonobese type 2 diabetic rats. We tested the hypothesis that caffeine would enhance insulin secretion capacity and pancreatic beta-cell mass through enhancing insulin/insulin-like growth factor 1 (IGF-1) signaling via inducing insulin receptor substrate (IRS) 2 expression in beta cells in type 2 diabetic animal models, whereas sucrose would exacerbate them. To test the hypothesis, we provided a 0.01% caffeine solution (CAF), 11% sucrose solution (SU), and CAF + SU to 90% pancreatectomized (Px) rats for 12 weeks.

2. Materials and methods

2.1. Animals

Male Sprague-Dawley rats, weighing 268 ± 19 g, had 90% of their pancreas removed using the technique of Hosokawa et al [14] or received a sham pancreatectomy operation. After a 90% pancreatectomy, the pancreas that remained was within 2 mm of the common bile duct and extended from the duct to the first part of the duodenum. Two weeks after surgery, Px rats with random-fed serum glucose levels of less than 9.4 mmol/L were excluded from the experiments. The Px rats retained in the experiments exhibited mild type 2 diabetic symptoms. A sham pancreatectomy was performed by disengaging the pancreas from the mesentery and gently rubbing it between the fingers. The sham-operated (sham) rats did not exhibit any diabetic symptoms. All rats were housed individually in stainless steel cages in a controlled environment (23°C and a 12-hour 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, Asan-Si, Korea.

2.2. Diets

Pancreatectomized rats were randomly assigned to 4 different groups; each group had water (control), CAF (Sigma-Aldrich, St Louis, MO), SU (CJ, Seoul, Korea), or CAF + SU for 12 weeks. Each group had free access to the designated solution. The amounts of caffeine and sucrose in the drinking water were the same as in the composition of cola. All rats consumed a semipurified diet, made by modifying a base AIN-93 formulation for experimental diets [15]. The diet consisted of 38 En% carbohydrates, 22 En% protein, and 40 En% fats. The sources of carbohydrates, protein, and fats were starch, casein, and shortening, respectively. Overnight-fasted serum glucose levels, food and water intakes, and body weight were measured every Tuesday at 10:00 AM. Sham rats were freely provided water and high-fat diets, as normal controls, during the experimental periods.

2.3. Insulin secretion and insulin resistance

After 11 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 and 10 mg/kg of body weight, respectively). After 5 to 6 days of implantation, a hyperglycemic clamp was performed in conscious and overnight-fasted rats to determine insulin secretion capacity, as described previously [16]. Briefly, 25% glucose was continuously infused for 120 minutes to maintain blood glucose levels at 5.5 mmol/L above fasting levels at a hyperglycemic state, and serum glucose and insulin levels were measured from arterial blood at 0, 2, 5, 10, 60, 90, and 120 minutes. During hyperglycemic clamp, first- and second-phase insulin secretions were defined as the average of serum insulin levels at 2 and 5 minutes and the average of serum insulin levels at 60, 90, and 120 minutes, respectively.

Two days later, peripheral insulin resistance was determined via a euglycemic hyperinsulinemic clamp, described previously [17], under the same conditions as the hyperglycemic clamp. While regular human insulin (Humulin, Eli Lilly and Co, Indianapolis, IN) was continuously infused at a rate of 20 pmol kg^{-1} min^{-1} to raise plasma insulin concentration to approximately 1100 pmol/L, 25% glucose was simultaneously infused to maintain euglycemia (6 mmol/L). The glucose infusion rate was calculated and expressed in milligrams of glucose per kilogram of body weight per minute. The glucose infusion rate represented the whole-body glucose disposable rate, an index of whole-body response to exogenous insulin. After completing the clamp, the rats were anesthetized with 35 mg/kg of body weight sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL) and were decapitated. Their tissues were rapidly dissected, weighed, frozen in liquid nitrogen, and stored at −70°C, until further analysis could be performed.

Serum glucose levels were analyzed with a Glucose Analyzer II (Beckman, Palo Alto, CA). Serum insulin and leptin levels were measured by radioimmunoassay (Linco Research, St Charles, MO). Advanced glycated end products (AGEs) of subcutaneous tissues were measured using fluorescence methods [18]. The subcutaneous tissues were homogenized in phosphate-buffered saline (PBS) and the lysates centrifuged at 10 000*g* for 30 minutes at 4°C. The pellets were defatted with chloroform and methanol (2:1, vol:vol) and digested with collagenase type 7 and proteinase K (Sigma-Aldrich) in PBS for 48 hours. This was followed by overnight incubation with an equal amount of 0.2N NaOH at 4°C. After centrifugation, half of the supernatant was used for fluorescence determination at 370 nm excitation and 440 nm emission to measure general AGE-associated fluorescence. The remainder was used for assaying the content levels of hydroxyproline, which were determined by colorimetric measurement.

2.4. Immunohistochemistry and islet morphometry

At the end of the 12-week experimental period, 9 to 10 rats from each group were injected with 5-bromo-2-deoxyuridine (BrdU) (100 μg/kg of body weight). Six hours

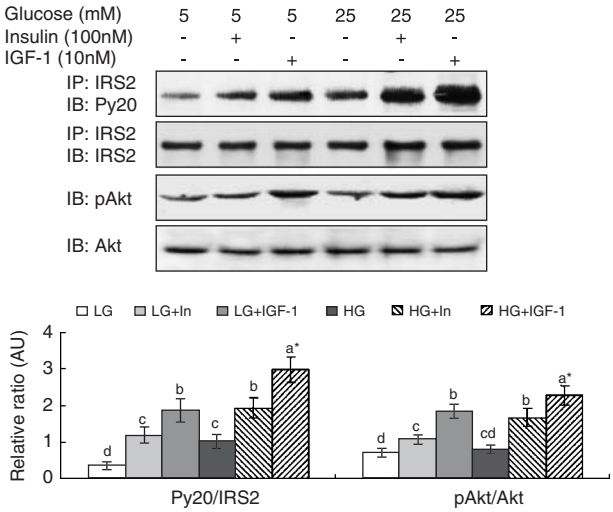

Fig. 1. Insulin signaling in islets isolated in low-glucose or high-glucose media and treated with insulin or IGF-1. Islets were isolated with low-glucose (LG) or high-glucose (HG) DMEM media and promptly administered 100 nmol/L insulin or 10 nmol/L of IGF-1 for 10 minutes. The islets were immediately lysed with a lysis buffer, and the phosphorylation and expression levels of the specific proteins were determined by immunoblotting with specific antibodies. The intensity of protein expression was determined using Imagequant TL (Amersham Biosciences, Piscataway, NJ). These experiments were repeated 4 times for islets, and the values are expressed as mean ± SD. Bars with different letters (a, b, c, d) were significantly different at $P < .05$. *Significantly different among Px groups at $P < .05$.

postinjection, rats were anesthetized with intraperitoneal injections of ketamine and xylazine, and the pancreas was immediately dissected. The pancreas was fixed with 4% paraformaldehyde and paraffin-embedded, as described in previous studies [19,20]. Two serial 5-μm paraffin-embedded tissue sections were selected out of the 7th or 8th sections to avoid counting the same islets twice in measuring beta-cell area, BrdU incorporation, and apoptosis. Endocrine beta and alpha cells were identified by applying guinea pig anti-insulin and rabbit antiglucagon antibodies to the sections. Incorporation of BrdU in beta cells was determined by staining rehydrated paraffin sections with anti-insulin and anti-BrdU antibodies [19]. Apoptosis of beta cells was measured by TUNEL kit (Roche Molecular Biochemicals, Indianapolis, IN) and counterstained with hematoxylin and eosin to visualize islets [20].

Pancreatic beta-cell area was measured by examining all nonoverlapping images in 2 insulin-stained sections of each rat at a magnification of ×10 with a Zeiss Axiovert microscope (Carl Zeiss Microimaging, Thornwood, NY). Results of beta-cell quantification were expressed as the percentage of the total surveyed area containing insulin-positive cells, measured by IP Lab Spectrum software (Scanalytics, Fairfax, VA). Pancreatic beta-cell mass was calculated by multiplying the percentage of insulin-positive area by the weight of the corresponding pancreatic portion [19,20]. The individual beta-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 beta-cell size indicates the induction of beta-cell hypertrophy [21]. Beta-cell proliferation was calculated as the total $BrdU^+$ nuclei in beta-cell nuclei per pancreas section [19]. Apoptosis of beta cells was measured by the total number of apoptotic bodies in beta-cell nuclei per pancreas section [20].

2.5. Islet isolation

Pancreatic islets from 9 to 10 rats of each group were isolated by collagenase digestion at the end of a 12-week treatment of water, CAF, SU, or CAF + SU solutions [19]. Through the pancreatic duct, 3 mL of 1.0 mg/mL collagenase (Sigma-Aldrich) in high-glucose Dulbecco modified Eagle medium (DMEM) was injected into the pancreas of rats anesthetized with sodium pentobarbital. The pancreas was immediately excised, and after incubation at 37°C for 15 minutes, the digested pancreas was washed 4 times with high-glucose DMEM on ice. Islets were isolated with a separation medium consisting of Ficoll reagent (Sigma-Aldrich). The islets washed with cold high-glucose DMEM were pooled from 2 to 3 rats from each group. The pooled islets were promptly administered 10 nmol/L of IGF-1 for 10 minutes to determine insulin/IGF-1 signaling cascade. Instead of insulin, IGF-1 was used for stimulating insulin/IGF-1 signaling cascade in islets for 2 reasons: (1) some studies showed that IGF-1 receptors played a more crucial role in promoting beta-cell development and survival through the IRS-2 signaling pathway than did insulin receptors [22,23], and (2) tyrosine phosphorylation of IRS-2 and serine phosphorylation of protein kinase B (PKB, Akt) were enhanced without additional insulin treatment in high-glucose media, in our preliminary study (Fig. 1). Indeed, additional insulin administration (100 nmol/L) and IGF-1 (10 nmol/L) improved the IRS-2 and Akt phosphorylation of islets in both low- and high-glucose media, but the intensity of the phosphorylation was greater with IGF-1 than with insulin (Fig. 1).

2.6. Immunoblot analysis

As previously mentioned, the IGF-1–treated islets were lysed with a 20-mmol/L Tris buffer (pH, 7.4) containing 2 mmol/L EDTA, 137 mmol/L NaCl, 1% NP40, 10% glycerol, and 12 mmol/L α-glycerol phosphate and protease inhibitors. After 30 minutes on ice, the lysates were centrifuged for 10 minutes at 12 000 rpm at 4°C. The supernatant with equivalent amounts of protein (400 μg) was immunoprecipitated with anti–IRS-1 and anti–IRS-2 antibodies (UBI, Waltham, MA) or resolved directly by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Lysates with equal amounts of protein (30-50 μg) were used for immunoblotting with specific antibodies against insulin receptor, IGF-1 receptor (Santa Cruz Biotech, Santa Cruz, CA), IRS-1, IRS-2 (Upstate Biotechnology, Charlottesville, VA), PKB, phosphorylated PKB^{Ser473}, forkhead transcription factor (FKHR), phosphorylated $FKHR^{Ser256}$ (Cell

Table 1
Average daily dietary intakes for 12 weeks

	CAF + SU (n = 10)	CAF (n = 9)	SU (n = 9)	Px control (n = 10)	Sham control (n = 10)
Energy (kJ)	35.5 ± 4.5	34.3 ± 4.2	38.4 ± 4.2	35.6 ± 4.0	28.8 ± 3.9†
Fluid intake (mL)	71.7 ± 9.5	67.8 ± 9.3	75.6 ± 9.8	64.5 ± 8.5	44.5 ± 10.1††
Caffeine intake (mg)	7.9 ± 1.0	7.3 ± 0.9	–	–	–
Complex CHO (g)	11.2 ± 1.4[b]	13.6 ± 1.6[a]	11.7 ± 1.5[b]	14.1 ± 1.7[a,*]	11.4 ± 1.5†
(En% of total energy)	(30.5)	(37.9)	(30.0)	(37.9)	(37.9)
Sucrose (g)	7.9 ± 1.0	–	8.3 ± 1.0	–	–
(En% of total energy)	(21.1)	–	(21.2)	–	–
Protein (g)	6.4 ± 0.7[b]	7.8 ± 0.8[a]	7.0 ± 0.8[b]	8.0 ± 0.8[a,*]	6.6 ± 0.8†
(En% of total energy)	(17.1)	(22.1)	(17.3)	(22.0)	(22.0)
Fat (g)	5.2 ± 0.6[b]	6.4 ± 0.7[a]	5.7 ± 0.7[b]	6.6 ± 0.7[a,*]	5.4 ± 0.7†
(En% of total energy)	(31.3)	(40.1)	(31.2)	(39.9)	(40.3)

Values are expressed as mean ± SD. Values on the same row with different superscripts (a, b, c) were significantly different at $P < .05$ by Tukey test. CAF and SU represent administration of 0.01% caffeine and 11% sucrose solution in 90% Px diabetic rats, respectively. Energy intake is calculated by the sum of daily consumption of food and sucrose from drink.

* Significantly different among Px groups at $P < .05$.

† Significantly different from Px control at $P < .05$.

†† Significantly different from Px control at $P < .01$.

Signaling Technology, Beverly, MA), and pancreatic homeodomain protein (PDX-1, Santa Cruz Biotech), as previously described [19,23]. The intensity of protein expression was determined using Imagequant TL (Amersham Biosciences, Piscataway, NJ). These experiments were repeated 4 times for each group.

2.7. Statistical analysis

All results were expressed as mean ± SD. Statistical analysis was performed using the SAS statistical analysis program (SAS, Cary, NC) [24]. Effects of CAF and SU were determined by 1-way analysis of variance. Significant differences in the main effects among groups were identified by Tukey tests. Differences with $P < .05$ were considered statistically significant in Tukey tests.

3. Results

3.1. Caffeine suppressed an increase of body weight and epididymal fat pads

All Px rats consumed a similar amount of fluid and energy, regardless of CAF and SU ingestion, whereas sham rats consumed less than Px rats in the same pattern (Table 1). The rats of the CAF + SU group consumed less energy from foods, and fat consumption was 10% less than in the control group because of energy intake from the SU solution. However, rats that consumed only SU tended to maintain a food intake similar to that of the control group, resulting in elevation of their total energy intake ($P = .1$). The CAF and CAF + SU groups consumed 7.6 ± 0.9 mg of caffeine from the fluids daily (Table 1).

Although total daily energy intake was not different among all groups of Px rats, body weight was significantly lower in rats that had continually ingested CAF, regardless of SU addition (Table 2). Along with body weight, epididymal fat pads were significantly reduced by supplementing CAF in Px rats (Table 2). In contrast, consumption of SU tended to raise body weight and fat, but not significantly, which was consistent with slightly elevated total energy intake in the SU group. As a result of the reduced body fat, serum leptin concentrations were lower in diabetic rats that consumed CAF, regardless of SU. Sham rats consumed less energy daily, but their body weight was higher than that of Px rats (Table 2) because Px rats had urinary glucose excretion. Epididymal fat pads were not

Table 2
Physiologic parameters of overnight-fasted states at the end of 12-week experimental periods

	CAF + SU (n = 10)	CAF (n = 9)	SU (n = 9)	Px control (n = 10)	Sham control (n=10)
Body weight (g)	336.1 ± 12.7[b]	328.4 ± 15.6[b]	412.5 ± 21.9[a]	392.6 ± 24.2[a,*]	435.4 ± 29.6†
Epididymal fat pad (g)	4.2 ± 0.6[b]	4.1 ± 0.5[b]	5.4 ± 0.6[a]	4.9 ± 0.6[a,*]	5.2 ± 0.7
Serum leptin (ng/mL)	3.2 ± 0.5[b]	3.1 ± 0.5[b]	4.3 ± 0.6[a]	3.9 ± 0.6[a,*]	6.2 ± 0.8††
Serum glucose (mmol/L)	6.5 ± 0.7	6.6 ± 0.9	7.0 ± 0.9	6.8 ± 0.8	4.8 ± 0.7†
Serum insulin (pmol/L)	366 ± 48[b]	357 ± 50[b]	443 ± 58[a]	378 ± 53[b,*]	559 ± 74††
AGE (arbitrary unit/mg collagen)	5.6 ± 0.6[c]	5.5 ± 0.6[c]	7.1 ± 0.7[a]	6.3 ± 0.7[b,*]	4.6 ± 0.6†

Values are expressed as mean ± SD. Values on the same row with different superscripts (a, b, c) were significantly different at $P < .05$ by Tukey test. CAF and SU represent administration of 0.01% caffeine and 11% sucrose solution in 90% Px diabetic rats, respectively.

* Significantly different among groups at $P < .05$.

† Significantly different from Px control at $P < .05$.

†† Significantly different from Px control at $P < .01$.

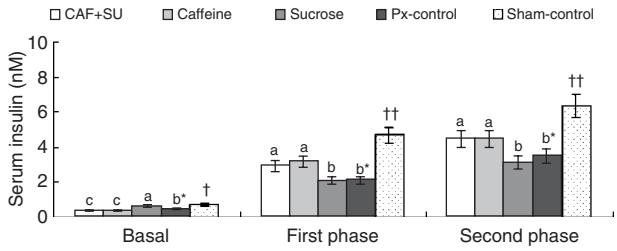

Fig. 2. Serum insulin levels during hyperglycemic clamp. A hyperglycemic clamp was performed in overnight-fasted mice to determine insulin secretion patterns and capacity at the end of experimental periods. We examined the effects of CAF, sugar, and CAF + sugar (SU) on insulin secretion capacity needed to maintain blood glucose levels at 5.5 mmol/L above fasting levels during hyperglycemic clamp in 90% Px diabetic rats. The rats in the control group were administered water. Serum insulin levels at first and second insulin secretion during hyperglycemic clamp in Px and sham rats are shown. First- and second-phase insulin secretions were defined as the average of serum insulin levels at 2 and 5 minutes during hyperglycemic clamp and the average of serum insulin levels at 60, 90, and 120 minutes, respectively. Sample size of each group was the same as in Table 1. Bars with different letters (a, b, c) were significantly different at $P < .05$. *Significantly different among Px groups at $P < .05$. †Significantly different from Px control at $P < .05$. ††Significantly different from Px control at $P < .01$.

significantly different between sham and Px rats. However, serum leptin levels were significantly higher in sham rats than in Px rats.

3.2. Caffeine improved peripheral glucose utilization with insulin sensitivity

To determine the effect of CAF and SU on glucose homeostasis, overnight-fasted serum glucose levels, insulin levels, and AGE were measured in Px rats. Serum glucose levels were the same among all groups of Px rats during the experimental periods (data not shown). Serum glucose levels of Px rats were higher than in sham rats, whereas serum insulin levels of Px rats were approximately half of those in sham rats (Table 2). Thus, Px rats exhibited mild diabetic symptoms due to decreased insulin secretion capacity, whereas sham rats did not. Advanced glycated end product in subcutaneous tissues, an indicator of long-term glucose homeostasis, increased significantly in the SU group of Px rats ($P < .05$), whereas it decreased significantly in the CAF groups. Overnight-fasted serum insulin levels were elevated in the SU group of Px rats, compared with the other groups (Table 2). During a euglycemic hyperinsulinemic clamp, whole-body glucose disposal rates increased in the CAF groups of Px rats, regardless of SU addition, whereas they decreased in the SU group (Fig. 2).

3.3. Caffeine enhanced first- and second-phase insulin secretion during hyperglycemic clamp

Long-term CAF consumption elevated serum insulin levels in Px rats during first- and second-phase insulin secretion at hyperglycemic clamp (Fig. 3). Ingestion of the SU solution did not alter serum insulin levels during first- and second-phase insulin secretion in Px rats, compared with the control group. The effect of SU on insulin secretion was cancelled out with CAF supplementation. Serum insulin levels in Px rats were half of those in sham rats during first- and second-phase insulin secretion at hyperglycemic clamp (Fig. 3).

3.4. Caffeine increased the expression of glucokinase involved in the glucose-sensing mechanism

The expression of glucokinase and glucose transporter 2 (GLUT2) in beta cells was involved in the glucose sensing mechanism, which modulated insulin secretion capacity [25]. Glucokinase expression increased in the CAF-supplemented groups of Px rats but was unchanged by the addition of SU. Caffeine and CAF + SU did not alter GLUT2 expression in Px rats, but SU alone tended to decrease it (Fig. 4). Glucokinase expression was parallel with first-phase insulin secretion rates. This suggested that CAF improved peripheral insulin sensitivity by accelerating first-phase insulin secretion, which was partly related to elevated glucokinase expression.

3.5. Caffeine increased pancreatic beta-cell mass via elevated proliferation

Decompensatory insulin secretion is reported to be associated with the expansion of pancreatic beta cells [19,26]. Pancreatic beta-cell mass, which is calculated by multiplying the pancreas weight by the beta-cell area, was increased by CAF and SU in Px rats compared with the control. Because the pancreas weight was not significantly

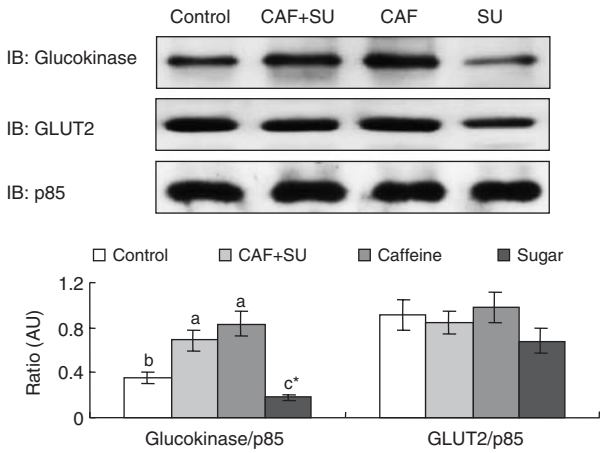

Fig. 3. The modulation of glucokinase and GLUT2 expression in islets isolated from 90% Px rats treated with caffeine and sucrose for 12 weeks. Islets isolated from the rats of each group were immediately lysed with a lysis buffer, and the phosphorylation and expression levels of the specific proteins were determined by immunoblotting with specific antibodies. The intensity of protein expression was determined using Imagequant TL (Amersham Biosciences, Piscataway, NJ). These experiments were repeated 4 times for islets, and the values are expressed as mean ± SD. Bars with different letters were significantly different at $P < .05$. **Significantly different among the Px groups at $P < .01$.

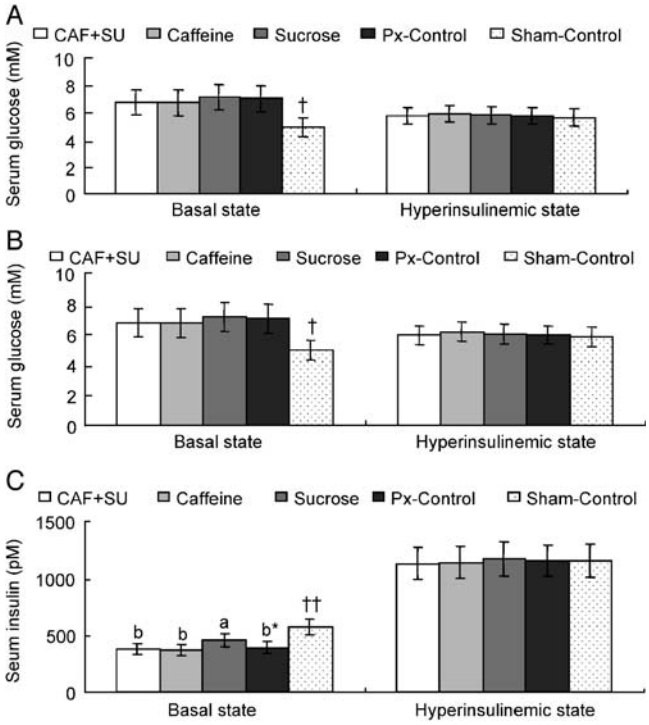

Fig. 4. Serum glucose and insulin levels and glucose disposal rates during a euglycemic hyperinsulinemic clamp. After a 12-week treatment of CAF, sugar, CAF + sugar (SU), or water (control), a euglycemic hyperinsulinemic clamp was performed in conscious, free-moving, and overnight-fasted rats to determine whole-body insulin resistance. A, Whole-body glucose disposal rates. B, Serum glucose at basal and hyperinsulinemic states. C, Serum insulin levels at basal and hyperinsulinemic states. Bars with different letters (a, b) were significantly different at $P < .05$. *Significantly different among Px groups at $P < .05$. †Significantly different from Px control at $P < .05$. ††Significantly different from Px control at $P < .01$.

different among all groups of Px rats, beta-cell mass exhibited the same result as the beta-cell area. Both CAF and SU would increase beta-cell mass (Table 3). However, the mechanism that expanded beta cells was different between the CAF and SU groups. Long-term consumption of CAF elevated beta-cell proliferation and reduced apoptosis in Px rats, resulting in hyperplasia (Table 3). Although SU reduced the number of beta cells by decreasing proliferation and increasing apoptosis (Table 3), it expanded beta-cell area by enlarging individual beta-cell size, determined by the number of nuclei in the given areas.

3.6. Caffeine enhanced insulin/IGF-1 signaling

To investigate the molecular pathways associated with the increase of beta-cell mass, insulin receptor, IGF-1 receptor, IRS-2, Akt, FKHR, and PDX-1 expression, and phosphorylation of IRS-2, we measured Akt and FKHR. As we expected from the results of our preliminary study (Fig. 1), basal phosphorylation of IRS-2, Akt, and FKHR existed in islets because they were isolated and incubated with high-glucose media. However, the intensity was lower than that of IGF-1 stimulation. The effect of caffeine and sucrose on insulin signaling in basal states exhibited the same trends of IGF-1 stimulation, but the activation was less potent by approximately 25% to 35% (data not shown). The expression of insulin and IGF-1 receptors was not different among the groups (Fig. 5). Caffeine increased IRS-2 protein levels, whereas sucrose did not (Fig. 5). The ratio of tyrosine phosphorylation of IRS-2 and its protein levels did not differ according to CAF and SU treatments, suggesting that tyrosine phosphorylation of IRS-2 was augmented by elevated IRS-2 protein levels. Along with an absolute increment of tyrosine phosphorylation of IRS-2, serine^{473} phosphorylation of Akt was potentiated in islets treated with CAF, yet they were unchanged by SU, compared with the control (Fig. 5). The expression of PDX-1 was consistent with phosphorylation of IRS-2 and Akt (Fig. 5). Caffeine elevated serine^{256} phosphorylation of FKHR, which contributed to the prevention of apoptosis in beta cells (Fig. 5). Therefore, the activation of insulin/IGF-1 signaling cascade in islets of CAF-fed rats was associated with increased tyrosine phosphorylation of IRS-2 due to IRS-2 induction.

4. Discussion

A recent epidemiological study and systemic and systematic review supports the hypothesis that habitual coffee consumption is associated with a substantially lower risk of type 2 diabetes mellitus [11,27]. However, the molecular mechanisms responsible for glucose regulation due to long-term coffee consumption remain to be eluci-

Table 3
The modulation of islet morphometry after caffeine and sucrose ingestion in 90% Px rats

	CAF + SU (n = 10)	CAF (n = 9)	SU (n = 9)	Px control (n = 10)	Sham control (n = 10)
Beta-cell area (%)	8.5 ± 1.0^{a}	8.7 ± 0.9^{a}	8.0 ± 1.0^{a}	6.9 ± 0.9^{b,*}	5.6 ± 0.8
Absolute beta-cell mass (mg)	25.9 ± 3.5^{a}	26.8 ± 3.7^{a}	24.6 ± 3.4^{a}	19.9 ± 2.8^{b,*}	33.9 ± 4.1†
Individual beta-cell size (μm^2)	211.5 ± 29.3^{b}	205.6 ± 31.2^{b}	269.5 ± 34.7^{a}	218.5 ± 28.4^{b}	179.4 ± 26.9†
Ratio of beta cells to alpha cells	5.6 ± 0.7^{a}	5.3 ± 0.6^{a}	4.3 ± 0.6^{b}	4.7 ± 0.6^{b,*}	5.1 ± 0.7
$BrdU^+$ cells (% $BrdU^+$ cells of islets)	1.06 ± 0.18^{a}	1.12 ± 0.21^{a}	0.88 ± 0.15^{b}	0.87 ± 0.12^{b,*}	0.78 ± 0.14
Apoptosis (% apoptotic bodies of islets)	0.54 ± 0.08^{c}	0.56 ± 0.07^{c}	0.73 ± 0.09^{a}	0.64 ± 0.08^{b,*}	0.56 ± 0.09

Data are expressed as mean ± SD. Values on the same row with different superscripts (a, b, c) were significantly different at $P < .05$ by Tukey test. CAF and SU represent administration of 0.01% caffeine and 11% sucrose solution in 90% Px diabetic rats, respectively.

* Significantly different among Px groups at $P < .05$.

† Significantly different from Px control at $P < .01$.

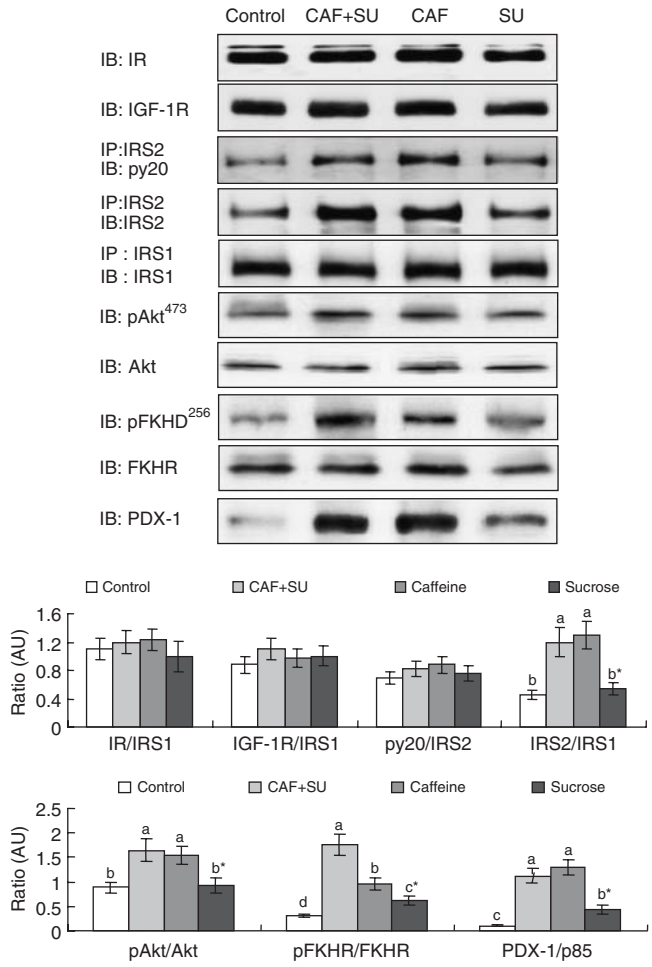

Fig. 5. The modulation of IGF-1/insulin signaling in islets isolated from 90% Px rats treated with caffeine and sucrose for 12 weeks. Islets isolated from the rats of each group were administered 10 nmol/L IGF-1 and immediately lysed with a lysis buffer, and the phosphorylation and expression levels of the specific proteins were determined by immunoblotting with specific antibodies. The intensity of protein expression was determined using Imagequant TL (Amersham Biosciences, Piscataway, NJ). These experiments were repeated 4 times for islets, and the values are expressed as mean ± SD. Bars with different letters (a, b, c, d) were significantly different at $P < .05$. *Significantly different among Px groups at $P < .05$. **Significantly different among Px groups at $P < .01$.

dated. This study demonstrated that in diabetic Px rats with insulin resistance and secretory deficit, long-term consumption of CAF, a major component of coffee, improved both insulin sensitivity and glucose-stimulated insulin secretion, regardless of SU addition, whereas sucrose itself aggravated insulin resistance.

Our observation of reduced insulin resistance by long-term consumption of CAF + SU in diabetic Px rats compares with our previous study [6] regarding cola consumption in normal rats. Sucrose alone increased insulin resistance slightly with body weight gain in the present study. Improved insulin sensitivity resulting from long-term CAF consumption, in both normal and diabetic Px rats, is related to reduced body weight and fat. Therefore, long-term CAF consumption had beneficial effects on peripheral insulin sensitivity by protecting against obesity, as demonstrated in our previous and present studies [6]. However, most CAF studies on glucose metabolism have investigated acute response with high dosage, not prolonged response. Most studies [7-9] showed that short-term CAF consumption reduced glucose utilization and insulin sensitivity in the liver and adipose tissues mainly because of the increased release of catecholamines. Activated epinephrine signaling elevated intracellular cyclic adenosine monophosphate (cAMP) concentrations, which accelerated gluconeogenesis and glycogenolysis, producing glucose in the liver, and stimulated lipolysis, decreasing glucose utilization. Therefore, a short-term and strong CAF consumption disrupted glucose homeostasis.

Recent studies demonstrated that increased intracellular cAMP did not disrupt glucose regulation but enhanced it in the liver and pancreatic beta cells [22,27]. Intracellular cAMP activates cAMP-responding element-binding protein, leading to increased IRS-2 expression in the liver and pancreatic islets [23,28,29]. In 2005, exenatide, a glucagon-like peptide 1 receptor agonist, was approved as a new hypoglycemic agent by working via insulinotropic action [30]. Our previous study demonstrated that exenatide enhanced glucose-stimulated insulin secretion and beta-cell hyperplasia by inducing IRS-2 expression [22]. Thus, IRS-2 has been known to play an important role in hepatic glucose production and pancreatic beta-cell growth and survival [19,23,28].

Consistent with the findings of other studies [22,27], CAF increased IRS-2 expression through cAMP-responding element-binding protein activation. Elevated IRS-2 protein levels have been known to enhance glucose homeostasis by improving insulin/IGF-1 signaling in islets via potentiating tyrosine phosphorylation, which activates Akt, downstream of IRS-2 [19,22]. Serine^{473} phosphorylation of Akt activates serine^{256} phosphorylation of FKHR. This phosphorylation of Akt and FKHR elevated PDX-1 expression, resulting in increased beta-cell proliferation. In addition, serine^{256} phosphorylation of FKHR contributed to preventing apoptosis of beta cells because FKHR plays a pro-apoptotic role by binding to DNA target sites in the nucleus [31]. Caffeine activated phosphorylation of AKT and FKHR via increasing IRS-2 induction in islets, which was associated with increased pancreatic beta-cell mass.

The direct relationship between pancreatic beta-cell mass and insulin secretion capacity was revealed through IRS-1 and IRS-2 knockout mice. The results from IRS-1 and IRS-2 knockout mice demonstrated that enlargement of pancreatic beta cells played an important role in the compensation for insulin resistance [32,33]. In IRS-1 knockout mice, insulin resistance increased by attenuating insulin signaling in muscle and adipose tissues. They did not develop diabetes because of elevated insulin secretion [32]. This was related to greater beta-cell mass

with elevated IRS-2 expression, consistent with the present study and other studies [19,20,23]. However, disruption of IRS-2 could not compensate for increased insulin resistance due to limited insulin secretion, which was directly associated with decreased beta-cell mass [19,33]. Maintaining sufficient beta-cell mass is important in compensating for insulin-resistant states by regulating insulin secretion.

With the exception of IRS-2 knockout mice, elevated insulin resistance states exhibited an expansion of pancreatic beta cells [21,26,34]. This enlargement could be achieved by hypertrophy and neogenesis of precursor cells, such as ductal cells [34]. However, insulin resistance decreased hyperplasia of beta cells via attenuated insulin/IGF-1 signaling. Insulin resistance can be compensated for by beta-cell hypertrophy. However, beta-cell hypertrophy cannot be sustained for long periods [26]. Our present study demonstrated that long-term consumption of SU increased insulin resistance and elevated pancreatic beta-cell mass by hypertrophy, not by hyperplasia. In contrast, long-term consumption of CAF promoted beta-cell hyperplasia in reduced insulin-resistant states, which had resulted from a potentiation of insulin/IGF-1 signaling through the induction of IRS-2 expression in diabetic Px rats. Thus, long-term consumption of CAF and SU increased pancreatic beta-cell mass in Px rats in independent pathways. However, beta-cell mass did not increase further in the CAF + SU group because CAF nullified SU effects on beta-cell hypertrophy.

In conclusion, habitual consumption of moderate caffeine–containing drinks induces IRS-2 expression. Induction of IRS-2 potentiates an insulin/IGF-1 signaling cascade in islets, leading to glucose-stimulated insulin secretion and increased pancreatic beta-cell mass through elevated proliferation and decreased apoptosis. Improved beta-cell function and mass results in ameliorating diabetic symptoms in insulin-deficient diabetic Px rats.

Acknowledgment

This work was supported by grant KRF-2005-041-C00510 from the Korea Research Foundation in Korea.

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