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Chlorogenic Acid and Caffeine in Coffee Restore Insulin Signaling in Pancreatic Beta Cells

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The incidence of type 2 diabetes is reported to be lower in frequent coffee drinkers than in non-coffee drinkers. To elucidate the mechanism by which coffee prevents the onset of type 2 diabetes, we analyzed how caffeine and chlorogenic acid, which are components of coffee, alter insulin signaling in MIN6 cells, a mouse pancreatic β cell line. The results showed that caffeine improved insulin signaling under endoplasmic reticulum stress, and chlorogenic acid protected pancreatic β cells by enhancing the expression of insulin receptor substrate 2 via cAMP response element-binding protein and promoting insulin signaling downstream of insulin receptor substrate 2. In addition, chlorogenic acid was a potent antioxidant for the protection of pancreatic β cells. Furthermore, *in vivo* and *in vitro* analyses revealed that the pancreatic β cell-protective effect of chlorogenic acid was mediated by the alleviation of endoplasmic reticulum stress. The results suggest that these components of coffee have the potential to reduce the pathogenesis of type 2 diabetes and improve pancreatic β cell insufficiency.

In recent years, the number of patients with type 2 diabetes has been increasing worldwide (1). The main pathogenic mechanisms of type 2 diabetes can be divided into two categories: insulin resistance and insulin insufficiency. Furthermore, insulin insufficiency is thought to be caused by abnormalities in the secretory mechanism of pancreatic β cells and a decrease in the number of pancreatic β cells (2). Our laboratory has previously reported that decreased insulin signaling markedly reduces pancreatic β cell volume, resulting in impaired glucose tolerance (3,4). We have also shown that pancreatic β cell insufficiency is caused by autophagy disorders (5) and that the accumulation of cell cycle inhibitory factors reduces pancreatic β cell volume (6,7). If substances that alleviate these causative factors of pancreatic β cell insufficiency are identified, they are expected to make a significant contribution to the treatment and prevention of type 2 diabetes mellitus. Recently, novel diabetes medications such as incretin and sodium/glucose cotransporter 2 inhibitors have become widely used, but because drugs are associated with side effects, we are focusing on the therapeutic potential of nutrients contained in food. The advantage of nutrients is their established safety when administered over long periods of time.

Coffee is a beverage that has long been the subject of numerous health-related studies. It has been reported that long-term coffee consumption reduces the risk of ischemic heart disease (8) and that coffee intake has a protective effect on cerebral infarction (9). Several other studies have indicated that coffee prevents Alzheimer's disease, osteoporosis, and cancer (10,11,12).

Multiple studies have also reported an association between type 2 diabetes and coffee consumption (13,14,15). The mechanism by which these effects of coffee occur is still under investigation, but caffeine and chlorogenic acid, which are components of coffee, are thought to play an important role. Caffeine and chlorogenic acid are both nutrients whose effects on the body have been widely reported, and although their association with health status and disease has attracted attention, there are few reports on their effects on pancreatic β cells. In this study, we examined the effects of caffeine and chlorogenic acid on pancreatic β cells.

MATERIALS AND METHODS

Mice

Pancreatic β cell-specific C/EBPβ TG mice on the C57BL/6J background were generated as previously described by inserting the C/EBPβ gene under the control of the rat insulin promoter (16). The animals were

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maintained in a 12-h light/dark cycle and fed normal chow from weaning at 3 weeks of age. All experiments were performed with male mice. After intraperitoneal injection of 10-week-old C/EBPβ TG mice with 40 mg/kg/day chlorogenic acid or saline for 2 weeks, the pancreas was removed and islet morphology was observed. This study was approved by the Institutional Animal Care and Use Committee (Permission number: P190503) and carried out in accordance with the Kobe University Animal Experimentation Regulations.

Cell culture

MIN6 cells, a murine pancreatic β cell line, was routinely maintained in Dulbecco's modified Eagle's medium (Sigma-Aldrich) containing 15% heat-inactivated fetal calf serum at 37°C with 5% CO_2. The medium was replaced every 3–4 days. Chlorogenic acid loading of MIN6 cells was performed for 24 h at the concentrations indicated. Caffeine loading of MIN6 cells was performed for 48 h at the concentrations indicated.

Immunoblot analysis

Lysates of isolated islets and MIN6 cells were prepared as previously described (17) and probed with antibodies to β-actin (Sigma-Aldrich), phosphorylated (phospho)-Akt (Thr308), phospho-Akt (Ser473), Akt, phospho-p70 S6 kinase, p70 S6 kinase, phospho-S6, S6, phospho-glycogen synthase kinase 3 beta (GSK3β), GSK3β, insulin receptor substrate 2 (IRS2), phospho-cAMP response element-binding protein (CREB), and CREB (Cell Signaling Technology).

Real-time reverse transcription (RT)-PCR analysis

Total RNA was extracted from MIN6 cells using a RNeasy Kit (Qiagen). RNA was subjected to reverse transcription (RT), and real-time polymerase chain reaction (PCR) analysis was performed as previously described (18). The cDNA synthesized from the RNA was analyzed using a sequence detector (model 7500; Applied Biosystems) with specific primers and SYBR Green PCR Master Mix (Qiagen). The relative abundance of each mRNA was normalized to the mRNA of the housekeeping gene cyclophilin A. Primers (sense and antisense, respectively) were as follows: cyclophilin A, 5′-CAGACGCCACTGTCGCTTT-3′ and 5′-TGTCTTTGGAACTTTGTCTGCAA-3′; insulin receptor, 5′-TTTGTCATGGATGGAGGCTA-3′ and 5′-CCTCATCTTGGGGTTGAACT-3′; *Irs2,* 5′-AGTCCCACATCGGGCTTGAAG-3′ and 5′-GGTCTGCACGGATGACCTTAG-3′; and *PI3K-p85a*, 5′-CCTTGTCCGGGAGAGCAGTA-3′ and 5′-TTGACTTCGCCGTCTACCACT-3′.

Statistical analysis

Quantitative data are presented as the mean ± standard error of the mean, and differences between means were assessed with Student's *t*-test (two-tailed). A P-value <0.05 was considered statistically significant.

RESULTS

First, we examined the effect of chlorogenic acid in a pancreatic β cell line. Western blotting showed that treatment of MIN6 cells with 0.1 mM chlorogenic acid promoted the phosphorylation of Akt, GSK3β, and S6K compared with controls (Fig. 1A).

Next, we examined how chlorogenic acid affects pancreatic β cells in the pathogenesis of diabetes. We performed endoplasmic reticulum (ER) stress loading as a mimic of diabetic pathology. Previously, our laboratory has shown that the islets of the db/db diabetic mouse model are exposed to ER stress loading (15,19). Other groups have reported that ER stress is an important factor in pancreatic β cell failure (20). Therefore, we loaded MIN6 cells with 2 μg/ml tunicamycin, a drug that induces ER stress, for 6 hours (16). We found that tunicamycin loading attenuated Akt phosphorylation in MIN6 cells, which was restored by the addition of 0.1 mM chlorogenic acid (Fig. 1B)(21). This suggests that chlorogenic acid may contribute to the improvement of pancreatic β cell insufficiency in the diabetic state in addition to the normal state.

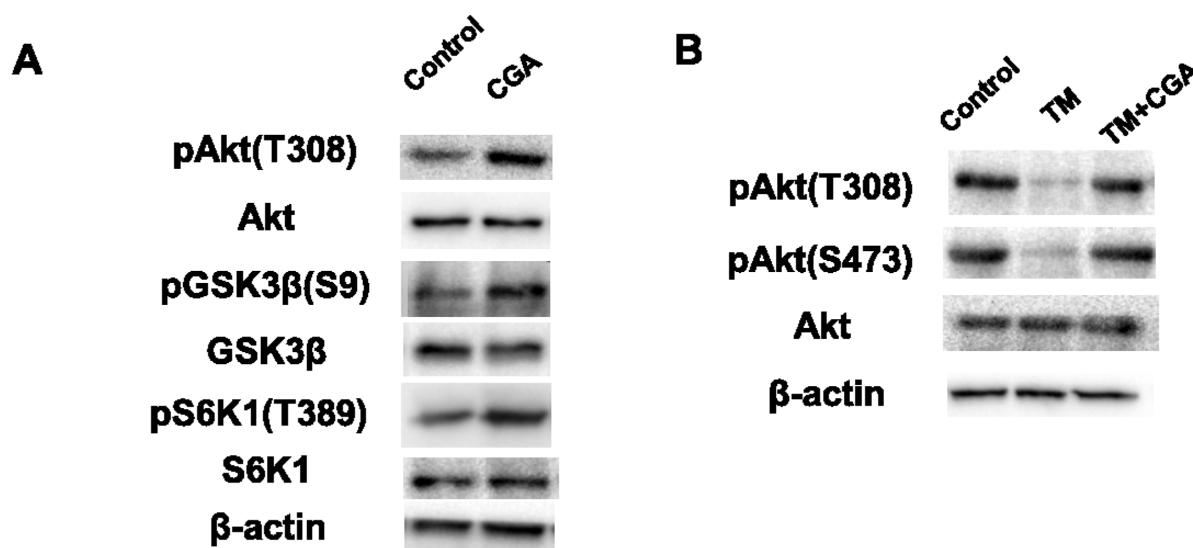

Fig. 1. Effect of chlorogenic acid on insulin signaling in a pancreatic β cell line
(A) Immunoblot analysis of insulin signaling with or without 0.1 mM chlorogenic acid in MIN6 cells
(B) Immunoblot analysis of insulin signaling with or without 0.1 mM chlorogenic acid in 2 μg/ml tunicamycin-treated MIN6 cells. CGA, chlorogenic acid; TM, tunicamycin.

In order to confirm the mechanism by which insulin signaling is upregulated, we examined the expression of insulin receptor, *PI3K*, and *Irs2* by real-time RT-PCR and found that only *Irs2* expression was significantly increased in the chlorogenic acid-treated group (Fig. 2A). To further investigate the mechanism underlying the increased expression of *Irs2*, the phosphorylation of CREB, a transcription factor of IRS2, was examined by western blotting. We found that chlorogenic acid promoted CREB phosphorylation in pancreatic β cells (Fig. 2B). These results suggest that chlorogenic acid enhances IRS2 expression and insulin signaling by promoting the phosphorylation of CREB, a transcription factor of IRS2.

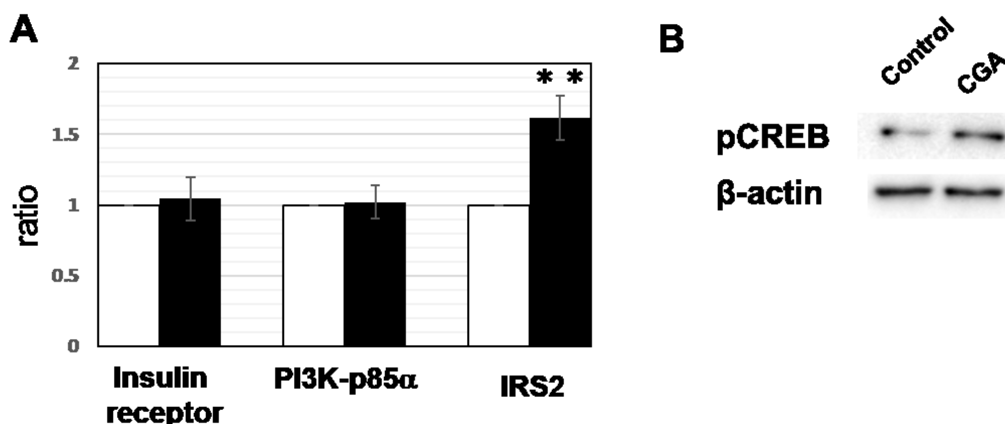

Fig. 2. Chlorogenic acid treatment increases *Irs2* expression upstream of insulin signaling
(A) Quantitative RT-PCR of molecules upstream of insulin signaling with or without 0.1 mM chlorogenic acid (black and white bars, respectively) in MIN6 cells (n = 6, respectively)
(B) Immunoblot analysis of phosphorylated CREB with or without 0.1 mM chlorogenic acid in MIN6 cells. CGA, chlorogenic acid.
Quantitative data in (A) are the mean ± SEM (n = 6). *P < 0.05, **P < 0.01 (2-tailed Student's *t*-test).

Next, to examine the effect of caffeine on insulin signaling, MIN6 cells were loaded with 0, 50, or 100 μM caffeine and protein quantification was performed. Under normal conditions, caffeine had no effect on insulin signaling (Fig. 3A). In contrast, the administration of tunicamycin induced ER stress, which reduced insulin signaling, and further administration of caffeine improved insulin signaling (Fig. 3B). Therefore, caffeine may have the potential to restore insulin signaling in pathological conditions. We further tested whether chlorogenic acid loading on tunicamycin-treated MIN6 cells, in which insulin signaling was restored by caffeine, further enhanced insulin signaling. However, no additive or synergistic effects were observed when chlorogenic acid and caffeine were loaded simultaneously (Fig. 3C).

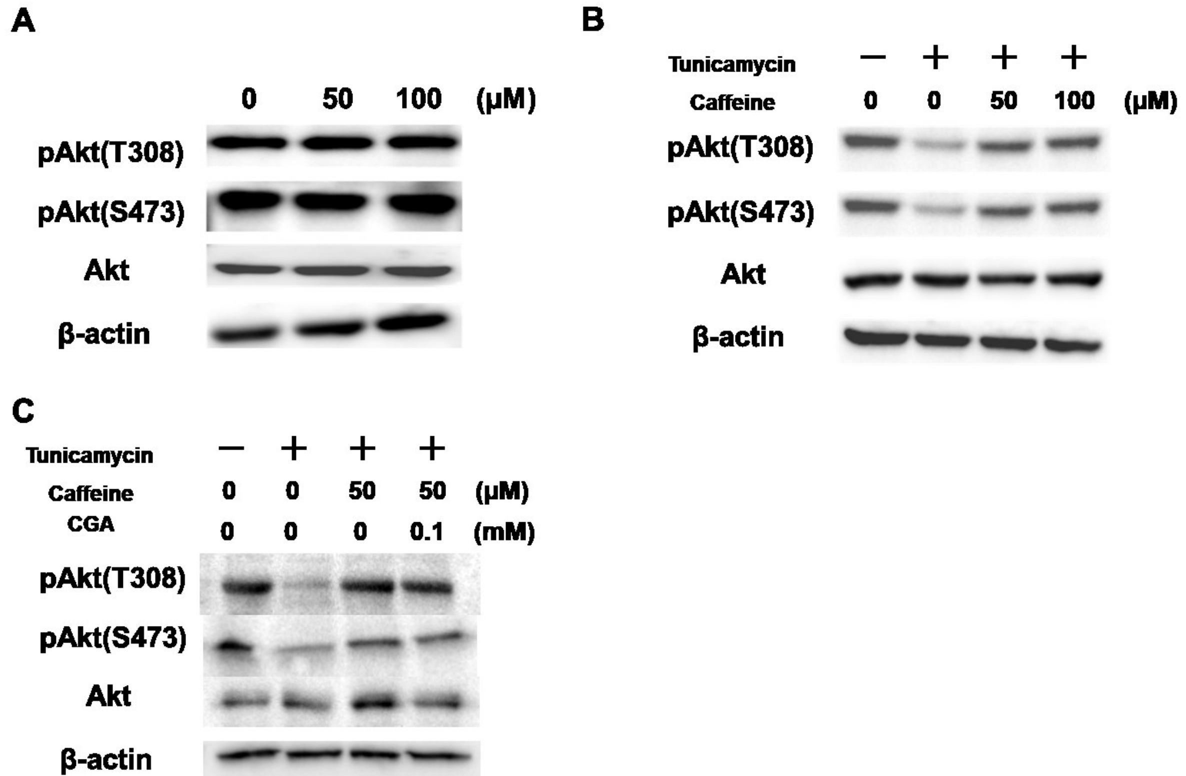

Fig. 3. Effect of caffeine on insulin signaling in MIN6 cells
(A) Immunoblot analysis of insulin signaling with 0, 50, or 100 μM caffeine in MIN6 cells
(B) Immunoblot analysis of insulin signaling with 0, 50, or 100 μM caffeine in tunicamycin-treated MIN6 cells
(C) Immunoblot analysis of insulin signaling with or without 50 μM caffeine and 0.1 mM chlorogenic acid in tunicamycin-treated MIN6 cells

Finally, to examine the effects of chlorogenic acid *in vivo*, we administered chlorogenic acid to C/EBPβ transgenic (TG) mice, a murine model of diabetes that overexpresses the transcription factor C/EBPβ specifically in pancreatic β cells. In a previous report, ER stress was shown to reduce pancreatic β cell volume and hyperglycemia (19). These mice were administered 40 mg/kg/day chlorogenic acid or saline by intraperitoneal injection for 2 weeks (22). As a result, a significant improvement in blood glucose levels was observed, although the body weight of the C/EBPβ TG mice did not change (Fig. 4A, 4B). Furthermore, the volume of pancreatic β cells in C/EBPβ TG mice was increased in the chlorogenic acid-treated group, and islet structure was also improved (Fig. 4C). Isolated islets from these mice showed elevated Akt phosphorylation and improved insulin signaling (Fig. 4D).

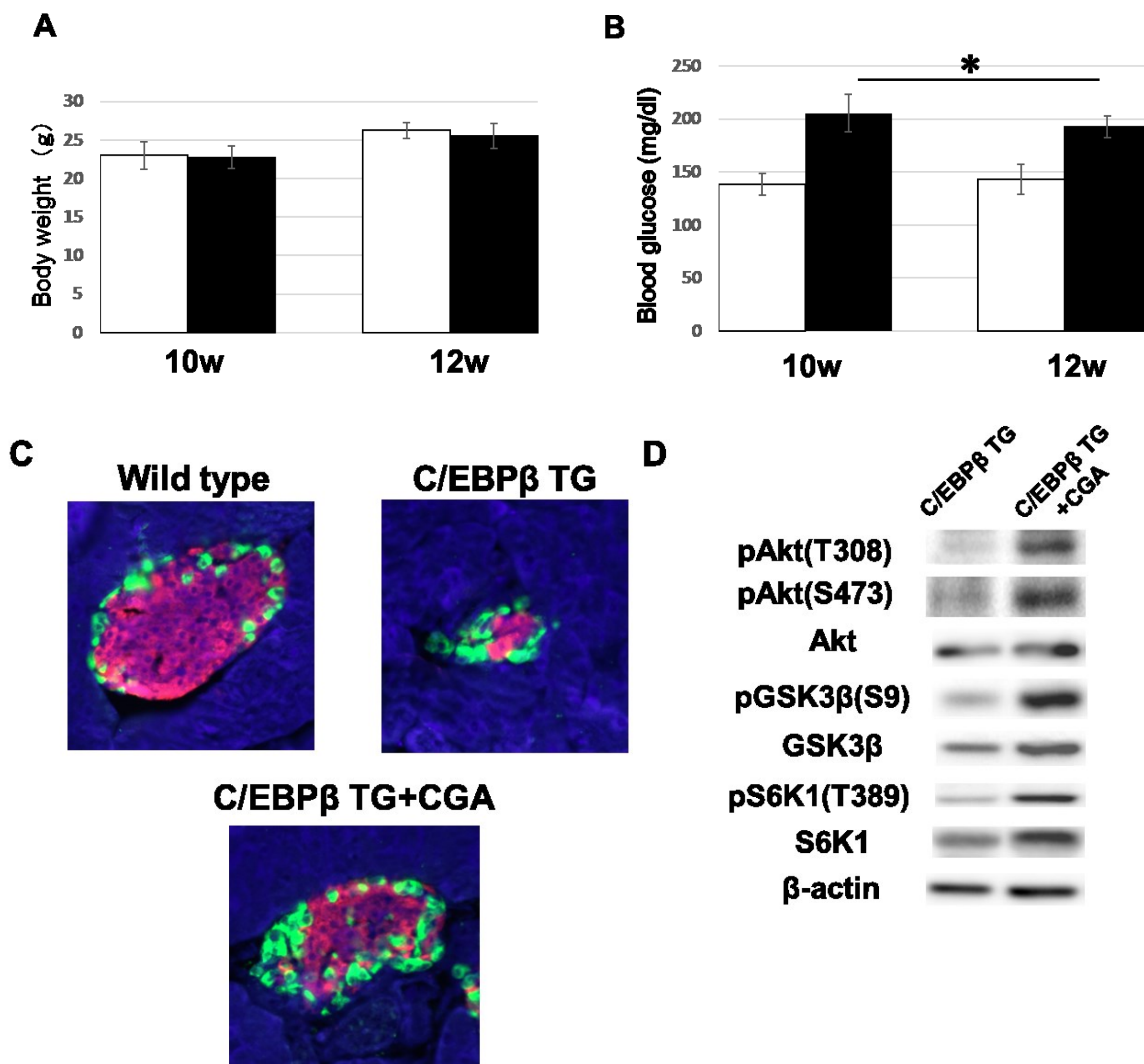

Fig. 4. Effects of chlorogenic acid on pancreatic β cells in the diabetic C/EBPβ TG mouse model

(A) Body weight (n = 12, respectively) at 10 and 12 weeks of age. White bars: wild-type mice as control; Black bars: C/EBPβ TG mice.

(B) Blood glucose levels (n = 12, respectively) at 10 and 12 weeks of age. White bars: wild-type mice as control; Black bars: C/EBPβ TG mice.

(C) Immunostaining of pancreatic islets in wild-type mice, C/EBPβ TG mice, and 40 mg/kg/day chlorogenic acid-treated C/EBPβ TG mice. Green: glucagon; Red: insulin.

(D) Immunoblot analysis of insulin signaling in pancreatic islets from C/EBPβ TG mice with or without 40 mg/kg/day chlorogenic acid.

Quantitative data in (A) and (B) are the mean ± SEM (n = 12). *P < 0.05, **P < 0.01 (2-tailed Student's *t*-test).

DISCUSSION

The effects of coffee on the human body have long been the focus of attention, and its effects on various organs have been reported. Coffee consumption is also reported to suppress the onset of psychiatric disorders such as depression (23) and cardiovascular disease (24); however, it is known to cause hypertension (25). A meta-analysis has also shown that coffee consumption reduces the incidence of cancer (26). Furthermore, coffee consumption reduces mortality from all causes (27), and there seems to be no doubt that it contributes to the health of coffee lovers.

A cohort study of 17,111 Dutch subjects was conducted in 2002 to investigate the relationship between coffee and the development of type 2 diabetes. The results showed that the incidence of type 2 diabetes was reduced in those who drank more than 7 cups of coffee per day compared with those who drank less than 2 cups per day (28). Since that report, experiments using db/db mice, a typical murine model of diabetes, and various epidemiological studies have been conducted (29,30). Based on the results of these studies, it seems safe to say that there is near-epidemiological agreement that the higher the level of coffee consumption, the lower the risk of developing type

2 diabetes. A meta-analysis reported that coffee and caffeine consumption are associated with a lower incidence of type 2 diabetes (31). Furthermore, another meta-analysis found that coffee consumption, both caffeinated and decaffeinated, was associated with a lower incidence of type 2 diabetes depending on the amount of coffee consumed (32). In addition, many reports suggest that the protective effect of coffee is due to some component other than caffeine, based on the comparison between caffeinated and decaffeinated coffee, and there are various theories on the actual component and its mechanism.

Chlorogenic acid is attracting attention as the component of coffee with the highest potential for preventing type 2 diabetes. In fact, chlorogenic acid has been shown to reduce blood glucose levels in animal and human studies (33,34). Therefore, we examined the effects of chlorogenic acid and caffeine on the survival and maintenance of pancreatic β cells. Our results showed that chlorogenic acid enhances insulin signaling in pancreatic β cells. Of particular interest was the restoration of insulin signaling in mouse islets and MIN6 cells, which was attenuated by ER stress. In a previous report, chlorogenic acid was shown to reduce ER stress in the testis, heart, and brain (35), and although the detailed molecular mechanism is unknown, the results were sufficiently reliable to indicate the reduction of ER stress in pancreatic β cells. Furthermore, chlorogenic acid is reported to enhance insulin secretion and glucose uptake in a KATP channel-independent manner in INS-1 cells, a rat pancreatic β cell line (36). In the present study, CREB phosphorylation, which regulates IRS2 expression, was enhanced by chlorogenic acid, which in turn enhanced insulin signaling. It has also been reported that chlorogenic acid loading of human neuroblastoma SH-SY5Y cells inhibits PARP-1, a molecule associated with Parkinson's disease, by enhancing CREB phosphorylation (37). This suggests that chlorogenic acid also contributes to cell proliferation via CREB in pancreatic β cells. Incidentally, although oral administration of chlorogenic acid to mice is more physiological, intraperitoneal administration was also used in the present study, since it has been adopted in many previous reports (22,35,36).

In contrast, caffeine is the most well-known component of coffee and has been shown to antagonize adenosine action through competitive inhibition (38), which has an excitatory effect on the central nervous system and also causes diuretic and heart rate elevation effects (39,40). Caffeine intake is known to be detrimental to diabetes mellitus, as it elevates blood glucose levels in association with decreased insulin sensitivity in skeletal muscle and liver (41,42), but in pancreatic β cells, caffeine increases insulin secretion and pancreatic β cell volume by enhancing insulin signaling (43). In the present study, caffeine ameliorated the ER stress-induced decrease in insulin signaling, suggesting that it may have a protective effect in pancreatic β cells.

In summary, coffee is a beverage that can be expected to have a protective effect on pancreatic β cells through the actions of chlorogenic acid and caffeine, but decaffeinated coffee may be preferable for stable blood glucose control. Further development of coffee research is expected to play a greater role in the prevention and treatment of diabetes, leading to the provision of safe and inexpensive medical care.

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