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Coffee Improves Insulin-Stimulated Akt Phosphorylation in Liver and Skeletal Muscle in Diabetic KK-A^y Mice

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Summary Coffee has an anti-diabetic effect, specifically the amelioration of both hyperglycemia and insulin resistance, in KK-A^y mice, a type 2 diabetes animal model. To investigate coffee's effect on insulin signaling in liver, skeletal muscle, and adipose tissue (epididymal fat), we assayed the tyrosine phosphorylation of insulin receptor (IR) and serine phosphorylation of Akt. In Expt. 1, we assayed insulin signaling under nonfasting conditions in KK-A^y mice that ingested water or coffee for 4 wk. Coffee ingestion ameliorated the development of hyperglycemia but did not affect insulin signaling in liver or skeletal muscle under such conditions. In Expt. 2, we assayed insulin signaling under basal and insulin-stimulated conditions in KK-A^y mice that ingested water or coffee for 3 wk. The levels of tyrosine phosphorylation of insulin receptor in response to insulin injection in insulin-sensitive tissues were not different between mice that drank water and those that drank coffee. Coffee ingestion significantly increased the insulin-induced serine phosphorylation of Akt in liver and skeletal muscle, but not in epididymal fat, of KK-A^y mice. Our results also indicated that coffee ingestion may contribute to the improvement of insulin resistance and hyperglycemia in KK-A^y mice via the activation of Akt in insulin signaling in liver and skeletal muscle.
Key Words diabetes, coffee, KK-A^y mice, Akt, insulin signaling

The associations between coffee intake and decreased risk of type 2 diabetes have been reported in a meta-analysis of human studies (*1*). In addition, in 2009, a meta-analysis of the association between coffee intake and the risk of diabetes was performed using data from 18 published studies and demonstrated that the high intake of coffee or decaffeinated coffee was associated with a reduced risk of new-onset type 2 diabetes (*2*). Type 2 diabetes is a complex disease caused by the combination of β-cell dysfunction and insulin resistance. Recently, it was reported that the intake of caffeinated coffee, but not of decaffeinated coffee, was positively related to insulin sensitivity in humans (*3*). However, the underlying mechanism by which coffee suppresses the development of type 2 diabetes remained unclear.

We recently examined the anti-diabetic effect of coffee using KK-A^y mice, a type 2 diabetes animal model, and demonstrated that coffee or caffeine ingested as drinking water ameliorated hyperglycemia, insulin resistance, and fatty liver in those mice (*4*). In another experiment, we showed that coffee or caffeine ingestion prevented the development of glucose intolerance and insulin resistance in C57BL/6J mice fed a high-fat diet for 17 wk (*5*). In KK-A^y mice, the accumulation of triglycerides in liver was reduced by coffee ingestion (*4*). As fatty liver provokes insulin resistance in liver, coffee might decrease insulin resistance in liver by ameliorating fatty liver. In KK-A^y mice, coffee ingestion also decreased the expression of inflammatory cytokine genes, such as monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor α (TNF-α), and interleukin 6 (IL-6), in epididymal fat (*4*). Similar to the results in KK-A^y mice, coffee ingestion reduced the gene expression of inflammatory cytokines in epididymal fat of C57BL/6J mice fed a high-fat diet. As these inflammatory cytokines produced in adipose tissues cause insulin resistance in insulin-sensitive tissues, including adipose tissue, skeletal muscle, and liver, it is suggested that coffee or caffeine ameliorates insulin resistance in these tissues by suppressing inflammatory cytokine expression (*6–11*). Insulin resistance is characterized by a decrease in insulin-stimulated glucose uptake in adipocytes and skeletal muscle via glucose transporter 4 (GLUT4) and by impaired suppression of glucose production in liver (*12*).

The binding of insulin to the insulin receptor leads to insulin receptor (IR) autophosphorylation and tyrosine phosphorylation of insulin receptor substrates (IRS).

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Abbreviations: GLUT4, glucose transporter 4; IL-6, interleukin 6; IR, insulin receptor; IRS-1, insulin receptor substrate-1; MCP-1, monocyte chemoattractant protein-1; PI3K, phosphatidylinositol 3-kinase; TNF-α, tumor necrosis factor α.

Subsequently, phosphorylated IRS activates phosphatidylinositol 3-kinase (PI3K), and then activated PI3K phosphorylates Ser/Thr kinase Akt (also called Protein Kinase B) (*13*). This activation of Akt promotes the translocation of GLUT4 vesicles to plasma membrane, resulting in the stimulation of glucose uptake into cells. In addition, Akt activation in liver contributes to the insulin-mediated suppression of gluconeogenesis and glycogenolysis.

The aim of this study is to reveal the tissue or organ in which coffee ingestion improves insulin sensitivity, by analyzing the phosphorylation states of IR and Akt. We first examined the effect of coffee ingestion on the phosphorylation state of IR and Akt in liver and skeletal muscle under non-fasting conditions. Secondly, we examined the effect of coffee ingestion on the insulin-stimulated phosphorylation of IR and Akt in liver, skeletal muscle, and adipose tissue under fasting conditions.

MATERIALS AND METHODS

Animals. Four-week-old male KK-A^y mice (CLEA Japan, Inc., Tokyo, Japan) were used for all experiments and were maintained at a controlled temperature of 23±3°C and 55±5% humidity on a 12-h light/dark cycle (light, 8:00–20:00). The mice were allowed free access to water and a standard laboratory diet (CE-2; CLEA Japan, Inc.) for 3 d before the experiments began. The composition of the diet was as follows: protein, 254 g/kg; fat 51 g/kg; non-nitrogenous substances, 506 g/kg; crude fiber, 35 g/kg, crude ash, 67 g/kg; energy 15.2 MJ/kg; and sufficient minerals and vitamins to maintain the health of the mice.

Experimental procedures. Black regular canned coffee was a gift from Pokka Corporation (Aichi, Japan). The coffee was used after 2.5-fold dilution with water. Expt. 1: Mice were divided into two groups and given water (control group, 8 mice) or diluted black coffee (coffee group, 8 mice) as drinking water. The mice were allowed free access to drinking water and diet (CE-2; CLEA) for 4 wk. Expt. 2: Mice were divided into two groups and given water (control group, 12 mice) or diluted black coffee (coffee group, 12 mice) as drinking water. The mice were allowed free access to drinking water and diet (CE-2; CLEA) for 3 wk. Coffee was prepared every second day. Blood samples were collected from the tail vein once a week for measurement of the serum glucose concentration. The collected blood was kept at room temperature for 15 min for coagulation. Then, the serum was obtained from the coagulated blood by centrifugation at 2,430 ×*g* for 10 min at 4°C. The serum was kept at −30°C prior to use. Serum glucose was measured by an assay kit using a glucose oxidase method, the Glucose C II-test (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The animal care and experimental procedures were approved by the Animal Research Committee of Nagoya University and were conducted according to the Regulations for Animal Experiments at Nagoya University.

Insulin signaling analysis. Expt. 1: At the end of the 4-wk experimental period, we analyzed the insulin signaling in mice. The day of sampling, all mice were deprived of diet at 9:00. After 1 hour of deprivation (at 10:00), the mice were killed by decapitation, and the liver and gastrocnemius muscle were collected. Expt. 2: At the end of the 3-wk experimental period, we performed the insulin signaling assay. The day before the sampling, all mice in the control and coffee groups were deprived of diet at 19:00. In each group, the mice were divided again into saline and insulin groups, and the final four groups were control-saline (n=5), control-insulin (n=7), coffee-saline (n=5), and coffee-insulin (n=7). After 14 h of fasting (at 9:00), saline or insulin (2 U/kg body weight; Humulin, Lily, Indianapolis, IN, USA) was intraperitoneally injected into mice. The mice were killed by decapitation at 15 min after injection. Liver, epididymal fat, and gastrocnemius muscle were quickly removed, immediately frozen with liquid nitrogen, and kept at −80°C until use.

For protein extraction, tissues were placed in a cold lysis buffer containing 10 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM Na_3VO_4, and protein inhibitor cocktail (Complete, Roche Diagnostics, Indianapolis, IN, USA) (*14*). The tissue homogenates were kept on ice for 1 h after homogenization, and the supernatants were obtained from the homogenates by centrifugation at 10,000 ×*g* for 20 min at 4°C. The lysates were stored at −80°C until the assays were performed.

Western blot analysis. The lysates (8 or 10 μg of protein) were subjected to SDS-PAGE on 7.5% acrylamide gel, and the proteins in the gel were transferred onto PVDF membranes (Hybond P, GE Healthcare, Tokyo, Japan). The membranes were incubated for 30 min at room temperature with Blocking One (Nacalai Tesque Inc., Tokyo, Japan) and incubated overnight at 4°C with the first antibody, rabbit polyclonal anti-insulin receptor beta (IRβ, sc-711, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-pIR (Tyr1162/1163) (sc-25103, Santa Cruz Biotechnology), anti-Akt (#sc-8312, Santa Cruz Biotechnology), anti-pAkt (Ser473) (#9271, Cell Signaling Technology, Beverly, MA, USA), anti-β-actin (#4967, Cell Signaling Technology), or anti-α tubulin (#2144, Cell Signaling Technology) and washed with Tris-buffered saline containing 0.1% Tween 20. The membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Santa Cruz Biotechnology) for 1 h at room temperature and washed. Each antibody was diluted with Canget Signal (Toyobo, Tokyo, Japan). The first antibodies were diluted as follows: IRβ (1 : 5,000), pIR (Tyr1162/1163, 1 : 4,000), Akt (1 : 12,000), pAkt (Ser473, 1 : 1,500), β-actin (1 : 4,500), and α-tubulin (1 : 10,000). The secondary antibodies for IR, pIR, Akt, and α-tubulin were diluted to 1 : 50,000 and those for pAkt and β-actin were diluted to 1 : 15,000. The membranes were autographed with the Supersignal West Dura Western blotting detection kit using the ECL method (Thermo Fisher Scientific, Waltham, MA, USA). Each protein on the band was quantified with Image J software.

Statistical analysis. All results are expressed as

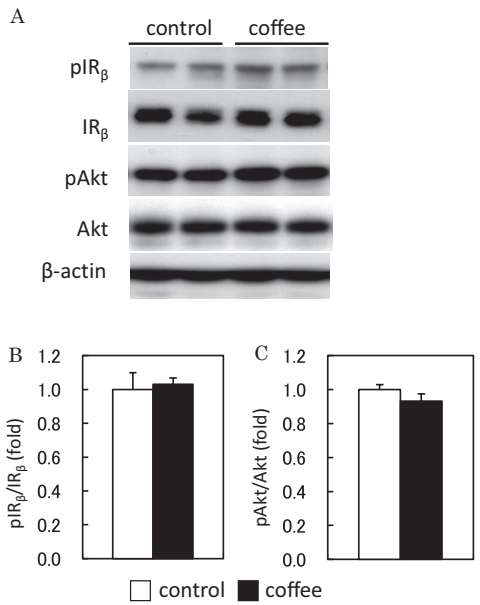


Fig. 1. Insulin signaling in liver of mice that ingested water or diluted coffee (Expt. 1). Mice were dissected under nonfasting (at 1 h after food deprivation) conditions at 4 wk into the experiment. The 8 μg of protein lysate was applied to SDS-PAGE. Fold levels are expressed relative to the level of signal in control liver. Control: n=8; Coffee: n=8.

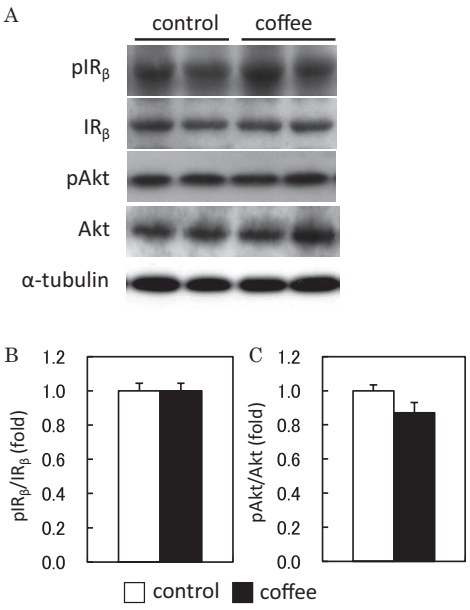


Fig. 2. The insulin signaling in skeletal muscle of mice that ingested water or diluted coffee (Expt. 1). Mice were dissected under nonfasting (at 1 h after food deprivation) conditions at 4 wk into the experiment. The 8 μg of protein lysate was applied to SDS-PAGE. Fold levels are expressed relative to the level of signal in control muscle. Control: n=8; Coffee: n=8.

Table 1. Body compositions and serum parameters in KK-A^y mice at 0 and 4 wk of experiment under non-fasting condition (Expt. 1).

Ingestion group	Control (n=8)	Coffee (n=8)
Initial (0 wk)		
Body weight (g)	18.6±0.5	18.4±0.3
Initial Blood glucose (mg/dL)	216±12	211±7
Final (4 wk)		
Body weight (g)	40.5±1.2	37.9±0.9
Food intake (g/100 gbw/d)	18.0±0.8	17.7±0.6
Blood glucose (mg/dL)	484±37	360±29*
Serum insulin (ng/mL)	16.5±2.4	11.3±1.5
Liver weight (g/100 gbw)	6.27±0.15	5.89±0.18
Epididymal fat weight (g/100 gbw)	3.27±0.04	3.13±0.15

Data are expressed as mean±SEM.
*p<0.05 vs. the control group.

means±SEM. Phenotypic data were statistically analyzed by either Student's t-test or Welch's test (Expt. 1). When the variances of each group were equal, mean values were compared using the former test. When the variances of each group were unequal, significance of differences was determined using the latter test. Phenotypic data were statistically analyzed by two-way ANOVA (Expt. 2). If the interaction effect of two components (coffee and insulin) was significant, then one-way ANOVA and a subsequent Tukey-Kramer test were carried out to compare the means of all groups (StatView; SAS Institute, Cary, NC). p values <0.05 were considered statistically significant.

RESULTS

Insulin signaling in liver and skeletal muscle of mice that ingested water or diluted coffee under nonfasting conditions (Expt. 1)

In mice that ingested water or coffee, we assayed the phosphorylation levels of IR and Akt in liver and muscle under nonfasting conditions (Figs. 1 and 2). Blood glucose concentrations at 4 wk into the experiment were significantly lower in the coffee than in the control (water) group (Table 1). In addition, final body weight and food intake (at 4 wk into the experiment) did not differ between two groups (Table 1). Liver weight

Table 2. Body compositions and serum parameters before and after the insulin injection (Expt. 2).

Ingestion group	Control (n=12)		Coffee (n=12)	
Initial (0 wk)				
Body weight (g)	18.2±0.3		18.1±0.2	
Blood glucose (non-fasting, mg/dL)	223±9		216±8	
Before insulin injection (3 wk)				
Body weight (g)	34.4±0.7		33.1±0.5	
Food intake (g/100 g bw/d)	18.6±0.5		17.7±0.4	
Blood glucose (non-fasting, mg/dL)	476±19		282±14*	
At 15 min after insulin injection				
Injection group	Saline		Insulin	
	Control (n=5)	Coffee (n=5)	Control (n=7)	Coffee (n=7)
Serum insulin (fasting, ng/mL)	1.05±0.06	0.99±0.15	ND	ND
Blood glucose (fasting, mg/dL)	164±12	158±15	80±6	53±6**

ND, not determined. Data are expressed as mean±SEM.
* $p<0.05$ vs. the control group. ** $p<0.05$ vs. the control group after the insulin injection.

and epididymal fat weight did not differ significantly between two groups (Table 1). Nonfasting serum insulin concentrations tended to be lower in the coffee compared to the control group (Table 1). In liver, the levels of pIR and pAkt did not differ between the control and coffee groups (Fig. 1A–C). Similar to the results in liver, the levels of pIR and pAkt in skeletal muscle were not affected by coffee ingestion (Fig. 2A–C).

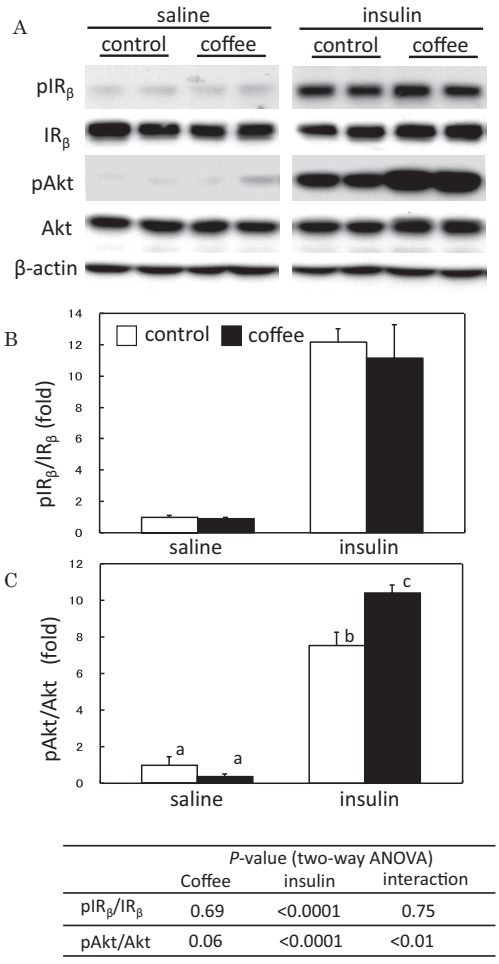
Phenotypes of mice that ingested water or diluted coffee before and after the insulin injection (Expt. 2)

We previously showed that coffee ingestion significantly reduced blood glucose concentration during the development of hyperglycemia in KK-A^y mice (*4*). In the present study, as well, coffee ingestion decreased the blood glucose concentration compared to the control groups at 2 wk into the experiment (data not shown). At 3 wk into the experiment, blood glucose concentration in the coffee group was significantly lower than that in the control group (Table 2), and no differences in body weight or food intake were observed between the control and coffee groups.

In mice injected with saline, the serum insulin concentration at 15 min after injection (that is, fasting insulin concentration) did not differ between the control and coffee groups (Table 2). In mice injected with insulin, we did not determine the serum insulin concentration because we injected human insulin but not mouse insulin. The blood glucose concentrations after the saline injection did not differ between these two groups. At 15 min after insulin injection, blood glucose concentration was significantly lower in the coffee group than in the control group. This result showed that insulin's glucose-lowering effect was greater in the coffee group than in the control group.

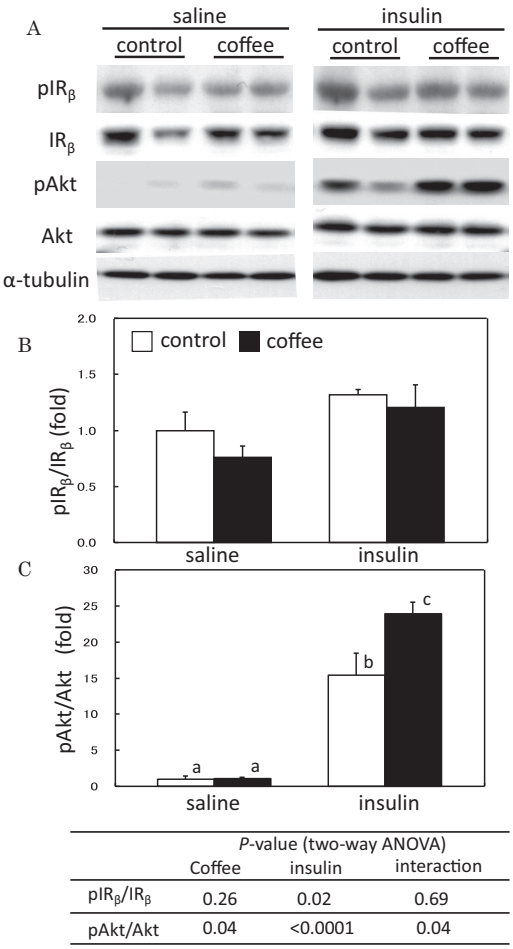
Insulin signaling in liver

As shown in Fig. 3A, 3B, and 3C, at 15 min after the injection, the insulin treatment markedly elevated both the level of tyrosine phosphorylation of IR and



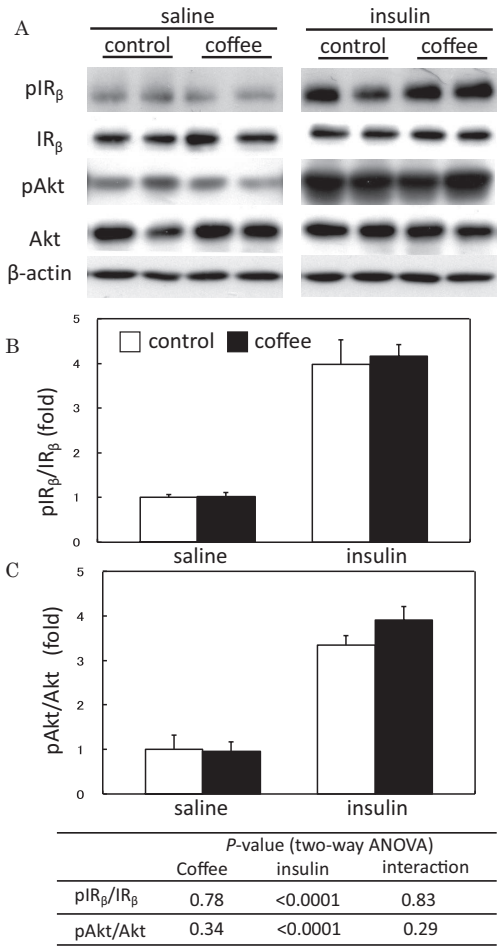
	P-value (two-way ANOVA)		
	Coffee	insulin	interaction
pIR_β/IR_β	0.69	<0.0001	0.75
pAkt/Akt	0.06	<0.0001	<0.01

Fig. 3. Insulin signaling in liver of mice ingested with water or diluted coffee (Expt. 2). Mice were fasted overnight and intraperitoneally injected with saline or insulin (2 U/kg body weight). After 15 min, liver was isolated and subjected to Western blot analysis. The 8 μg of protein lysate was applied to SDS-PAGE. Fold levels are expressed relative to the level of signal in control liver with saline. Control: n=12 (saline: n=5; insulin: n=7); Coffee: n=12 (saline: n=5; insulin: n=7).



	P-value (two-way ANOVA)		
	Coffee	insulin	interaction
pIR_β/IR_β	0.26	0.02	0.69
pAkt/Akt	0.04	<0.0001	0.04

Fig. 4. Insulin signaling in skeletal muscle of mice that ingested water or diluted coffee (Expt. 2). Mice were fasted overnight and intraperitoneally injected with saline or insulin (2 U/kg body weight). After 15 min, gastrocnemius muscles were isolated and subjected to Western blot analysis. The 8 μg of protein lysate was applied to SDS-PAGE. Fold levels are expressed relative to the level of signal in control muscle with saline. Control: n=12 (saline: n=5; insulin: n=7); Coffee: n=12 (saline: n=5; insulin: n=7).



	P-value (two-way ANOVA)		
	Coffee	insulin	interaction
pIR_β/IR_β	0.78	<0.0001	0.83
pAkt/Akt	0.34	<0.0001	0.29

Fig. 5. Insulin signaling in epididymal fat of mice that ingested water or diluted coffee (Expt. 2). Mice were fasted overnight and intraperitoneally injected with saline or insulin (2 U/kg body weight). After 15 min, epididymal fat as white adipose tissue was isolated and subjected to Western blot analysis. The 10 μg of protein lysate was applied to SDS-PAGE. Fold levels were expressed relative to the level of signal in control epididymal fat with saline. Values are mean±SEM. Control: n=12 (saline: n=5; insulin: n=7); Coffee: n=12 (saline: n=5; insulin: n=7).

the level of serine phosphorylation of Akt in liver. Akt is an insulin-signaling transduction molecule existing downstream of IR, and its serine residue (Ser473) is phosphorylated by insulin signaling. The phosphorylation level of IR after the insulin injection did not differ between the control and coffee groups (Fig. 3A and B). On the other hand, the level of pAkt after the insulin injection was significantly higher in the coffee group than in the control group (Fig. 3A and C).

Insulin signaling in skeletal muscle

In skeletal muscle at 15 min after the injection, the levels of IR phosphorylation were significantly higher in mice treated with insulin than in mice treated with saline (Fig. 4A and B). The level of pIR after the insulin injection did not differ between the control and coffee groups. On the other hand, insulin treatment remarkably increased the level of Akt phosphorylation. The levels of pAkt in mice treated with saline did not differ between the control and coffee groups. The level of pAkt after the insulin injection was significantly higher in the coffee group than in the control group (Fig. 4A and C). In the control and coffee groups, the levels of pAkt after the insulin injection were increased by 15-fold and 24-fold compared to those after the saline injection, respectively.

Insulin signaling in epididymal fat

In epididymal fat, the levels of pIR and pAkt after the saline injection did not differ between the control and coffee groups (Fig. 5A and B). The levels of pIR and pAkt in insulin-treated mice were increased by 3.5–4-fold compared to the respective value in mice treated with saline. After the insulin injection, however, there were no differences in the levels of pIR or pAkt between the control and coffee groups.

DISCUSSION

We previously demonstrated that coffee ingestion

enhanced insulin sensitivity in both diabetic KK-A^y mice and C57BL/6J mice fed a high-fat diet (*4*, *5*), both of which develop insulin resistance in peripheral tissues. However, the mechanisms underlying the improvement of insulin sensitivity by coffee ingestion remained to be clarified in these diabetic models. In the present study, we revealed for the first time that coffee ingestion improved the insulin signaling in liver and skeletal muscle at the level of Akt phosphorylation in diabetic KK-A^y mice.

In the first experiment, under non-fasting conditions, we measured the levels of phosphorylation of both IR and Akt in liver and skeletal muscle. As observed in the previous study (*4*), coffee ingestion ameliorated the development of hyperglycemia compared to the control group. However, the levels of pIR and pAkt in liver and skeletal muscle were not changed with coffee ingestion in nonfasting KK-A^y mice (Figs. 1 and 2). Because nonfasting serum insulin concentrations tended to be lower in the coffee group (11.3 ± 1.5 ng/mL, $p=0.089$) than in the control group (16.5 ± 2.4 ng/mL), we consider that coffee ingestion could enable the maintenance of similar levels of IR and Akt phosphorylation with lower levels of serum insulin compared to the mice in the control group.

In the second experiment, we assayed the insulin-stimulated insulin signaling in fasting mice. Although blood glucose concentrations before the insulin injection did not differ between the control and coffee groups, the blood glucose concentration at 15 min after insulin injection was significantly lower in the coffee group than in the control (Table 2). This result clearly showed that coffee ingestion improved systemic insulin sensitivity in KK-A^y mice.

In fact, we detected the activation of insulin signaling—that is, the enhancement of Akt phosphorylation—in liver and skeletal muscle by coffee ingestion. In the previous study, we showed that coffee ingestion significantly decreased the mRNA levels of IL-6, MCP-1, and TNF-α in epididymal fat and decreased serum IL-6 concentrations (*4*). These proinflammatory adipocytokines secreted from adipose tissue induce insulin resistance in liver and skeletal muscle in a paracrine and endocrine manner (*7*). Therefore, we speculated that the reduction in inflammatory adipocytokine production by coffee ingestion might help to improve the insulin signaling in liver and skeletal muscle. Although we expected that coffee ingestion could improve insulin signaling in adipose tissues, this study did not show a change of insulin signaling in epididymal fat. From the previous and present results, coffee ingestion improved the insulin signaling in liver and ameliorated the development of fatty liver in KK-A^y mice (*4*). We reported that coffee ingestion suppressed the hepatic expressions of sterol regulatory element binding protein-1 and fatty acid synthase genes (*4*). The activation of hepatic Akt by coffee ingestion might contribute to the suppression of lipogenesis and the decrease of hepatic triglyceride content in KK-A^y mice. On the other hand, we also think that the direct effect of coffee in liver may suppress lipogenesis and improve insulin sensitivity. We need to examine whether or not the components of coffee directly act on hepatocytes and suppress the expression of lipogenic genes.

In liver and skeletal muscle, the activation of Akt by insulin was enhanced by coffee ingestion, but the activation of insulin receptor in liver and skeletal muscle did not differ between the water- and coffee-ingestion groups (Figs. 3A and 4A). This result showed that coffee ingestion improved the downstream signaling of IR tyrosine phosphorylation and led to Akt activation.

In a mouse preadipocyte cell line, it was reported that caffeine inhibited insulin-induced Akt phosphorylation and subsequently caused a decrease in glucose uptake via the blocking of GLUT4 translocation (*15*). In ex vivo skeletal muscle incubation, some groups reported that caffeine decreased insulin sensitivity and glucose transport by inhibiting insulin signaling (*16–18*). Kolnes et al. reported that the insulin-stimulated Ser^{473} and Thr^{308} phosphorylations of Akt in skeletal muscle were blocked by caffeine (*17*). At present, there has been no report that caffeine has a positive effect on insulin signaling. However, the present study showed that caffeinated coffee ingestion increased insulin-induced Akt phosphorylation in skeletal muscle and did not inhibit insulin-stimulated Akt activation in epididymal fat tissue (Figs. 4 and 5 in Expt. 2). Coffee also contains chlorogenic acids, caffeic acids, ferulic acids, and melanoidins (*19*). Thus, we think that the other components of coffee, besides caffeine, may have positive effects on insulin signaling. Actually, caffeic acid ingestion is reported to significantly reduce the blood glucose and glycosylated hemoglobin levels in C57BL/KsJ-*db*/*db* mice (*20*). Ferulic acid was also shown to reduce blood glucose concentrations in KK-A^y mice (*21*), C57BL/KsJ-*db*/*db* mice (*22*), and streptozotocin-induced diabetic mice (*21*) and rats (*23*). Caffeic acid and cinnamic acid, metabolites of chrologenic acids, were shown to improve the tyrosine phosphorylation of IR in the FL83B insulin-resistant hepatocyte cell line (*24*). Therefore, in a future study, we need to investigate the effect of caffeine and the mentioned compounds in insulin signaling and to examine whether or not this effect is identical to that of coffee.

In conclusion, this study revealed for the first time that coffee ingestion enhanced Akt phosphorylation in liver and skeletal muscle in diabetic KK-A^y mice. We think that liver and skeletal muscle contribute to coffee's ameliorative effect on systemic insulin sensitivity. This study provides valuable findings for elucidating the molecular mechanisms underlying coffee's antidiabetic effect.

Acknowledgments

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