



Original

Phenotypic and genotypic changes in obesity and type 2 diabetes of male KK mice with aging

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Abstract: Research into the prevention and treatment of age-related metabolic diseases are important in the present-day situation of the aging population. We propose that an elderly diabetic mouse model may be useful to such research as it exhibits deterioration of glucose and lipid metabolism. Although the KK mouse strain is commonly used as a model of moderate obesity and type 2 diabetes, the utility of this strain as an elderly obese and diabetic model mouse for research into aging remains unclear. The present study aimed to investigate age-related changes of glucose and lipid metabolism in male KK mice fed a standard chow diet. We demonstrate that 40 weeks KK mice exhibit age-related dysfunctions, such as development of insulin resistance associated with pancreatic islet hypertrophy and decreased lipolysis in white adipose tissue (WAT) compared with 15 weeks KK mice. However, aging does not appear to cause mitochondrial dysfunction of brown adipose tissue. Unexpectedly, hyperglycemia, potential glucose uptake in insulin-sensitive organs, hepatic lipid accumulation, hypertrophy of adipocytes, and inflammation in epididymal WAT did not worsen but rather compensated in 40 weeks KK mice. Our data indicate that the use of male KK mice as an elderly obese and diabetic mouse model has some limitations and in order to represent a useful elderly obese and diabetic animal model, it may be necessary to induce deterioration of glucose and lipid metabolism in KK mice through breeding with high-sucrose or high-fat diets.

Key words: age-related changes, glucose metabolism, KK strain, lipid metabolism, model mouse

Introduction

The current aging population has caused considerable problems to the health economy as there has been a concomitant increase in the prevalence of age-related metabolic diseases such as obesity, diabetes mellitus, dyslipidemia, hypertension, atherosclerosis, and cardiovascular disease [1–3]. Thus, research into the prevention and treatment of these diseases is important to be able to maintain patients' quality of life [4]. To address these problems, *in vitro* and *in vivo* studies, as

well as clinical research, are necessary. In the field of *in vivo* studies, appropriate animal models must be used to achieve adequate pathophysiological characterization.

The elderly diabetic model mouse is expected to be useful for studies into aging because of pathophysiological characterization, such as deterioration of glucose and lipid metabolism, associated with spontaneous aging. The KK mouse strain is commonly used as an animal model of moderate obesity and type 2 diabetes (T2D) as it displays insulin resistance and glucose intolerance [5]. This strain is well characterized; in particular, it is a

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polygenic animal model which closely reflects obesity and non-insulin-dependent diabetes mellitus through features such as polyuria, glucose intolerance, and hyperinsulinemia, which—in humans—are profoundly affected by the interaction between environment and multiple gene defects [6–9]. However, it remains controversial whether the KK mouse is appropriate for research into age-related glucose and lipid metabolic abnormalities.

Obesity is observed in both male and female KK mice; however, the manifestations of T2D are more severe in male than female mice [7]. In male KK mice fed with commercial pet food containing 19% protein and 11% fat, urinal sugar levels indicate that T2D appears by 3 months of age but begins to spontaneously improve at 10 months of age, almost disappearing after 12 months of age [10]. These findings suggest that KK mice experience partial remission of T2D (at least glycosuria) with increasing age. Therefore, the utility of elderly KK mice as an animal model for age-related metabolic diseases including obesity, T2D, and dyslipidemia has its limit. It is also unclear whether the pathophysiological features of elderly KK mice are comparable with that of aged humans.

The present study aimed to provide further basic characterization of elderly KK mice and evaluate the suitability of this strain as an elderly obese and diabetic model mouse for research into aging including glucose and lipid metabolism. We assessed age-related changes in phenotype and gene expression in insulin-response organs of both young and elderly male KK mice relative to C57BL/6J (B6) mice as a lean control under standard laboratory chow-feeding conditions.

Materials and Methods

Animals and diets

We used C57BL/6Jcl (B6) and KK/TaJcl (KK) male mice aged 6 weeks (CLEA Japan, Tokyo, Japan) in the present study. Mice were housed in individual cages with free access to water and commercial chow diet (CE2; CLEA Japan) in an animal room (Josai University Life Science Center) under a controlled 12-h light-dark cycle, at a temperature of $22 \pm 2^\circ\text{C}$, and humidity of $55 \pm 10\%$. At 7 weeks of age, all mice were weighed and divided into four groups ($n=5/\text{group}$) based on body weight: B6 mice were divided into young (BL/Y) and old (BL/O) groups; KK mice were similarly divided into KK/Y and KK/O groups. The young and old groups were fed a CE2 diet for 8 and 33 weeks, respectively. During the experimental period, we monitored food intake, body weight, and blood biochemical parameters. Food intake

(g/mouse/week) was represented by average amount of a mouse per week. The present study was performed in accordance with the “Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions” (Ministry of Education, Culture, Sports, Science and Technology, Japan, Notice No. 71, dated June 1, 2006) and approved by the Animal Care and Use Committee of the Josai University.

Biochemical parameters

To evaluate age-dependent changes in blood glucose, plasma insulin, and oxidative stress in B6 and KK strains, blood samples were collected every 4 weeks from the tail vein after 12-h overnight fasting. Blood glucose levels were immediately measured using a blood-glucose-monitoring system (One Touch Ultra; Johnson & Johnson), then the blood sample was centrifuged at $900 \times g$ for 10 min to separate plasma and stored at -80°C until analysis. We performed the diacron of reactive oxygen metabolites (d-ROMs) test using the Free Radical Elective Evaluator (Diacron International, Grosseto, Italy) with plasma samples and commercial kits (Diacron International). Plasma d-ROMs levels are expressed as arbitrary units (U. CARR), with 1 U. CARR equivalent to 0.08 mg/dl hydrogen peroxide (H_2O_2). The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) index was calculated using the following formula: fasting blood glucose (mg/dl) \times fasting plasma insulin ($\mu\text{U}/\text{ml}$)/405.

Sample collection and measurement of biochemical parameters

At the end of the experimental period, young (15 weeks of age) and old (40 weeks of age) 12-h overnight-fasted mice were euthanized by intraperitoneal injection of pentobarbital sodium (Kyoritsuseiyaku, Tokyo, Japan), weighed, and blood glucose levels measured as described above. Blood samples were collected from the inferior vena cava using a heparin-anticoagulated syringe attached to a needle and centrifuged as above to separate plasma. The livers, epididymal white adipose tissue (WAT), interscapular brown adipose tissue (BAT), and gastrocnemius muscles were subsequently removed, weighed, and frozen in liquid nitrogen. Additionally, the pancreases were removed and fixed for histological and immunohistochemical analysis. Plasma and tissue samples were stored at -80°C until analysis. Plasma insulin levels were measured using an insulin ELISA kit (Moriguchi Institute of Biological Science, Tokyo, Japan). Plasma adiponectin levels were measured using a mouse/rat adiponectin ELISA kit (Otsuka Pharmaceutical, To-

kyo, Japan). Hepatic total lipid levels were evaluated using the method described by Folch *et al.* [11]. Triacylglycerol (TG) levels in the plasma and liver were measured by Wako E-Test kit (Wako Pure Chemical Industries, Tokyo, Japan).

Morphological analysis

For histopathological and morphometric analyses, epididymal WAT and pancreas tissues were fixed in 10% neutral-buffered formalin (Wako Pure Chemical Industries), embedded in paraffin, and stained with hematoxylin and eosin (H&E) by Kotobiken Medical Laboratories (Tokyo, Japan). The mean adipocyte size of each group was evaluated by evaluating 4–11 randomly chosen fields of epididymal WAT specimens. Adipocyte size was measured by analyzing more than 1,000 cells per group. The mean islet area (μm^2) and distribution was determined from H&E-stained sections using ImageJ software, version 1.52a (Wayne Rasband, NIH). Immunohistochemical staining of glucagon and insulin in pancreas was also performed by Kotobiken Medical Laboratories using anti-glucagon and anti-insulin antibody (Takara Bio, Shiga, Japan). Antibodies used for immunohistochemical staining are described in Supplementary Table 1. The estimated percentages of glucagon- and insulin-stained areas were calculated as follows: glucagon- or insulin-positive area (μm^2)/islet area (μm^2) \times 100. More than 14 visual fields of pancreatic tissue sections and an average of 54 islets were analyzed per group. In detail, quantitative analyses of pancreas samples were performed according to our previously published protocols [12].

Immunoblot analysis

Tissue samples were homogenized by lysis buffer with a protease inhibitor cocktail (cOmplete Mini; Roche, Mannheim, Germany) then centrifuging at $18,000 \times g$ at 4°C for 30 min. Proteins (25–50 μg) in the supernatant were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred on to polyvinylidene fluoride membranes. Membranes were blocked with 5% skimmed milk for 1 h at room temperature then probed with primary antibodies against glucose transporter 4 (GLUT4; Cell Signaling Technology, Beverly, MA, USA), adipose triglyceride lipase (ATGL; Proteintech Group, Rosemont, IL, USA), hormone-sensitive lipase (HSL; Proteintech Group), uncoupling protein 1 (UCP 1; Abcam, Cambridge, UK), cytochrome c oxidase IV (COX IV; Proteintech Group), β -actin (Cell Signaling Technology), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Proteintech Group), then incubated with horseradish peroxidase-conjugated anti-rabbit and

anti-mouse immunoglobulin G (IgG; Cell Signaling Technology). Detailed information of antibodies is described in Supplementary Table 2. Target proteins were detected using a chemiluminescence reagent (Clarity™ Western ECL Substrate; Bio-Rad) and captured with Lumicube (Liponics, Tokyo, Japan). The chemiluminescence intensity of bands was quantified using ImageJ software, version 1.52a (Wayne Rasband, NIH) and presented as fold-change of the mean value of the BL/Y group.

Real-time polymerase chain reaction

Total RNA was isolated from liver and WAT using the TRIzol® reagent (Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. One-step quantitative real-time polymerase chain reaction (RT-PCR) was performed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) with QuantiTect SYBR Green RT-PCR kits (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Thermal cycling conditions were as follows: 1 cycle of reverse transcription at 50°C for 30 min; initial activation at 95°C for 15 min; then 40 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for one min. Primer sequence is shown in Supplementary Table 3. We analyzed β -actin for normalization [13, 14]. Data were analyzed using the $2^{-\Delta\Delta\text{CT}}$ method and shown as fold-change of the mean value of the BL/Y group.

Statistical analysis

Data are presented as mean \pm SE. A Student's paired *t*-test was used to evaluate successive changes in each group. Comparisons between the young and old groups of the same strain were carried out using a Student's unpaired *t*-test. We defined *P* values of <0.05 as statistically significant. All statistical analyses were performed using the Microsoft Excel software.

Results

Liver and epididymal WAT weights and food intake decreased with age, while body weight did not change in KK mice

Figure 1 illustrates the changes in food intake and body weight during the experimental period. In the first week of the experiment (7 weeks of age), the food intake of the BL/O group was 27.9 ± 0.4 g, which remarkably decreased between 21 and 31 weeks of age. The difference in the food intake of the BL/O group at 40 weeks of age (27.5 ± 0.4 g) compared with that at 7 weeks of age was not significant. In the KK/O group, the food

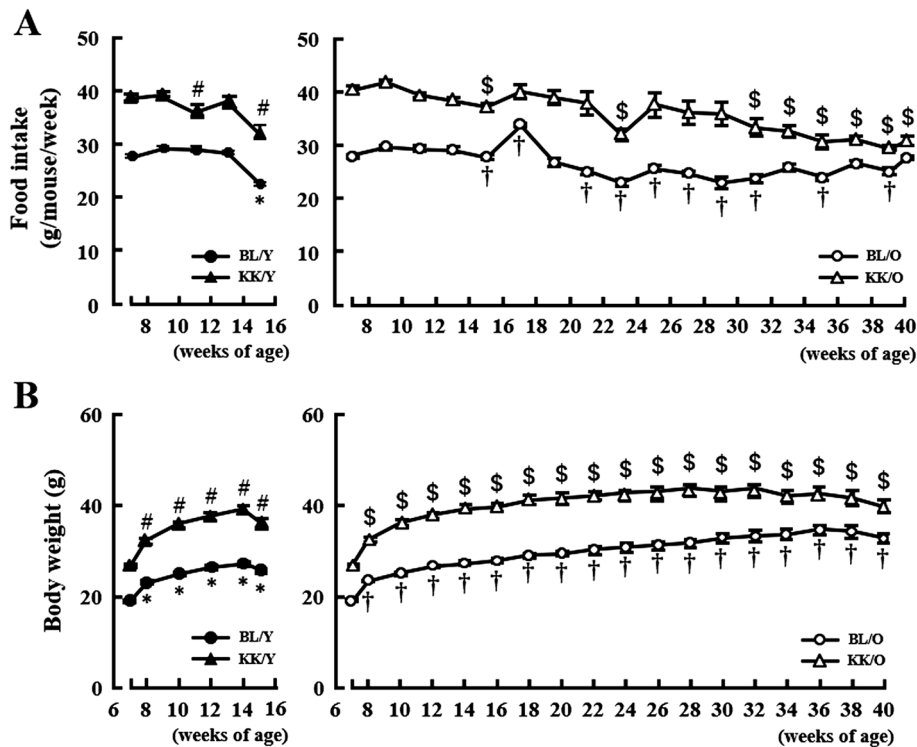


Fig. 1. Changes of food intake (A) and body weight (B) in C57BL/6J and KK strains with aging. Data are represented as mean \pm SE (n=5). * P <0.05 compared to 7 weeks of age in BL/Y group, † P <0.05 compared to 7 weeks of age in BL/O group, # P <0.05 compared to 7 weeks of age in KK/Y group, and \$ P <0.05 compared to 7 weeks of age in KK/O group by Student's paired t -test.

Table 1. Body weight and tissue weight in C57BL/6J and KK strains at 15 and 40 weeks of age

Group	BL/Y	BL/O	KK/Y	KK/O
Initial body weight (g)	19.0 \pm 0.2	19.0 \pm 0.1	26.7 \pm 0.5	26.7 \pm 0.4
Final body weight (g)	24.8 \pm 0.6	32.6 \pm 1.0*	36.2 \pm 1.1	39.1 \pm 1.2
Liver weight (g/100g BW)	4.23 \pm 0.07	3.97 \pm 0.11	4.13 \pm 0.10	3.55 \pm 0.04#
Epididymal WAT weight (g/100g BW)	1.41 \pm 0.20	3.74 \pm 0.49*	3.44 \pm 0.09	2.28 \pm 0.21#
BAT weight (g/100g BW)	0.23 \pm 0.02	0.29 \pm 0.02	0.54 \pm 0.04	0.58 \pm 0.05
Gastrocnemius weight (g/100g BW)	0.88 \pm 0.05	0.95 \pm 0.08	0.52 \pm 0.03	0.60 \pm 0.02

Data are represented as mean \pm SE (n=5). * P <0.05 compared to BL/Y group and # P <0.05 compared to KK/Y group by Student's unpaired t -test.

intake was 40.2 ± 0.7 g at 7 weeks of age and gradually decreased to reach 30.8 ± 0.9 g at 40 weeks of age. In both B6 and KK mice, body weight increased with age, until the end of the experiment.

Table 1 shows the body and tissue weights for each strain at time points between 15 and 40 weeks of age. Although the final body weight was significantly higher in the BL/O group than the BL/Y group, there was no difference between the KK/Y and KK/O groups. However, the liver and epididymal WAT weights of KK/O group were significantly lower than KK/Y group. The BAT and gastrocnemius weights of young and old groups were not significantly different in either B6 or KK mice.

The KK strain exhibited diabetic features after 15 weeks of age with increased oxidative stress

Figure 2 illustrates the changes over time in fasting blood glucose, insulin resistance, and plasma reactive oxygen metabolites during the experimental period. Compared with the beginning of the experiment (7 weeks of age), blood glucose levels of KK mice significantly increased between 11 and 19 weeks of age. The blood glucose levels of B6 mice were higher at 15 and 31–39 weeks of age compared with 7 weeks of age. Although the plasma insulin level and HOMA-IR index of B6 mice did not largely change throughout the experiment, these parameters were obviously higher in KK mice at 19, 27, and 39 weeks of age compared with 7 weeks of age.

The d-ROMs test is simple method of measuring the

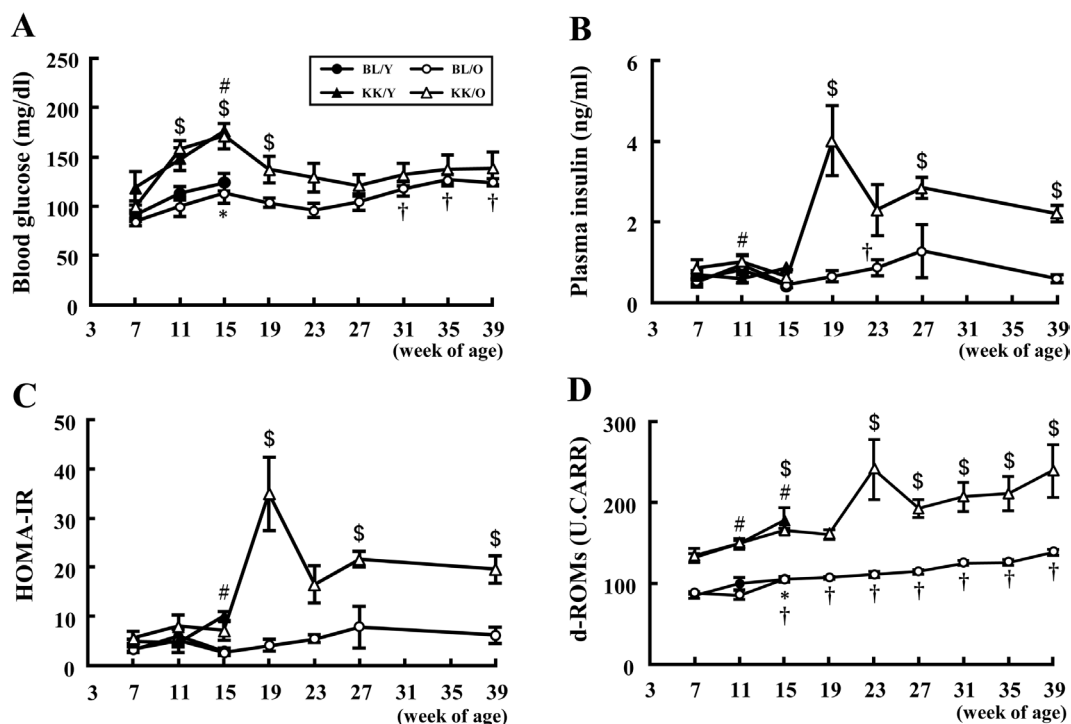


Fig. 2. Changes of blood glucose (A), plasma insulin (B), HOMA-IR index (C), plasma d-ROMs (D) in C57BL/6J and KK strains with aging. Data are represented as mean \pm SE (n=4–5). * P <0.05 compared to 7 weeks of age in BL/Y group, † P <0.05 compared to 7 weeks of age in BL/O group, # P <0.05 compared to 7 weeks of age in KK/Y group, and \$ P <0.05 compared to 7 weeks of age in KK/O group by Student's paired t -test.

Table 2. Biochemical parameter in C57BL/6J and KK strains at 15 and 40 weeks of age

Group	BL/Y	BL/O	KK/Y	KK/O
Blood glucose (mg/dl)	202 \pm 22	140 \pm 10*	217 \pm 34	146 \pm 15
Plasma insulin (ng/ml)	0.28 \pm 0.05	0.55 \pm 0.04*	0.69 \pm 0.12	3.51 \pm 0.76#
HOMA-IR	3.8 \pm 0.8	5.0 \pm 0.6	10.0 \pm 2.7	32.7 \pm 8.6#
Plasma adiponectin (μ g/ml)	12.3 \pm 0.2	14.7 \pm 0.8*	9.2 \pm 0.3	7.7 \pm 0.3#
Plasma TG (mg/dl)	45.2 \pm 3.6	57.0 \pm 4.9	106.7 \pm 12.0	91.9 \pm 4.7
Hepatic TG (mg/g liver)	27.7 \pm 3.1	48.7 \pm 7.1*	37.0 \pm 3.7	20.8 \pm 5.6#

Data are represented as mean \pm SE (n=5). * P <0.05 compared to BL/Y group and # P <0.05 compared to KK/Y group by Student's unpaired t -test.

free radical levels *in vivo* and a high correlation with other oxidative stress markers [15]. At the beginning of the experiment, plasma d-ROMs levels of the BL/O and KK/O groups were 88.6 ± 2.2 and 134.8 ± 8.4 U. CARR, respectively. At the end, these levels had increased significantly to 138.8 ± 3.9 and 239.4 ± 33.1 U. CARR, respectively.

Table 2 shows the results of analysis of the parameters that were used to evaluate severity of T2D at 15 and 40 weeks of age. Fasting blood glucose levels were significantly lower in the BL/O and KK/O groups than the BL/Y and KK/Y groups. And, the plasma insulin levels of the BL/O and KK/O groups were significantly increased compared with the BL/Y and KK/Y groups, by 96% and 409%, respectively. The HOMA-IR index was significantly higher in the KK/O group than the KK/Y

group, although there was no difference between the BL/Y and BL/O groups. In contrast to the B6 strain, plasma adiponectin levels of KK strain were significantly decreased at 40 weeks of age compared with 15 weeks of age.

In the present study, there were differences in glucose levels at 15 weeks of age between Fig. 2A (BL/Y, 124 ± 9 mg/dl; KK/Y, 176 ± 11 mg/dl) and Table 2 (BL/Y, 202 ± 22 mg/dl; KK/Y, 217 ± 34 mg/dl). This may be related to the presence or absence of anesthesia by pentobarbital sodium before the blood sampling.

The KK strain exhibited pancreatic hypertrophy at 40 weeks of age

Figure 3 illustrates morphological differences of the pancreatic islets that were observed in B6 and KK mice

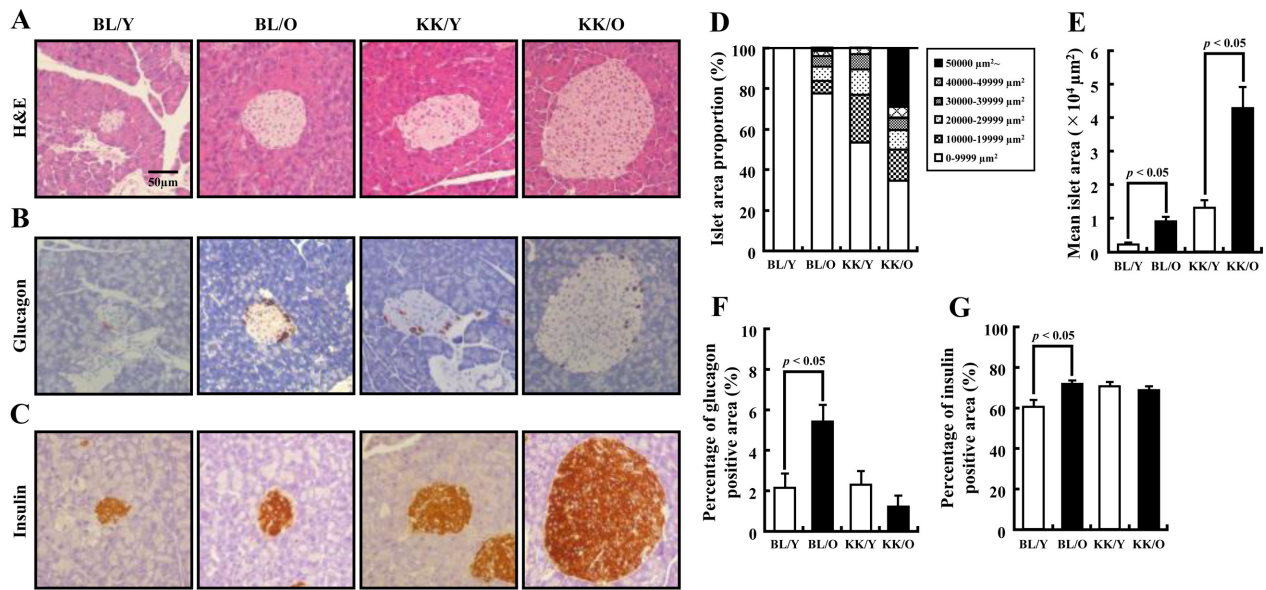


Fig. 3. Islet images of H&E staining (A), glucagon immunostaining (B), insulin immunostaining (C), mean islet area (D), islet area proportion (E), percentage of glucagon positive area (F), percentage of insulin positive area (G) in C57BL/6J and KK strains at 15 and 40 weeks of age. Data are represented as mean \pm SE (n=5). The images of stained section were captured under a microscope at 200-fold magnification (scale bar, 50 μm). Comparison of parameters between young and old groups in an identical strain was analyzed by Student's unpaired *t*-test.

between 15 and 40 weeks of age. In the KK/O group, there were many hypertrophic pancreatic islets ($>50,000 \mu\text{m}^2$) and fewer small islets ($<9,999 \mu\text{m}^2$) compared with others. The mean islet areas of the KK/O and BL/O groups were significantly larger than the KK/Y and BL/Y groups, respectively. Specifically, the mean islet area of the KK/O group was approximately three times larger than that of the KK/Y group. Immunohistochemical analysis revealed glucagon and insulin expression to be significantly increased in the BL/O group compared with the BL/Y group; however, these were not significantly different between the KK/Y and KK/O groups.

Hepatic triglyceride content decreased with age while lipogenesis was enhanced in KK mice

Parameters related to lipid metabolism are shown in Table 2 and Fig. 4. Plasma TG levels were not significantly different in either B6 or KK mice. Hepatic TG levels were significantly lower in the KK/O group compared with the KK/Y group, while those of the BL/O group were higher than BL/Y group.

Figure 4 shows the results of analysis on hepatic gene expression at 15 and 40 weeks of age. In the gene expressions of lipogenesis-related enzyme, the levels of acetyl-CoA carboxylase (*ACC*), fatty acid synthase (*FAS*), and stearoyl-CoA desaturase-1 (*SCD-1*) mRNA levels tended to be high in BL/O group, but there were not significantly different between the BL/Y and BL/O groups. On the other hand, the *ACC* and *FAS* mRNA levels, except

for the *SCD-1* mRNA, were significantly higher in the KK/O group than the KK/Y group. In terms of fatty acid β -oxidation related, the levels of carnitine palmitoyl-transferase 1 (*CPT1*) and medium chain acyl-CoA dehydrogenase (*MCAD*) mRNA did not significantly change in either the B6 or KK strain. However, in BL/O group, the levels of *CPT1* and *MCAD* mRNA tended to be high relative to the BL/Y group.

Fat accumulation and inflammation response in epididymal WAT improved with age whereas lipolysis-related protein expression decreased in KK mice

Figure 5 illustrates the results of morphological analysis, cytokine gene expression measurement, and expression of glucose- and lipid-metabolism-related proteins in epididymal WAT. Although the mean adipocyte size was significantly larger in the BL/O group than in the BL/Y group, the reverse was observed in the KK strain (Fig. 5B). In the BL/Y group, smaller adipocytes (2,500–3,600 μm^2) were frequently observed, while larger adipocytes ($>14,400 \mu\text{m}^2$) were not detected. The size distribution was shifted from a peak at 2,500–3,600 μm^2 in the BL/Y group to 8,100–10,000 μm^2 in the BL/O group. In the KK/O group, the percentage of larger adipocytes ($>14,400 \mu\text{m}^2$) was decreased compared with the KK/Y group (Fig. 5C).

In the gene expressions of adipose-derived cytokine, the level of tumor necrosis factor- α (*TNF- α*) mRNA was

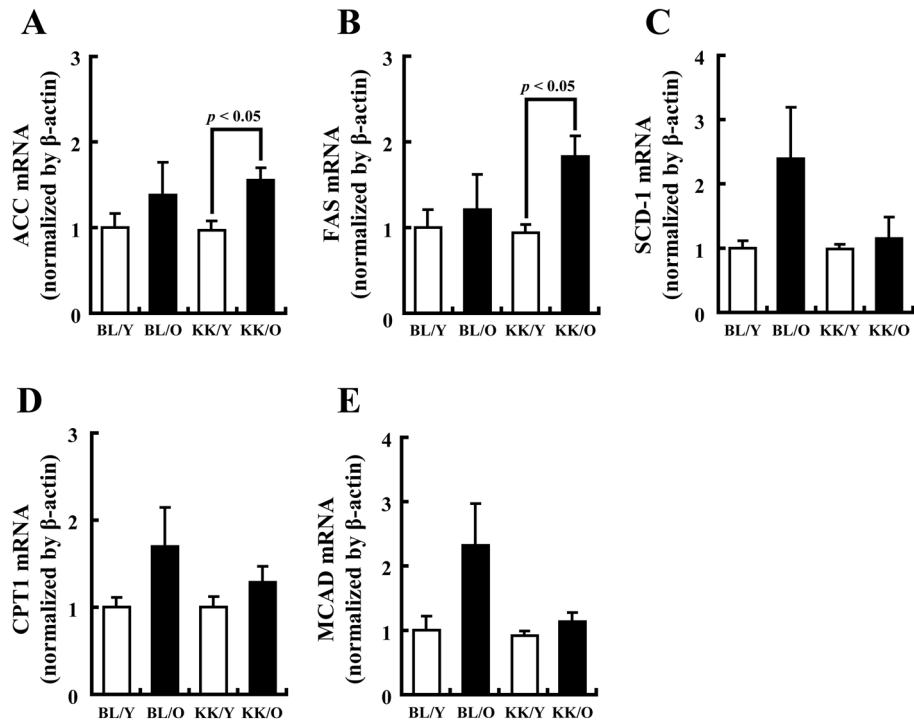


Fig. 4. mRNA expression levels of *ACC* (A), *FAS* (B), *SCD-1* (C), *CPT1* (D), and *MCAD* (E) in liver of C57BL/6J and KK strains at 15 and 40 weeks of age. Data are represented as mean \pm SE (n=5). Comparison of parameters between young and old groups in an identical strain was analyzed by Student's unpaired *t*-test.

also not significantly different between the BL/Y and BL/O groups. However, the *TNF- α* mRNA level of the KK/O group was significantly lower compared with the KK/Y group (Fig. 5D). The monocyte chemoattractant protein-1 (*MCP-1*) mRNA level was significantly higher in the BL/O group than the BL/Y group, although this was not significantly different between the KK/Y and KK/O groups (Fig. 5E).

There were no significant differences in the protein expression levels of ATGL or HSL between the BL/Y and BL/O groups; however, the ATGL expression was lower in KK/O group than KK/Y group ($P=0.0812$) (Fig. 5H). The expression of HSL was significantly lower in the KK/O group than the KK/Y group (Fig. 5I).

Glucose transporter 4 expression in epididymal white adipose, brown adipose, and gastrocnemius muscle tissues increased with age in B6 and KK mice

The expression of GLUT4 was significantly increased in epididymal WAT, BAT, and gastrocnemius muscle tissue of BL/O and KK/O mice compared with BL/Y and KK/Y mice, respectively. Comparison between B6 and KK strains revealed that GLUT4 expression in epididymal WAT, BAT, and gastrocnemius muscle tissue was over tenfold higher in the KK/Y group compared with the BL/Y group (Figs. 5G, 6B, and 6E).

Mitochondrial dysfunction of BAT was unaffected with age in B6 or KK mice

The major function of brown adipocytes is the uncoupling of the respiratory chain to produce heat via the mitochondrial UCP1, which dissipates energy and prevents lifestyle-related diseases such as obesity, T2D, and dyslipidemia [16]. As Fig. 6C shows, protein expression of UCP1 in BAT did not change significantly with age in either the B6 or KK strain in the present study.

Discussion

The KK strain was firstly established by Kondo [17], and its characterization as a diabetic animal model was described by Nakamura and Yamada [7]. Glycosuria is representative of the diabetic condition and is typically observed between 3 and 10 months of age, spontaneously disappearing after 12 months of age, in male KK mice [10]. In the present study, KK mice fed a CE2 diet (including 12% fat) were found to have the highest 12-h-fasting blood glucose levels (over 170 mg/dl) at 15 weeks of age, followed by a slow decrease. Akagiri *et al.* [18] reported that KK mice fed basal chow including 13% fat exhibited relatively low blood glucose levels (below 100 mg/dl) between 6 and 22 weeks of age. These results of blood glucose levels with KK mice under low-

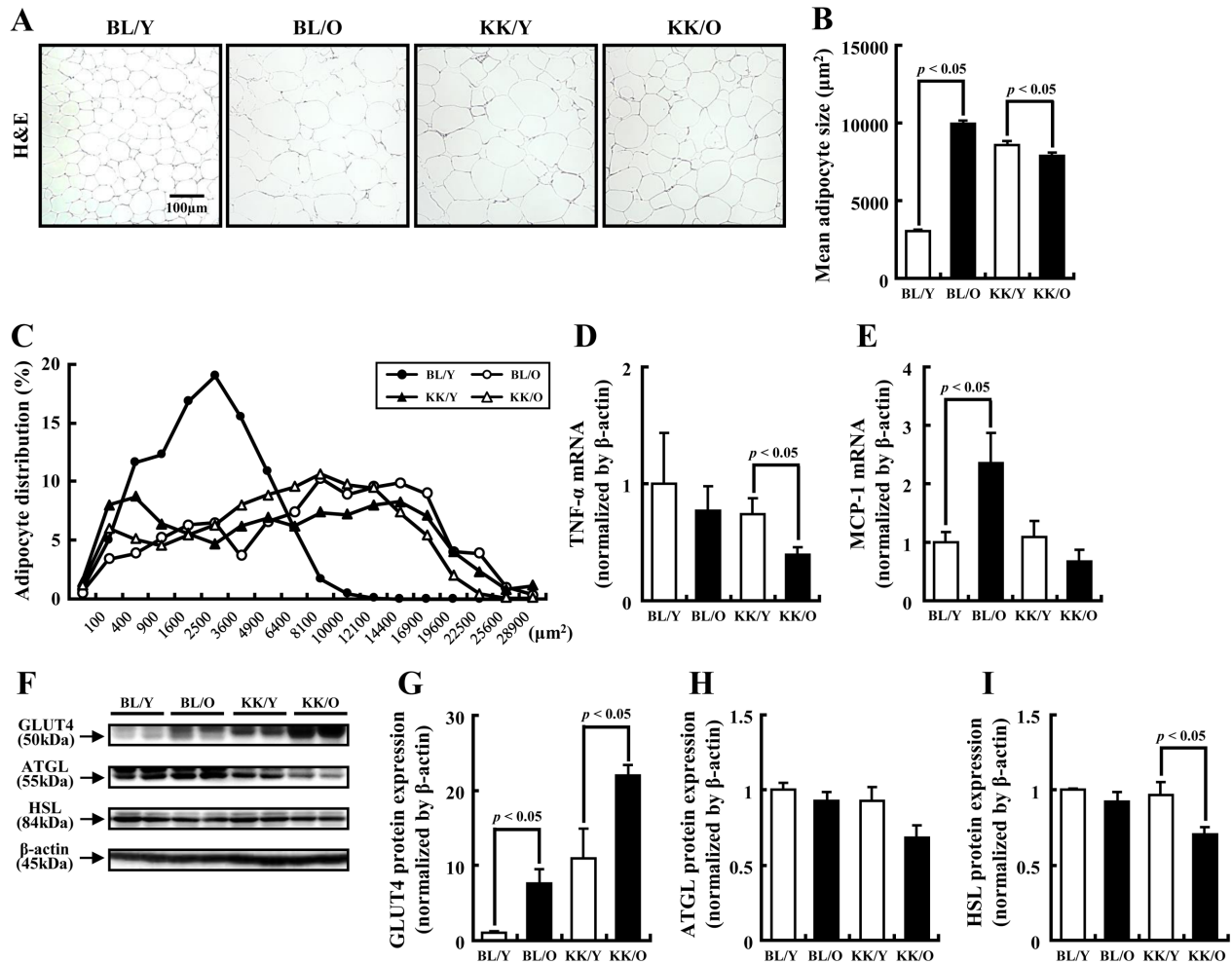


Fig. 5. Images of H&E staining (A), mean adipocyte size (B), adipocyte distribution (C), mRNA expression levels of *TNF-α* (D) and *MCP-1* (E), images of immunoblot analysis (F), and protein expression levels of GLUT4 (G), ATGL (H), and HSL (I) in epididymal WAT of C57BL/6J and KK strains at 15 and 40 weeks of age. Data are represented as mean ± SE (n=3–5). The images of stained section were captured under a microscope at 200-fold magnification (scale bar, 100 μm). Comparison of parameters between young and old groups in an identical strain was analyzed by Student's unpaired *t*-test.

fat feeding have been inconsistent.

Further investigations will be necessary in future studies.

Our data show the KK strain largely exhibited increased hyperinsulinemia and insulin resistance compared with the B6 strain between 19 and 39 weeks of age, suggesting that hyperglycemia did not worsen; however, the potential insulin resistance persisted in the aged KK mice. In the present study, age-induced insulin resistance and hypertrophy of the pancreatic islets were more frequently observed in the KK strain compared with the B6 strain. However, the proportion of the insulin-positive area did not change with aging in the KK strain. Tomita *et al.* [19] reported that obesity-related insulin resistance induced hypertrophy of islet cells and hyperinsulinemia. The increase of pancreatic islets with β-cell mass is the result of excess insulin biosynthesis due to peripheral insulin resistance, which induces

chronic activation of apoptotic pathways in β-cells and decreased β-cell mass [20, 21]. Consistent with these reports, we have previously demonstrated that the ratio of the insulin-positive area to total islet area was decreased to 60% in insulin-resistant male KK mice who were fed a 20% fat diet for 8 weeks [12]. These findings suggest that, although male KK mice experience an increase in pancreatic islets with β-cell mass due to insulin resistance by 40 weeks of age, this is not severe enough to impact on the survival rate of pancreatic β-cells when these mice are fed a low-fat diet.

Insulin resistance is characterized by obesity-induced excess of adipose tissue associated with an increase in adipocyte hypertrophy. In this condition, the adipose tissue recruits bone marrow-derived macrophages via secretion of MCP-1. The macrophage-infiltrated adipose tissue represents an inflammatory state involving chronic secretion of inflammatory cytokines such as TNF-α

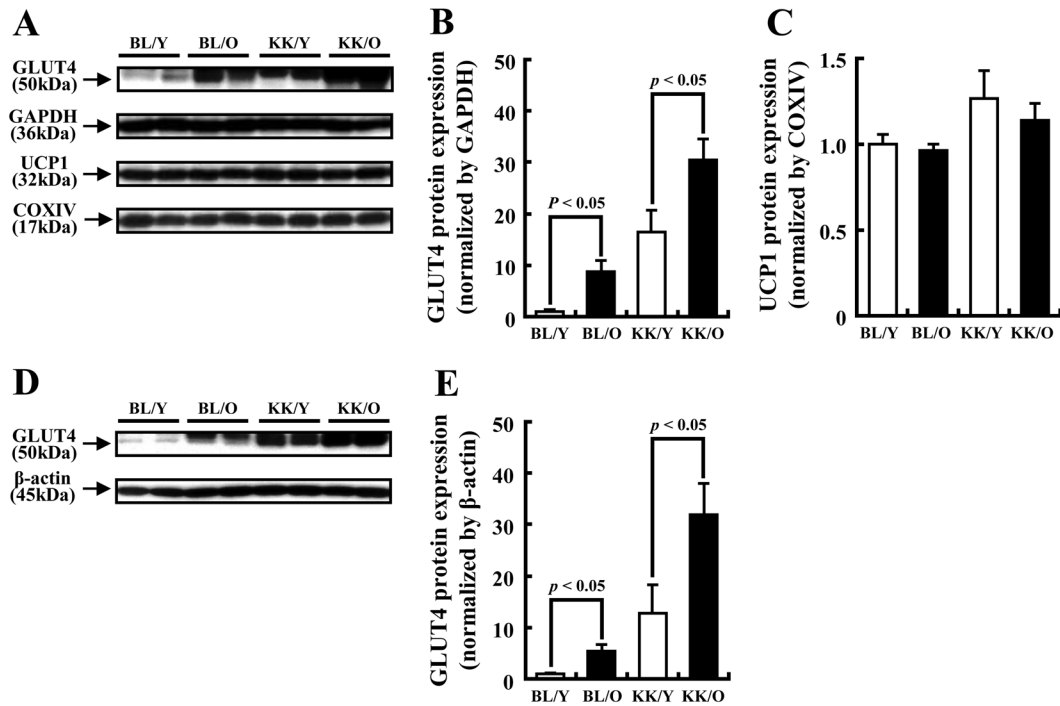


Fig. 6. Images of immunoblot analysis (A) and protein expression levels of GLUT4 (B), and UCP1 (C) in BAT, and images of immunoblot analysis (D) and protein expression level of GLUT4 (E) in gastrocnemius of C57BL/6J and KK strains at 15 and 40 weeks of age. Data are represented as mean \pm SE (n=5). Comparison of parameters between young and old groups in an identical strain was analyzed by Student's unpaired *t*-test.

and interleukin-1 β , which causes insulin resistance by impairing insulin signaling in insulin-sensitive organs [22]. Although adiponectin is an adipose-derived cytokine, it positively regulates insulin sensitivity of several organs [23]. Thus, reduced plasma adiponectin concentration is closely linked to the development of insulin resistance and T2D in humans and rodents [24]. Consistent with these findings, KK mice have been reported to develop insulin resistance in response to accumulation of visceral WAT, increased adipocyte size, and reduced plasma adiponectin levels at 22 weeks of age as a result of being fed a high-fat diet [18]. The present study also demonstrates that age-induced insulin resistance in KK mice was due to low plasma adiponectin level. Unexpectedly, these observations were independent of lipid accumulation in visceral fat/adipocytes and chronic inflammation in epididymal WAT. One explanation for this is that decreased energy intake after 25 weeks of age may contribute to anti-obesity events described above in KK mice. However, the mechanism underlying the reduction in obesity features in the context of insulin resistance in the KK strain remains unclear.

As the major glucose transporter of muscle and adipose cells, GLUT4 is key in insulin-mediated glucose uptake. We found that the expression of GLUT4 in epididymal WAT, BAT, and gastrocnemius muscle tissue of KK and B6 mice increases markedly with age. It has

been reported that glucose uptake in skeletal muscle under basal conditions declines with age in overnight-fasted Wistar rats and is associated with reduced GLUT4 expressions [25]. The basal amounts of GLUT4 and insulin sensitivity were also found to decrease with aging in epididymal WAT of Wistar rats [26]. In humans, the concentration of GLUT4 protein in skeletal muscle has also found to be negatively associated with age [27, 28]. Moreover, prolonged insulin exposure generates reactive oxygen species which activates transportation of GLUT4 to the lysosome for degradation in 3T3-L1 adipocytes [29]. These previous findings contradict our observations of aged KK mice, which suggest that these mice exhibited an increased GLUT4 expression in insulin-sensitive tissues. The present data indicate that aged KK mice may experience insulin resistance but hypersensitivity to glucose uptake by a high carbohydrate diet. It should be noted that the KK strain, at least in the potential glucose uptake of skeletal muscle, is different to elderly humans with T2D.

Aging is associated with obesity and metabolic dysfunction [30], and so we focused on the characterization of energy homeostasis, especially lipid metabolism, in the liver, WAT, and BAT. The liver plays an important role in lipid metabolism through regulation of lipogenesis and lipolysis. ACC and FAS are lipogenic enzymes which catalyzes a step of saturated fatty acid synthesis.

Our results showed that the *ACC* and *FAS* mRNA levels in liver increased with aging in KK mice. A similar finding was reported by Kuhla *et al.* [31], who demonstrated that senescence-accelerated mouse prone 8 mice expressed higher levels of hepatic *FAS* mRNA with increased age. Although fatty acid β -oxidation is a major pathway of hepatic lipolysis mediated by mitochondrial enzymes such as CPT1 and MCAD [32], aging was not found to affect these mRNA expressions in the liver of KK mice in the present study. In addition, hepatic TG decreased unexpectedly with age in KK mice. These findings suggest that some considerations, such as increased total amount of carbohydrate or fat, are required for using aged KK strain as an elderly obese and diabetic mouse model of aging.

WAT stores lipids in the form of TG, which are broken into fatty acids and glycerol by ATGL or HSL to be used as an energy source [33]. These enzymes play a central role in the mobilization of WAT TG stores. Mennes *et al.* [34] reported that male B6 mice exhibited increased epididymal WAT weight associated with reduced ATGL and HSL protein expression at 24 months compared with 11 weeks of age; however, the protein contents did not change with aging. In the present study, the ATGL and HSL expression in epididymal WAT of B6 mice was not significantly different at 40 weeks (10 months) compared with 15 weeks of age. In the KK strain, the expression of these proteins in epididymal WAT was decreased at 40 weeks of age, suggesting that lipolysis in epididymal WAT is impaired earlier in this strain compared with the B6 strain during aging. In addition, Narita *et al.* [35] demonstrated that calorie restriction decreases epididymal WAT weight but not protein expression of ATGL and HSL in Wistar rats at 9 months of age. Reduced food intake may therefore be independent of decreased lipolysis in the KK strain.

Although BAT activation is known to prevent age-related weight gain by increasing energy expenditure, BAT activity declines with age, with concomitant loss of mitochondrial function [30]. Some animal studies have addressed the association between dysfunction of BAT and aging. For example, male B6 mice fed standard laboratory chow have been reported to exhibit reduced UCP1 gene expression in BAT at 12 and 24 months of age relative to 4 months of age [36]. In contrast, both the mRNA and protein expression of UCP1 in the BAT of male *ob/ob* mice were not found to change significantly between 3.1 and 14.6 months of age [37]. These data suggest that reduced UCP1 expression in the BAT of obese mice occurs more slowly than in lean mice. The present study demonstrates that UCP1 protein expression in BAT did not change in either KK (obese) or B6 (lean)

mice with aging. Regarding UCP1 expression in BAT, the evaluation at 40 weeks of age may be insufficient for both strains fed a low-fat diet. Moreover, our results highlight the possibility that BAT may be less affected by aging than the liver and WAT in KK mice.

We have shown that the age-related dysfunction of glucose and lipid metabolism in aged KK mice, which includes the development of insulin resistance associated with hypertrophy of pancreatic islets and decreased lipolysis in WAT. However, hyperglycemia, potential glucose uptake in insulin-sensitive organs, hepatic lipid accumulation, hypertrophy of adipocytes, and inflammation in epididymal WAT did not worsen but rather improved in aged KK mice. On the other hand, several studies used a high-sucrose or high-fat diets to deteriorate an obese, insulin resistant, or hyperlipidemia-phenotype in KK mice at relatively young weeks of age [13, 18, 38]. In conclusion, our data indicate that the use of male KK mice as an elderly obese and diabetic mouse model has some limitations and in order to represent a useful elderly obese and diabetic animal model, it may be necessary to induce deterioration of glucose and lipid metabolism in KK mice through breeding with high-sucrose or high-fat diets.

Conflict of Interests

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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