Decreased Insulin Production and Increased Insulin Sensitivity in the *Klotho* Mutant Mouse, a Novel Animal Model for Human Aging

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We have recently identified a novel gene, *klotho* (*kl*), which may suppress several aging phenotypes. A defect of *kl* gene expression in the mouse results in a syndrome resembling human aging, such as arteriosclerosis, skin atrophy, osteoporosis, and pulmonary emphysema. To determine whether mouse homozygotes for the *kl* mutation (*kl*/*kl*) show abnormal glucose metabolism, an oral glucose tolerance test (OGTT) was performed at 6 to 8 weeks of age. Blood glucose levels during the OGTT were significantly lower in *kl*/*kl* mice versus wild-type mice. The insulin content of the pancreas was significantly lower in *kl*/*kl* mice compared with wild-type mice. Decreased insulin production was also supported by Northern blot analysis showing lower levels of insulin mRNA in *kl*/*kl* mice. To examine how lower blood glucose levels may exist in *kl*/*kl* mice despite decreased insulin production, insulin tolerance tests (ITTs) were performed. The glucose decline following insulin injection was more severe in *kl*/*kl* mice versus wild-type mice, suggesting that insulin sensitivity was higher in *kl*/*kl* mice versus wild-type mice. In *kl*/*kl* mice, an augmented expression of GLUT4 in skeletal muscle was demonstrated by both Northern blot analysis and Western blot analysis. Thus, we conclude that insulin production is decreased and insulin sensitivity is increased in the *klotho* mouse, a novel animal model for human aging.

A SERIES OF REPORTS have shown that aging is associated with impaired glucose tolerance and type 2 diabetes mellitus which may be caused by a decrease in insulin secretion and an increase in insulin resistance.[1-3] The reduction of insulin secretion with aging is attributed to a decreased responsiveness of the β cells to glucose stimulation.[4,5] However, the exact mechanisms by which insulin secretion decreases in older subjects remain ill defined because of a lack of a proper animal model for human aging.

Recently, we have established a new animal model for human aging that expresses several aging phenotypes such as arteriosclerosis, osteoporosis, skin atrophy, and pulmonary emphysema.[6] The *klotho* mouse was generated by an insertional mutation of a transgene through generating transgenic mice that overexpress the rabbit type-1 sodium-proton exchanger. The transgenic mice that did not express the transgene were independently mated to obtain any mutant mice that were homozygous for the transgene-inserted allele. One of these mice, now termed *klotho*, exhibited several phenotypes resembling human aging. As reported, the novel gene was cloned and encodes a membrane protein that shares sequence similarity with the β-glucosidase.

The precise mechanism by which the *klotho* gene product suppresses the aging syndrome is still ill defined. In this study, we sought to determine whether mutation of the *kl* gene is associated with abnormal glucose metabolism and insulin resistance as found in aged subjects.

MATERIALS AND METHODS

Animals

The mouse homozygote for the *klotho* mutation (*kl*/*kl*) and the heterozygote (*kl*/+) were used in this study. The wild-type (+/+) littermates were used as a control. All animals were maintained on regular rat chow and tap water in a specific pathogen–free facility at Gunma University, and were cared for according to the guidelines for animal care of Gunma University.

Oral Glucose Tolerance Test and Insulin Tolerance Test

An oral glucose tolerance test (OGTT) and intraperitoneal insulin tolerance test (ITT) were performed after 8 hours and 2 hours of fasting, respectively, at age 6 to 8 weeks. The OGTT was performed by oral administration of glucose (2 g/kg body weight) with determination of tail vein blood glucose and plasma immunoreactive insulin at 0, 30, 60, and 120 minutes by a glucose oxidase method using a Glutest E II (Sanwa Medical, Nagoya, Japan)[7] and by radioimmunoassay (RIA) using rat insulin as standard. The ITT was performed similarly, except for the peritoneal injection of human insulin (0.05 U/kg body weight) and sample times during the ITT at 0, 15, 30, 45, and 60 minutes after injection.

Insulin Measurement and Histopathology of the Pancreas

Male and female *kl*/*kl* mice aged 8 weeks were killed for measurement of the insulin and glucagon content of the pancreas and for histopathology together with age- and sex-matched *kl*/+ or +/+ mice. The pancreas was removed and dissected longitudinally. Half of the pancreas was frozen at −20°C and extracted with cold acidified ethanol, and the insulin and glucagon content of the pancreas was determined by RIA using rat insulin and human glucagon as standard, respectively.[8] Half of the pancreas was also fixed in Bouin's solution for immunopathological analysis. To detect insulin-producing β cells and glucagon-producing α cells of the pancreas, immunohistological examination was

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performed using anti-insulin antibody and anti-glucagon antibody, respectively (Japan, Kyoto, Japan) as previously described.[9]

Measurement of Growth Hormone, Thyrotropin, and Corticotropin

To determine the level of growth hormone (GH), thyrotropin (TSH), and corticotropin (ACTH), blood samples were obtained after 8 hours of fasting and these hormones were determined by RIA.

Expression of GLUT4 Protein in the Soleus Muscle

The soleus muscle was homogenized and subjected to centrifugation for 10 minutes at 1,200 × *g* (4°C) as reported by Klip et al.[10] The supernatant was used to assess GLUT4 by Western blot analysis as previously described.[11]

Northern Blot Analysis of GLUT4 and Insulin

Cellular total RNA was prepared from the soleus muscle and pancreas by the acid guanidium phenol chloroform procedure as described previously.[12] Total RNA (10 μg) was denatured with formaldehyde, separated by 1.2% agarose gel electrophoresis, and transferred onto a nitrocellulose filter. The *Dra*I-*Xba*I fragment of rat GLUT4 cDNA (nucleotides 143 to 1947) and full-length rat proinsulin cDNA were labeled with ^{32}P, and hybridization of the filters with probes was performed in 50% formamide, 5× SSC (1× SSC is 0.15 mol/L NaCl plus 0.015 mol/L sodium citrate), 5× Denhardt solution, 50 mmol/L sodium phosphate buffer, pH 7.4, and 1% sodium dodecyl sulfate at 42°C. The filters were washed and subjected to autoradiography.

Statistical Analyses

The data are presented as the mean ± SEM and were analyzed using the Statview 4.5 software package for Macintosh (Abacus Concepts, Berkeley, CA). One-way ANOVA and Fisher's protected least-significant difference (PLSD) test were performed for 3-group comparisons, and the Mann-Whitney *U* test was used for 2-group comparisons. *P* values less than .05 were statistically significant.

RESULTS

The Klotho Mouse Demonstrates a Hypoglycemic State

To determine glucose metabolism in *kl*/*kl* mice, an OGTT was performed after 8 hours of fasting (Fig 1). At the age of 8 weeks, blood glucose at 0, 30, 60, and 120 minutes after the glucose load in *kl*/*kl* mice was 71.0 ± 8.3, 116.7 ± 9.9, 64.3 ± 8.3, and 44.5 ± 3.8 mg/dL, respectively (n = 6 in each group). These were significantly lower than the values in +/+ mice (101.7 ± 6.8, 202.8 ± 16.6, 130.2 ± 15.5, and 105.2 ± 11.8 mg/dL, respectively, n = 6). Also, blood glucose levels in heterozygote (*kl*/+) mice were in an intermediate state and significantly higher at 60 and 120 minutes after the glucose load versus *kl*/*kl* mice (n = 6). Plasma insulin levels in *kl*/*kl* mice during the OGTT were too low to be detected constantly by a conventional RIA using rat insulin as standard, although plasma insulin at 0, 30, 60, and 120 minutes after the glucose load in +/+ mice was 3.0 ± 0.1, 4.2 ± 0.4, 3.4 ± 0.3, and 2.6 ± 0.3 ng/mL, respectively (n = 6).

Reduced Insulin Production of the Pancreas

The insulin content of the pancreas in *kl*/*kl* mice was 34.3 ± 3.3 mg/g pancreas, significantly lower versus *kl*/+ and +/+ mice (118.2 ± 19.5 and 171.6 ± 15.8 μg/g pancreas, $P < .01$ *v* *kl*/+ and $P < .01$ *v* +/+, respectively, n = 6 per group), and the insulin content of the pancreas was significantly lower in *kl*/+ versus +/+ mice ($P < .05$). In contrast, the glucagon content of the pancreas was not significantly different among these 3 groups (Fig 2). The reduced insulin production in the pancreas is supported by the Northern blot analysis (Fig 3). Histological findings showed that insulin-positive β cells in the islets of the pancreas were slightly diminished in number in *kl*/*kl* mice compared with +/+ mice, whereas glucagon-positive α cells seemed to be relatively increased in *kl*/*kl* mice (Fig 4).

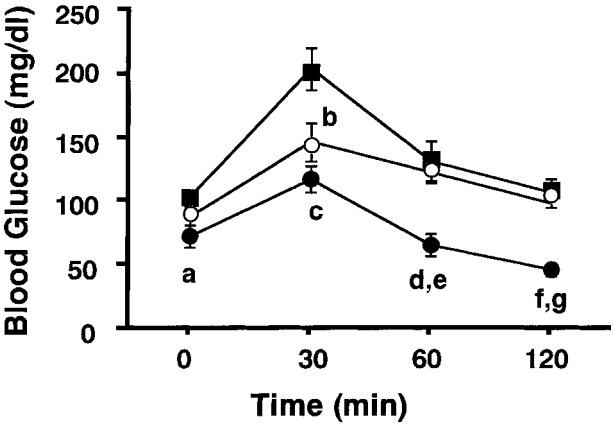

Fig 1. OGTT in *kl*/*kl* mice. *kl*/*kl* mice demonstrate a hypoglycemic state compared with *kl*/+ and +/+ mice. Results are the mean ± SEM for 6 animals and were analyzed by 1-way ANOVA and multiple-comparison methods of Fisher's PLSD. [a]*kl*/*kl* *v* +/+, $P < .01$; [b]*kl*/+ *v* +/+, $P < .01$; [c]*kl*/*kl* *v* +/+, $P < .01$; [d]*kl*/*kl* *v* +/+, $P < .01$; [e]*kl*/*kl* *v* *kl*/+, $P < .01$; [f]*kl*/*kl* *v* +/+, $P < .01$; [g]*kl*/*kl* *v* *kl*/+, $P < .01$. ■, +/+; ○, *kl*/+; ●, *kl*/*kl*.

The Klotho Mouse Demonstrates Increased Insulin Sensitivity

To determine how lower glucose levels may exist in *kl*/*kl* mice despite decreased insulin production, an ITT was performed. Blood glucose in *kl*/*kl* mice immediately decreased after insulin administration and this hypoglycemic state was maintained throughout the ITT at a dose of 0.05 U/kg, whereas blood glucose levels in +/+ mice did not significantly change at this insulin dose (Fig 5). When insulin was injected at a dose of 0.1 U/kg, blood glucose in *kl*/*kl* mice declined immediately, resulting in hypoglycemic coma or death, while levels in +/+ mice gradually decreased, suggesting enhanced insulin sensitivity in *kl*/*kl* mice compared with wild-type mice (data not shown).

Measurement of GH, TSH, and ACTH

To determine whether low blood glucose in *kl*/*kl* mice is related to the level of insulin counterregulatory hormones, some of the insulin counterregulatory hormones such as GH, TSH, and ACTH were measured after 8 hours of fasting when blood glucose levels in *kl*/*kl* and wild-type mice were 60.5 ± 7.8 and 109.0 ± 3.7 mg/dL, respectively. None of these hormones were lower in *kl*/*kl* mice versus wild-type mice (Table 1).

GLUT4 Expression in Skeletal Muscle

To understand the increased insulin sensitivity in *kl*/*kl* mice, we examined GLUT4 expression in the skeletal muscle of *kl*/*kl*

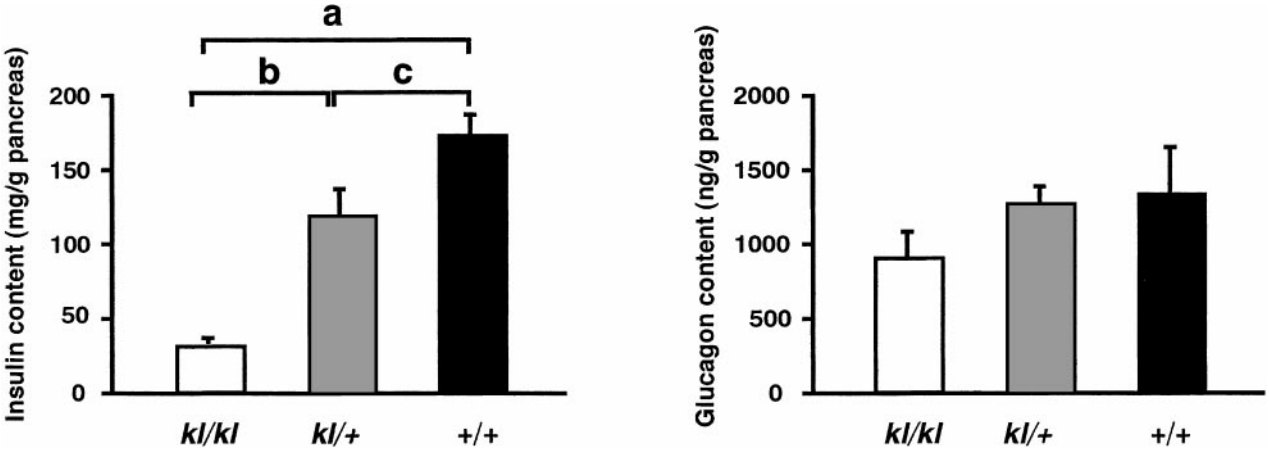

Fig 2. Insulin and glucagon content of the pancreas in *kl/kl* mice. Insulin and glucagon were determined by RIA, and data are the mean ± SEM for 6 animals. Mean values for insulin and glucagon were compared with 1-way ANOVA and PLSD. The insulin content of *kl/kl* mice was significantly lower *v kl/+* and *+/+* mice. [a]*kl/kl v +/+*, $P < .01$; [b]*kl/kl v kl/+*, $P < .01$; [c]*kl/+ v +/+*, $P < .05$. In contrast, the glucagon content of the pancreas was not significantly different in these 3 animals.

mice. Northern blot analysis using rat GLUT4 cDNA as a probe showed that the abundance of GLUT4 mRNA was increased in the soleus muscle of *kl*/*kl* mice compared with *kl*/+ and +/+ mice (Fig 6). In immunoblot analysis, GLUT4 protein was also increased in the soleus muscle of *kl*/*kl* mice compared with *kl*/+ and +/+ mice, when an equal amount of total membrane protein was applied to each lane (Fig 7).

DISCUSSION

In this study, we used the *klotho* mutant mouse (*kl*/*kl*), a novel animal model for human aging, to examine the hypothesis that a mutation of the *klotho* gene product leads to abnormal glucose metabolism and insulin resistance. We have demonstrated that *kl*/*kl* mice have decreased insulin production in the pancreas and increased insulin sensitivity.

Human studies have shown that insulin secretion decreases with aging, which is attributed to a decrease in glucose-stimulating insulin release.[13,14] In animal studies as well, old rats show a decrease in insulin secretion although the islets are large, the number of β cells per islet is high, and the insulin content in the islet is high in these old animals.[15,16] Several studies using animal models for type 2 diabetes, such as the GK rat[17] and the Akita mouse,[18] have shown that not only is insulin secretion impaired but pancreatic β cells are decreased in number and the insulin content of the pancreas is low in these animals. Thus, the *klotho* mutant mouse could be the first non-obese animal model for decreased insulin production coexisting with human aging–related disorders.

The precise mechanisms by which *kl*/*kl* mice have a reduction in the insulin production of the pancreas remain ill defined because the *klotho* protein has not been completely characterized yet. At the present time, there are several possible mechanisms. First, the *klotho* gene product may have insulinotropic actions. Histological studies demonstrate that pancreatic α cells seem to be unaffected, and the glucagon content of the pancreas was not different between +/+ and *kl*/*kl* mice. In

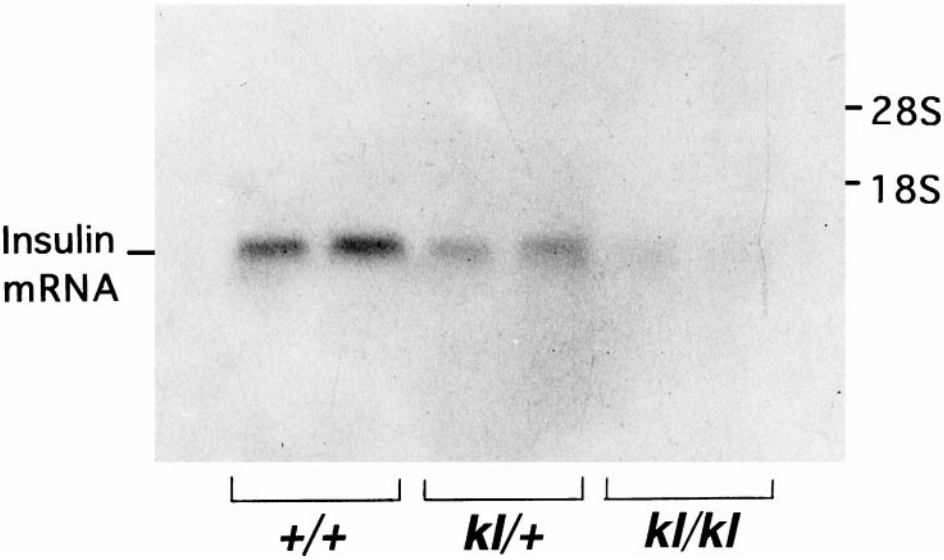

Fig 3. Northern blot analysis of insulin mRNA for the whole pancreas in *kl/kl* mice. Expression of insulin mRNA was low *v kl/+* and *+/+* mice.

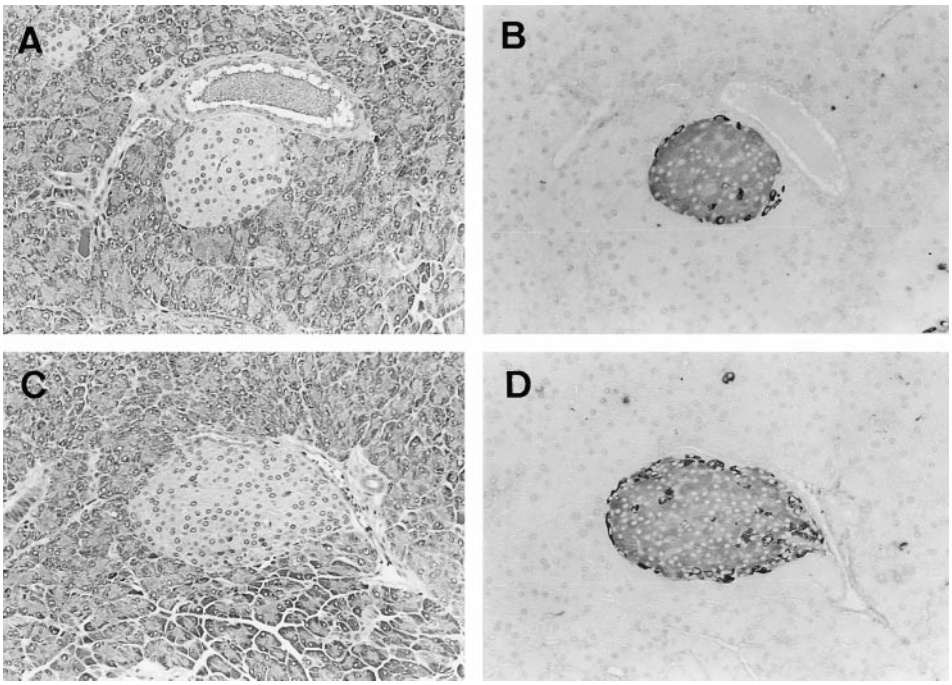

Fig 4. Histological findings in pancreatic islets of *kl/kl* mice at age 8 weeks. A and C, hematoxylin and eosin staining of an islet of the pancreas from +/+ or *kl/kl* mice, respectively. B and D, immunostaining with anti-insulin and anti-glucagon antibodies, respectively. Insulin-positive β cells are relatively few in *kl/kl* mice, whereas glucagon-positive α cells are relatively increased in *kl/kl* mice. Original magnification ×200.

contrast, serum insulin and the insulin content of the pancreas are remarkably low, suggesting that the *klotho* protein could specifically regulate the differentiation of β cells among the other endocrine cells in the pancreas. Second, the *klotho* protein may preserve the function of pancreatic β cells through an improvement of vascular endothelial function. As reported, nitric oxide production is impaired and the vasodilator response of the arteries to acetylcholine is attenuated in *kl*/*kl* mice.[19] Moreover, parabiosis of wild-type and heterozygous *klotho* mice results in a restoration of endothelial function in heterozygous *klotho* mice.[19] Since microvasculature forms the infrastructure of an islet of the pancreas and each islet cell is faced to both arterial and venous capillaries,[20] it is possible that decreased β-cell function in *kl*/*kl* mice is due to an impaired function of capillary endothelial cells in the islets. Third, reduced insulin production in *kl*/*kl* mice may result from increased insulin sensitivity. Exercise training leads not only to enhanced insulin sensitivity via an increase in GLUT4 expression in skeletal muscle but also to a reduction in insulin secretion.[21] The insulin requirement to maintain blood glucose is reduced in subjects after exercise.[22] However, this possibility is unlikely, because *kl*/*kl* mice did not show hyperactivity compared with *kl*/+ and wild-type mice. The fourth possibility is that hypoglycemia provides a reduced stimulation to β cells of the pancreas. As previously reported, glucopenia results in a decrease in endogenous insulin secretion.[23] Moreover, hypoglycemia produces a decrease in insulin gene expression but an increase in glucagon gene expression.[24]

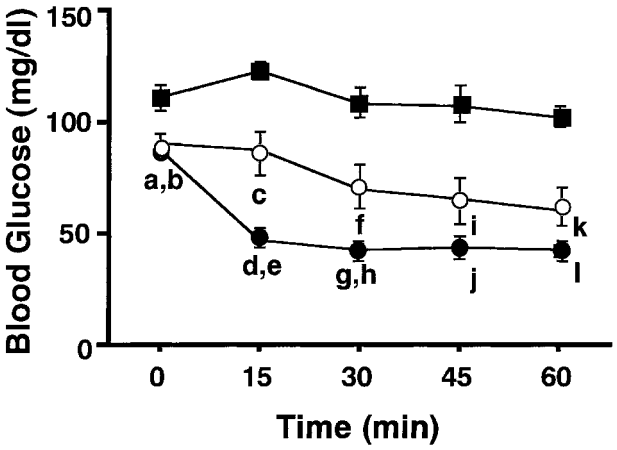

Fig 5. Intraperitoneal ITT in *kl/kl* mice. Insulin sensitivity was high in *kl/kl* mice *v kl*/+ and +/+ mice. Results are the mean ± SEM for 6 animals and were analyzed by 1-way ANOVA and multiple-comparison Fisher's PLSD. [a]*kl/kl v* +/+, $P < .01$; [b]*kl*/+ *v* +/+, $P < .01$; [c]*kl*/+ *v* +/+, $P < .01$; [d]*kl/kl v* +/+, $P < .01$; [e]*kl/kl v kl*/+, $P < .01$; [f]*kl*/+ *v* +/+, $P < .01$; [g]*kl/kl v* +/+, $P < .01$; [h]*kl/kl v kl*/+, $P < .05$; [i]*kl*/+ *v* +/+, $P < .01$; [j]*kl/kl v* +/+, $P < .01$; [k]*kl*/+ *v* +/+, $P < .01$; [l]*klkl v* +/+, $P < .01$. ■, +/+; ○, *kl*/+; ●, *kl/kl*.

Blood glucose in *kl*/*kl* mice was maintained at low levels throughout the OGTT. This glucopenia was not due to hyperinsulinemia or low levels of insulin counterregulatory hormones. As demonstrated in this report, serum insulin and the insulin

Table 1. Serum GH, TSH, and ACTH in *kl/kl* and +/+ Mice

Variable	*kl/kl*	+/+	*P*
GH (ng/mL)	15.5 ± 2.5 (4)	10.0 ± 1.4 (5)	.086
TSH (ng/mL)	2.4 ± 0.5 (4)	2.1 ± 0.3 (5)	.902
ACTH (pg/mL)	88.0 ± 11.5 (3)	46.3 ± 17.3 (3)	.127

NOTE. GH, TSH, and ACTH in serum were analyzed by RIA after 8 hours of fasting when blood glucose in *kl/kl* and wild-type mice was 60.5 ± 7.8 and 109.0 ± 3.7 mg/dL, respectively. None of these hormones were lower in *kl/kl v* wild-type mice. The number of mice is shown in parentheses.

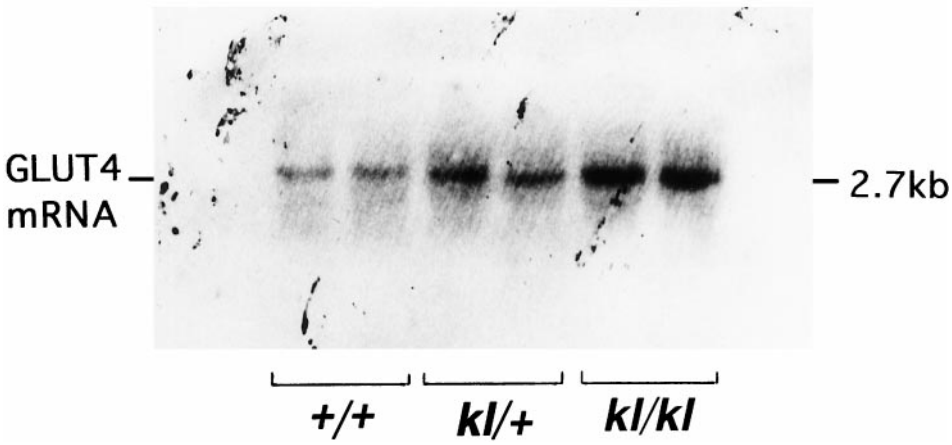

Fig 6. Northern blot analysis for GLUT4 expression in skeletal muscle of *kl/kl* mice. Expression of GLUT4 mRNA was increased *v* *kl/+* and *+/+* mice.

content of the pancreas were low in *kl*/*kl* mice, suggesting that insulin-independent processes for glucose uptake could contribute to the lower glucose levels observed in *kl*/*kl* mice. Glucose disposal occurs by both an insulin-dependent process and an insulin-independent process that interacts with insulin sensitivity in the peripheral tissue.[25] On the other hand, the levels of some insulin counterregulatory hormones such as GH, TSH, and ACTH were similar to those in wild-type mice. From these observations, glucopenia in *kl*/*kl* mice could be attributed to increased insulin sensitivity. Insulin sensitivity was evaluated by ITTs as previously reported.[26] When insulin was injected at a dose of 0.1 U/kg, blood glucose in *kl*/*kl* mice declined immediately, resulting in hypoglycemic coma or death, while blood glucose in +/+ mice gradually decreased, suggesting increased insulin sensitivity in *kl*/*kl* mice. This increased insulin sensitivity is explained in part by the increased expression of GLUT4 in the skeletal muscle of *kl*/*kl* mice.

The hypoglycemic condition is not uncommon in diabetic patients with advanced renal dysfunction, and may be associated with a decrease of caloric intake, a prolonged clearance of insulin, and a reduction of renal gluconeogenesis.[27] Renal function in *kl*/*kl* mice seems unaffected, because serum creatinine levels were normal and histological examination of the kidney was normal except for calcification of the small arteries in the kidney.[6] On the other hand, the expression of *klotho* mRNA is predominantly high in the kidney among different tissues in mice, rats, and humans.[6] Moreover, the expression of *klotho* mRNA was significantly low in the kidney of the Otsuka Long-Evans Tokushima fatty rat,[28] an animal model for type 2 diabetes mellitus and diabetic nephropathy.[29] From these studies, one would suspect that in diabetic patients with chronic renal dysfunction, the *klotho* gene product could be diminished, resulting in the development of systemic arteriosclerosis in these patients.

In conclusion, the *klotho* mutant mouse is a novel non-obese animal model for decreased insulin secretion and production coexisting with human aging–related diseases. Further studies to characterize the *klotho* protein show promise in understanding the relationship between aging and glucose metabolism.

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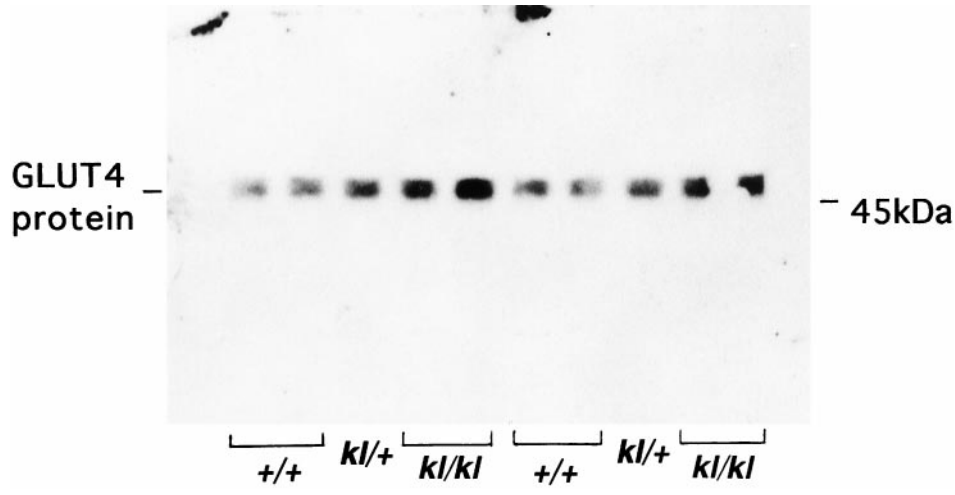

Fig 7. Western blot analysis for GLUT4 expression in skeletal muscle of *kl/kl* mice. Expression of GLUT4 protein in the soleus muscle was increased in *kl/kl* mice *v* *kl/+* and *+/+* mice.

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