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ORIGINAL CONTRIBUTION

Low-protein diet improves blood and urinary glucose levels and renal manifestations of diabetes in C57BLKS-*db/db* mice

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Abstract
Purpose Dietary protein content is related clinically to the development of diabetic nephropathy. Here, we investigated how dietary protein content (12–24 % energy) within the range used by humans affected renal manifestations including the expressions of genes involved in the renin-angiotensin (RA) system in control and diabetic mice. Moreover, we examined the effects of dietary protein content on HbA1c and urinary glucose.
Methods Control (CT) and leptin receptor-deficient obese (*db*) mice, 5 weeks old, were fed the diets below. Under ad libitum conditions, mice were fed 12, 18, and 24 % energy from protein (L-, M-, and H-diets) for 8 weeks. Under pair-feeding conditions, *db* mice were supplied H-diet (*db*-Hp) to the equivalent energy to that consumed by *db*-L mice. Renal manifestations and values related to glucose and insulin were examined biochemically and pathologically.
Results Under ad libitum conditions, *db* mice consumed food and water dose dependently of the dietary protein content, although they were consumed similarly by CT mice. CT-L mice showed lower urinary albumin and kidney weight, in association with lower mRNA levels of angiotensinogen and renin, than CT-H mice. Under pair-feeding conditions, *db*-L mice showed a lower ratio of kidney/body weight, $HbA1_C$, and urinary glucose, and a higher β-cell distribution rate in the pancreas than *db*-Hp mice.
Conclusions Low-protein intake in the range used by humans may relieve renal manifestations through the suppressed expression of genes in the renal RA system of CT mice. On the other hand, in *db* mice, low-protein intake improved hyperglycemia and the renal manifestations of diabetes.

Keywords Animal model · Diet therapy · Insulin insufficiency · Leptin receptor · Renin-angiotensin system

Abbreviations

ACE	Angiotensin I-converting enzyme
BW	Body weight
BUN	Blood urea nitrogen
Cr	Creatinine
FBG	Fasting blood glucose
PAS	Periodic acid-Schiff
RA system	Renin-angiotensin system
U-Alb	Urinary albumin

Introduction

During the past several decades, the prevalence of type 2 diabetes has been rising steadily in most parts of the world

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[1, 2]. Moreover, diabetic nephropathy, one of the complications of diabetes, is increasing [3–5]. Although diabetes and diabetic nephropathy have genetic predispositions, it is unlikely that the gene pool has changed appreciably over this short period of time [6, 7]. Thus, the increasing numbers of diabetes and diabetic nephropathy might be related to lifestyle rather than genetic factors. Lifestyle factors, including nutrition, have markedly altered in the past several decades [8, 9]; however, the impact of altered nutrition on the development of diabetes and diabetic nephropathy is not fully understood and remains controversial [2, 3].

Since 1986, the American Diabetes Association (ADA) has recommended that all diabetic patients should eat 0.8 g protein/kg body weight/day to protect against diabetic nephropathy, based on accumulated evidence from animal and human experiments [10–14]. Clinically, this intervention may sustain or improve measurements of renal function [11, 15]; however, dietary protein intake has not been fully monitored in diabetic patients in the early stage of nephropathy or without any renal manifestations [15]. In Asian countries, including Japan, people eat high-carbohydrate grains as a staple food. Thus, diabetic patients, especially Asian people, have been managed by a low-energy diet, resulting in relatively high-protein intake. There have been controversial reports that high-protein intake decreased [16–18] or increased [19, 20] insulin sensitivity in diabetic patients. It should be examined whether a low-protein diet is beneficial for patients with diabetes in the early stage; therefore, we examined how dietary protein contents affect renal manifestations and glucose levels under diabetic conditions in the early stage. To achieve this purpose, an appropriate experimental model was established.

To reveal the significance of a low-protein diet under type 2 diabetic conditions, there have been several reports using *db* mice, a leptin receptor-deficient mouse, as an animal model of type 2 diabetes and diabetic nephropathy [21–23]; however, the dietary protein content used might have been too high or low to evaluate the impact of the diet appropriately [2, 10–14]. Therefore, we set the range between 12 and 24 % protein content as the energy base, which is the range used in the regular diet of humans [2, 24–26]. Namely, a 12–24 % protein energy containing diet corresponds to the consumption of 0.9–1.8 g protein/kg of body weight (BW)/day, in the case of 30 kcal energy expenditure/kg of BW/day.

Recently, the effect of dietary protein, 12–20 % energy from protein, on *db* mice has been reported [27]. The objective of the study was to investigate the effects of protein types in the diet, such as animal protein and plant protein, on the renal manifestations and blood glucose levels of *db* mice. Unexpectedly, *db* mice fed 12 % plant protein showed weight loss under their experimental conditions, due to the possible deficiency of essential amino acids. More importantly, loss of fat free body mass must be avoided when eating a low-protein diet [10, 11]. *db* mice fed 12 % animal protein showed similar weight gain to mice fed 20 % animal or plant protein diet under their experimental conditions; therefore, we used a mixture of animal protein and plant protein. In addition, they measured blood glucose only, although it is necessary to measure biochemical parameters such as urinary glucose and HbA1c.

In the present study, we examined the impact of dietary protein composed of 50 % animal and 50 % plant protein sources within the range used by humans (12–24 % energy from protein in diet) on renal manifestations and measures related to glucose and insulin in *db* mice.

Materials and methods

Animals

Male diabetic [C57BLKS(BKS).Cg- $+Lepr^{db}/+Lepr^{db}$/J; *db*] mice, having a homozygous mutation of the leptin receptor, and control (BKS.Cg-Dock7^{m} +/Dock7^{m} +/J; CT, non-diabetic) mice at 4 weeks of age were purchased from Charles River Japan (Kanagawa, Japan) [28, 29]. The mice were housed individually for 1 week and received a standard diet (CE2: 29 % energy from protein composed of soy, fish, and yeast; CLEA Japan, Shizuoka, Japan). After one-week acclimation, the mice under ad libitum or pair-feeding conditions were fed the special diets (Table 1; Nosan Co., Kanagawa, Japan). AIN-76 vitamin and mineral mixture containing a relatively high content of phosphorus (0.40 g/100 g of the diet) was used to alleviate the influence of different phosphorus contents of the respective diets. Finally, L-, M-, and H-diets (100 g) contained 0.45, 0.48, and 0.51 g phosphorus, respectively (the calculation based on the Standard Tables of Food Composition in Japan, see supplementary Table 1). A humidity- and temperature-controlled (50 ± 10 %, 22 ± 2 °C) facility was used with a 12-h light/dark cycle (0700–1900 h). The mice had ad libitum access to water. The present study was approved (H20-079 and H21-009) by the Ethics Committee for Animal Experimentation at Kagoshima University.

Study design

For baseline experiments, 6 CT and 6 *db* mice purchased at 4 weeks of age were used. After one-week acclimation, the mice were killed. Under ad libitum conditions, 15 CT and 18 *db* mice acclimated for 1 week were randomly separated and then supplied with the respective diets (Table 1) for 8 weeks. The abbreviations for the respective diets are

Table 1 Composition of the diets used in the experiments

	Diets (g)		
	L	M	H
A			
Casein[a]	6.4	9.6	12.8
Soy protein[b]	6.4	9.6	12.8
Cornstarch[c]	49.6	43.1	36.6
Dextrinized cornstarch[d]	14.5	14.5	14.5
Sucrose[e]	10	10	10
(Sucrose from MX and VX)	(1.4)	(1.4)	(1.4)
Soybean oil[f]	8.25	8.25	8.25
Supplements			
L-cystine[g]	0.18	0.25	0.33
DL-methionine[g]	0.13	0.19	0.24
Mineral mix (MX)[h]	3.5	3.5	3.5
Vitamin mix (VX)[h]	1	1	1
Total[i]	100	100	100
Distribution of energy	Diets (% energy)		
	L	M	H
B			
Protein	12	18	24
Carbohydrates	71	65	59
Fat	17	17	17
Total	100	100	100
kcal/g	4.29	4.29	4.29

[a] Acid casein (Meggle Japan Co., Ltd)

[b] Soy flour (Nisshin Oillio Group, Ltd)

[c] Nisshoku Alstar E (Nihon Shokuhin Kako Co., Ltd.)

[d] Cornstarch W (Shikishima Starch Mfg. Co., Ltd.)

[e] Granulated sugar (Fuji Nihon Seito Co., Ltd)

[f] Daizu Hakko-yu (J-oil Mills Inc.)

[g] Sigma-Aldrich, Japan, Co., Ltd

[h] These mixtures were made of pure chemicals (Nosan Co., Ltd.)

[i] Rounded off to the second decimal place

as follows: 12 % (low protein, L), 18 % (moderate protein, M), and 24 % (high protein, H) of energy from protein sources composed of 50 % animal and 50 % plant protein (Table 1). Under pair-feeding conditions, 5 CT and 12 *db* mice were acclimated for 1 week. CT mice were supplied freely with L-diet. *db* Mice were randomly separated and then supplied with L- or H-diet (Table 1) for 10 weeks. It was expected that *db*-H mice would show growth retardation for 10 weeks under pair-feeding with the amount of food that CT-L mice consumed; therefore, *db* mice were supplied with H-diet (*db*-Hp) to the equivalent energy to that consumed by *db*-L mice. During the experiments, one *db* mice fed H-diet was euthanized due to anorexia. The BW, food and water intake were measured every week during 0700–0900 h. We measured the food consumption and water intake by subtracting the remaining values from the supplied values (weight), which we measured using a scale during the time indicated. On the last day of feeding experiments, the mice were anesthetized by pentobarbital (100 mg/kg) after 6-h fasting (0700-1300 h), and then, blood was taken from the heart. The blood (0.5 ml) was mixed with EDTA (10 µl of 200 mM). For glucagon measurement, 100 µl blood containing EDTA was mixed with 2 µl aprotinin (50 kallikrein inhibitor units; Sigma-Aldrich Japan, Tokyo, Japan). The blood was centrifuged, and then, the supernatant was stored at −80 °C. The organs, including the kidneys, heart, liver, and fat surrounding the epididymis, were weighed, quickly frozen by liquid nitrogen, and then stored at −80 °C until analysis. The pancreas was divided evenly in cross-section. The head, used for insulin measurement, was quickly frozen in liquid nitrogen and then stored at −80 °C until analysis. The caudal part for histological examinations underwent the fixation procedure described below.

Biochemical measurements

Fasting blood glucose (FBG), urinary glucose, and urinary albumin (U-Alb) were measured. For FBG at 5 weeks, blood was taken from the tail vein of mice fasting during 0700–1300 h and then measured with a glucose meter (Nipro, Tokyo, Japan). FBG, except at 5 weeks, and urinary glucose were measured by a commercial kit (glucose CII-test Wako; WAKO, Tokyo, Japan). Urine was taken during 0700-0900 h. Blood urea nitrogen (BUN) and creatinine (Cr) were measured by commercial kits (Urea BN test Wako, WAKO; CRE-EN, KAINOS Laboratories Inc., Tokyo, Japan). Phosphorus was measured by Dri-Chem 4,000 V (FUJIFILM Holdings Co., Tokyo, Japan). Leptin, insulin, glucagon, corticosterone, and albumin were

measured by the respective ELISA kits (R&D Systems, Minneapolis, MN; Morinaga Institute of Biological Science Inc., Kanagawa, Japan; Yanaihara Institute Inc., Shizuoka, Japan; Enzo Life Sciences Inc., Plymouth Meeting, PA; Exocell Inc., Philadelphia, PA). $HbA1_c$ was measured by an immunoassay (DCA 2000 system; Bayer Diagnostics, Elkhart, IN) [30]. The head of the pancreas was homogenized in acidic/ethanol solution (75 % ethanol, 23.5 % distilled water, and 1.5 % concentrated HCl; 1.8 ml/pancreas) with a Polytron homogenizer [31, 32]. The homogenates were stored at 4 °C for 16 h and centrifuged at 1,500*g* for 30 min at 4 °C. The supernatants were diluted to 1:20,000, and the insulin levels were measured by an ELISA kit (Morinaga).

Histological examinations

The right kidney and caudal part of the pancreas (divided evenly in cross-section) were used for histological examinations. They were fixed with 10 % phosphate-buffered formalin, dehydrated, embedded in paraffin, and sectioned at 5 μm. The kidney and pancreas were stained with periodic acid-Schiff (PAS) and Gomori trichrome [33], respectively, and then examined histopathologically as follows. Thirty glomeruli per kidney were quantitatively examined by software (IPAP-WIN; Sumika Technoservice Corporation, Hyogo, Japan). Extra-area (Bowman's capsule) and intra-area (glomerulus parenchyma) of the glomerulus were measured. PAS-positive area of the glomerulus was measured by the color intensity based on red-green-blue. In the pancreas, the β-cell distribution rate was calculated based on the following equation: [β-cell count/total cell count in the largest islet of pancreas] × 100 (%). β-cell was determined pathologically by Gomori trichrome staining.

Real-time PCR for quantification of mRNA

The left kidney was used for the isolation of total RNA [34]. Isolated RNA was treated with DNase treatment to remove genomic contamination. First-strand cDNA synthesis was performed using 5 μg total RNA with oligo-$(dT)_{20}$ primer following the manufacturer's instructions (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed using SYBR-green on a TAKARA detection system (TAKARA, Shiga, Japan) under the conditions recommended by the manufacturer. We measured mRNA levels of the genes involved in the development of diabetic nephropathy. The primary sequences of the genes are shown in supplementary Table 2 [35–37]. The relative level of mRNA was calculated using cycle time (Ct) values, which were normalized against the value of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Relative quantification (fold change) between

Table 2 Effects of diets on organ weights and blood parameters in CT and *db* mice under ad libitum conditions

Diet	CT				*db*			
	5 W	13 W			5 W	13 W		
	CE2	L	M	H	CE2	L	M	H
n	6	5	5	5	6	6	6	5
Organ weight (g)								
Kidney	0.125 ± 0.004	0.147 ± 0.003$^{\#,a}$	0.159 ± 0.006$^{\#,ab}$	0.166 ± 0.006$^{\#,b}$	0.138 ± 0.003*	0.169 ± 0.008$^{\#,*,\alpha}$	0.206 ± 0.008$^{\#,*,\beta}$	0.219 ± 0.015$^{\#,*,\beta}$
Liver	1.17 ± 0.04	0.99 ± 0.03$^{\#}$	1.01 ± 0.05$^{\#}$	1.00 ± 0.04$^{\#}$	1.79 ± 0.07*	1.97 ± 0.11*	1.89 ± 0.14*	1.75 ± 0.14*
Fat	0.11 ± 0.01	0.43 ± 0.03$^{\#}$	0.40 ± 0.03$^{\#}$	0.42 ± 0.03$^{\#}$	0.93 ± 0.03*	1.94 ± 0.09$^{\#,*}$	1.99 ± 0.09$^{\#,*}$	1.79 ± 0.18$^{\#,*}$
Heart	0.086 ± 0.002	0.124 ± 0.017$^{\#}$	0.133 ± 0.027$^{\#}$	0.127 ± 0.013$^{\#}$	0.095 ± 0.002*	0.128 ± 0.014$^{\#}$	0.137 ± 0.015$^{\#}$	0.136 ± 0.014$^{\#}$
Blood parameters								
BUN (mg/dl)	22.2 ± 0.7	13.1 ± 0.4$^{\#}$	15.0 ± 0.9$^{\#}$	15.1 ± 0.8$^{\#}$	26.7 ± 0.8*	12.4 ± 0.4$^{\#,\alpha}$	14.1 ± 0.2$^{\#,\beta}$	16.0 ± 0.7$^{\#,\beta}$
Cr (mg/dl)	0.16 ± 0.01	0.16 ± 0.01^{ab}	0.19 ± 0.02^{b}	0.12 ± 0.02$^{\#,a}$	0.17 ± 0.01	0.19 ± 0.01	0.20 ± 0.02	0.17 ± 0.03
Leptin (ng/ml)	1.9 ± 0.1	4.4 ± 0.8$^{\#}$	3.1 ± 0.6	3.5 ± 0.9	44.6 ± 1.5*	106.3 ± 9.1$^{\#,*}$	86.3 ± 6.6$^{\#,*}$	82.0 ± 17.7*
Insulin (ng/ml)	0.78 ± 0.07	0.98 ± 0.30	0.80 ± 0.11	0.92 ± 0.17	6.20 ± 1.48*	2.78 ± 0.44*	2.59 ± 0.61$^{\#,*}$	1.43 ± 0.32$^{\#}$

Data are the mean ± SE. * $P < 0.05$ compared with CT mice fed the respective diet, and $^{\#}$ $P < 0.05$ compared with the respective genotype mice at 5 W. Values sharing identical superscripts (alphabetical or Greek letters) are not significantly different

different samples was then determined according to the $2^{-\triangle\triangle Ct}$ method. The level of the genes in CT-L mice was set at 1.00.

Statistical analysis

Values are shown as the mean ± standard error (SE). Statistical analysis was performed using one-way or two-way (repeated measurement) analysis of variance (ANOVA) as appropriate. Significant differences were determined using Fisher's PSD test for multiple comparisons, the unpaired Student's *t* test, and the Mann-Whitney test (for *β*-cell distribution rate). $P < 0.05$ was considered significant (Ekuseru-Tokei 2008; Social Survey Research Information, Tokyo, Japan).

Results

Body weight, food and water intake under ad libitum conditions

db-L, *db*-M, and *db*-H mice showed a significantly higher BW than CT-L, CT-M, and CT-H mice during the feeding experiments, respectively. There were no significant differences in BW among *db* or CT mice fed the three diets, respectively (Fig. 1a). Since weight gain was suppressed in *db*-H mice at 10–13 weeks of age, we therefore determined the end of the experimental period as 13 weeks of age. For food and water intake during the period, *db* mice receiving the three different diets ate and drank a significantly higher amount than CT mice, respectively. There was a significant difference in food and water intake, respectively, between *db*-L and *db*-H mice (Fig. 1b, c).

Glucose in blood under ad libitum conditions

At 5 weeks of age, *db* and CT mice in the three groups (L, M, and H) fed the standard diet (CE-2) showed no significant differences in 6-h FBG. For 6-h FBG at 13 weeks, *db*-L, *db*-M, and *db*-H mice showed a significantly higher value than CT-L, CT-M, and CT-H mice, respectively. There were no significant differences in FBG in *db* or CT mice fed the three diets at 13 weeks of age, although FBG seemed to increase high dose dependently with the dietary protein content in *db* mice (Fig. 2a).

Hormones and biochemical parameters under ad libitum conditions

We examined leptin and insulin levels in *db* and CT mice at 13 weeks of age. For leptin levels, *db*-L, *db*-M, and *db*-H mice showed significantly higher values than CT-L, CT-M, and CT-H mice, respectively. There were no significant differences in leptin levels in *db* or CT mice fed the three diets, respectively. For insulin levels, *db*-L and *db*-M mice

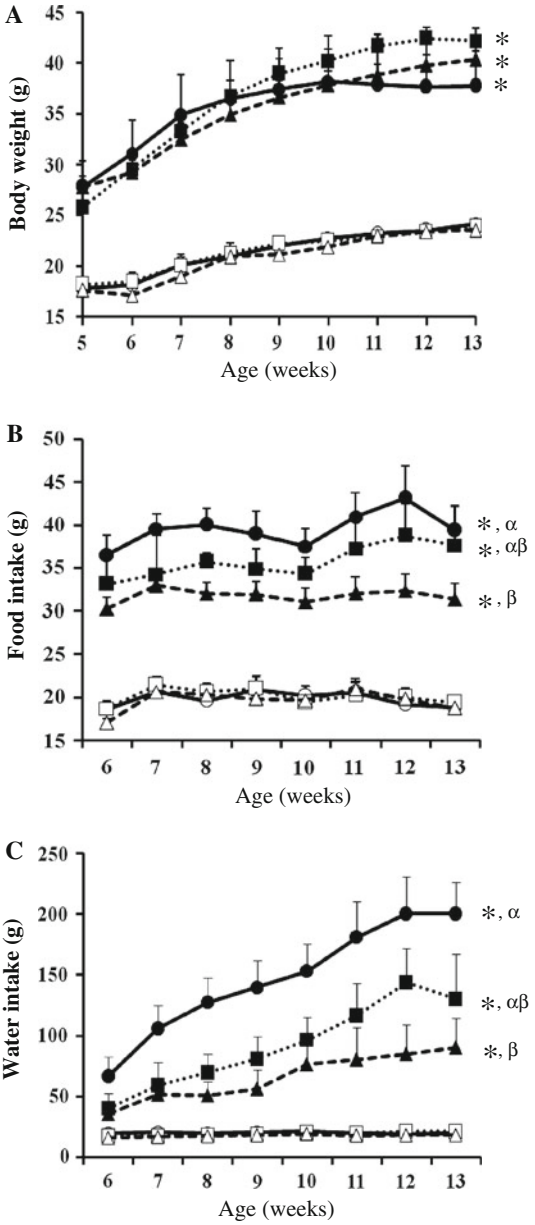


Fig. 1 Under ad libitum conditions, body weight (**a**), food intake (**b**), and water intake (**c**) of *db* (*closed symbol*) and CT (*open symbol*) mice fed different diets (L-diet, 12 % energy from protein, *triangle* and *long-dotted line*; M-diet, 18 % energy from protein, *square* and *short-dotted line*; H-diet, 24 % energy from protein, *circle* and *thick line*) were monitored during 8 weeks. The data are presented as the means + SE of 5–6 mice under the respective conditions. The data were analyzed statistically by two-way ANOVA (repeated measurement), and then, significant differences were determined using Fisher's PSD test for multiple comparisons among the same genetic mice fed the three different diets. *$P < 0.05$ compared with CT mice fed the respective diet. Values sharing identical superscripts (*Greek letters*) are not significantly different

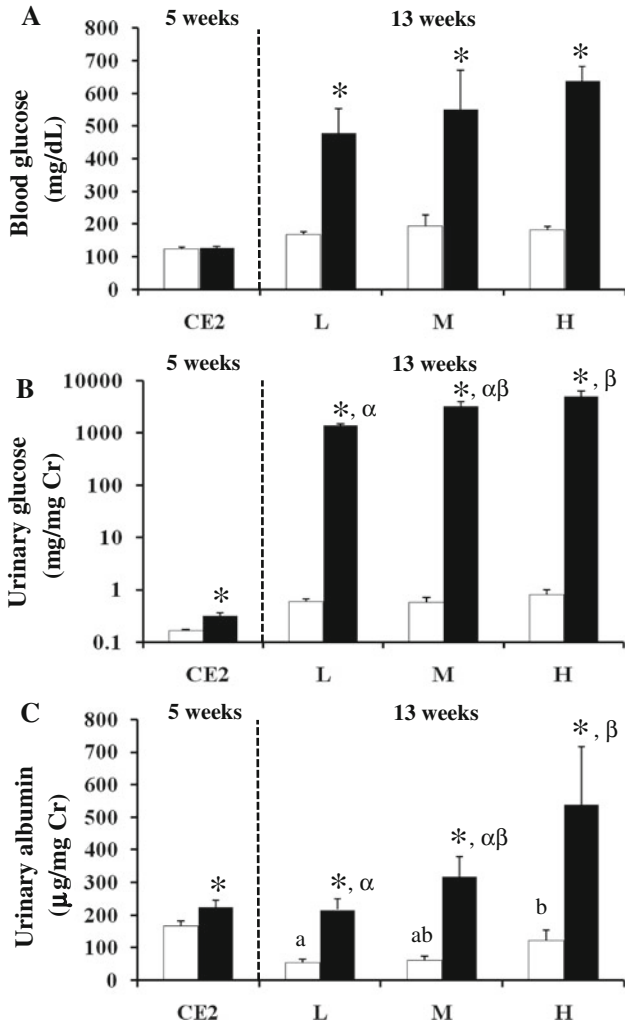


Fig. 2 Blood and urinary examinations under ad libitum conditions. Fasting blood glucose (**a**), urinary glucose (**b**), and urinary albumin (**c**) of *db* (*closed column*) and CT (*open column*) mice are shown at 5 (*left panel*) and 13 (*right panel*) weeks of age. *db* and CT mice were randomly separated into three groups at 4 weeks of age and then fed a standard diet for 1 week. L, M, and H denote the respective diets, as described in Fig. 1. The data are presented as the means + SE of 5-6 mice under the respective conditions. The data were analyzed statistically by one-way ANOVA, and then, the significant differences were determined using Fisher's PSD test for multiple comparisons among the same genetic mice fed the three different diets. Values sharing identical superscripts (*alphabetical* or *Greek letter*) are not significantly different. $*P < 0.05$ compared with CT mice fed the respective diet by Student's *t* test

showed significantly higher values than CT mice fed the diets, respectively. With the H-diet, there were no significant differences in insulin levels between *db* and CT mice. Compared with *db* mice at 5 weeks of age, *db*-M and *db*-H at 13 weeks of age showed significantly lower levels of insulin, indicating exhaustion of insulin secretion under high-protein diet conditions. At 5 weeks of age, *db* mice showed a significantly higher corticosterone level than CT mice (111.5 ± 8.4 vs. 80.7 ± 19.9 ng/ml). Glucagon

Fig. 3 Renal manifestations of *db* and CT mice at 13 weeks of age ▶ under ad libitum conditions. Representative microscopic appearance of glomeruli (PAS staining) for CT and *db* mice fed a low- or high-protein diet (CT-L, CT-H, *db*-L and *db*-H). *Bar* 20 μm (**a**). Kidney weight (**b**), extraglomerular (**c**), intraglomerular (**d**), and PAS-positive areas (**e**). L, M, and H denote the respective diets, as described in Fig. 1. The data are presented as the means + SE of 5-6 mice under the respective conditions. The data were analyzed statistically by one-way ANOVA, and then, significant differences were determined using Fisher's PSD test for multiple comparisons among the same genetic mice fed the three different diets. Values sharing identical superscripts (*alphabetical* or *Greek letter*) are not significantly different. $*P < 0.05$ compared with CT mice fed the respective diet by Student's *t* test

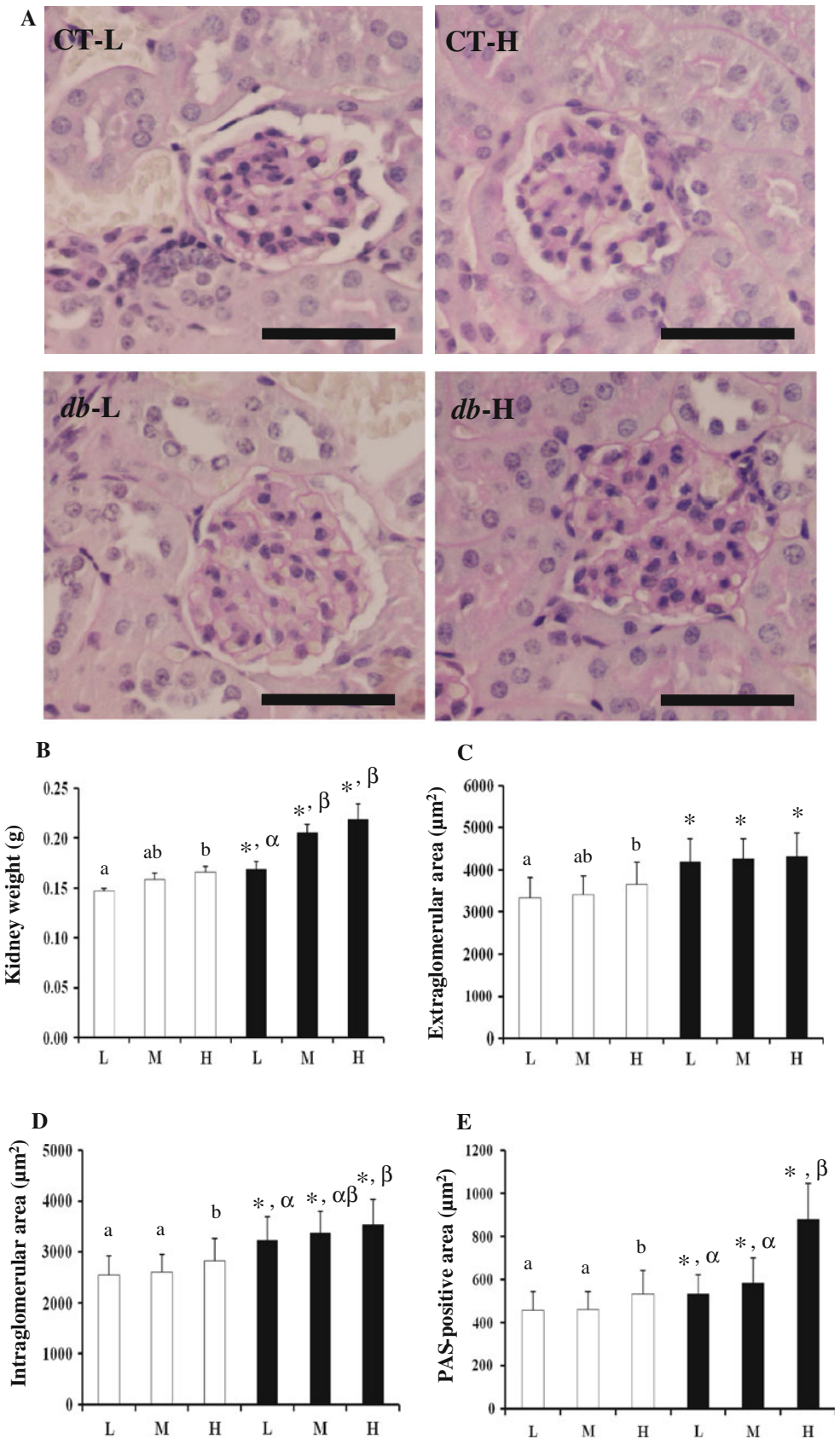
levels at 5 weeks of age were not reliable because the values of some CT and *db* mice were below the assay limitation (<10 pg/ml). In BUN and Cr, there were no significant differences between *db* and CT mice fed the three diets, respectively. Values of BUN in *db* mice altered significantly according to the dietary protein content. CT-H mice showed a significantly lower value of Cr than CT-M mice (Table 2).

Renal manifestations under ad libitum conditions

db mice showed significantly higher values of urinary glucose and U-Alb than CT mice at 5 weeks of age. For urinary glucose, and U-Alb at 13 weeks of age, *db*-L, *db*-M, and *db*-H mice showed significantly higher values than CT-L, CT-M, and CT-H mice, respectively (Fig. 2). There were significant differences in urinary glucose between *db*-L and *db*-H mice (Fig. 2b). For U-Alb, CT-L mice showed significantly lower values than CT-H as well as *db* mice (Fig. 2c).

For kidney weight, *db*-L, *db*-M, and *db*-H mice showed significantly higher values than CT-L, CT-M, and CT-H mice, respectively (Fig. 3b). *db*-L and CT-L mice showed significantly lower kidney weight than *db* and CT mice fed H-diet, respectively (Fig. 3b). There were no significant differences in the weight of organs, including the liver, fat, and heart, among *db* or CT mice fed the three diets, respectively. *db*-L, *db*-M, and *db*-H mice showed significantly higher liver and fat weights than CT-L, CT-M, and CT-H mice, respectively (Table 2).

We performed histological examinations of the kidney. As shown in Fig. 3, *db*-L, *db*-M, and *db*-H mice showed significantly higher values in extraglomerular, intraglomerular, and PAS-positive glomerular areas, than CT-L, CT-M, and CT-H mice, respectively. These findings suggested diabetic nephropathy, such as glomerular hypertrophy and glomerulosclerosis. In the extraglomerular area, there were no significant differences among *db* mice fed the three diets. On the other hand, CT-L mice showed significantly lower values in extraglomerular, intraglomerular, and PAS-


A
CT-L
CT-H
db-L
db-H
B
Kidney weight (g)
0.25
0.20
0.15
0.10
0.05
0.00
a
ab
b
*, α
*, β
*, β
L M H L M H
C
Extraglomerular area (μm²)
6000
5000
4000
3000
2000
1000
0
a
ab
b
*
*
*
L M H L M H
D
Intraglomerular area (μm²)
5000
4000
3000
2000
1000
0
a
a
b
*, α
*, αβ
*, β
L M H L M H
E
PAS-positive area (μm²)
1200
1000
800
600
400
200
0
a
a
b
*, α
*, α
* , β
L M H L M H

positive glomerular areas than CT-H mice. In intraglomerular and PAS-positive glomerular areas, CT-M mice showed significantly lower values than CT-H mice. Similarly to CT mice, *db*-L mice showed significantly lower values in intraglomerular and PAS-positive glomerular areas than *db*-H (Fig. 3).

db-L, *db*-M, and *db*-H mice showed significantly higher angiotensinogen (*Agt*) and lower angiotensin I-converting enzyme (*Ace*) expression than CT-L, CT-M, and CT-H mice, respectively. CT-L mice showed significantly lower values of *Agt* and renin (*Renin*) than CT-H mice. For advanced glycosylation end-product-specific receptor (*Ager*), a receptor of advanced glycosylated proteins, there were no significant differences among *db* and CT mice fed the three diets. Moreover, there were no significant differences in *Ager* expression among *db* or CT mice fed the three diets. The genes involved in inflammatory reactions, transforming growth factor beta 1 (*Tgfb1*), procollagen α1 chain of type I collagen (*Col 1a1*), and procollagen α1 chain of type IV collagen (*Col 4a1*), were evaluated, and no significant differences were found among *db* and CT mice fed the three diets. *db*-L mice showed significantly lower values of *Tgfb1* and *Col 4a1* than *db*-H mice. There were no significant differences in *Tgfb1*, *Col 1a1*, and *Col 4a1* among CT mice fed the diets (Table 3).

Body weight, water intake, and $HbA1_c$ under pair-feeding conditions

Under ad libitum conditions, *db*-H mice ate more food than *db*-L mice, resulting in more energy intake as calories (Fig. 1b). Without the influence of different energy intake, we sought to evaluate the impact of different protein content in the diet on renal manifestations and glucose metabolism; therefore, we performed pair-feeding experiments as follows. *db*-Hp: *db* mice were supplied with H-diet with the equivalent amount of food to that consumed by *db*-L mice (Fig. 4a inset). The energy per gram of food was the same (Table 1); therefore, *db*-L and *db*-Hp mice took in the same energy under this feeding condition. *db*-L mice showed significantly higher BW and lower water intake than *db*-H mice. For $HbA1_c$, *db*-L mice showed a significantly lower value at 15 weeks of age than *db*-Hp mice (Fig. 4c).

Organ weight, blood, and urine parameters when killed under pair-feeding conditions

db-L mice showed significantly higher weights of the liver and heart than *db*-Hp mice (Table 4). On the other hand, *db*-L mice showed significantly lower kidney/body weight and heart/body weight than *db*-Hp mice. Both *db*-L and *db*-Hp mice showed significantly higher glucose, leptin, insulin, and corticosterone in blood than CT-L (Table 4). *db*-L mice showed significantly lower FBG and BUN, and higher leptin and corticosterone than *db*-Hp mice. *db*-L, but not *db*-Hp mice, showed significantly higher glucagon than CT-L mice. There were no significant differences in plasma phosphorus between any two groups, suggesting that the different amounts of dietary phosphorus in L- and H-diets had no significantly different effects on the mice (Table 4). In urine parameters, *db*-L mice showed significantly lower glucose than *db*-Hp mice. For U-Alb excretion, *db*-L mice showed a significantly higher value than CT-L, but similar to that of *db*-Hp mice.

Insulin content and the β-cell distribution rate in the pancreas under pair-feeding conditions

db-Hp mice showed significantly lower insulin content in the pancreas than CT-L mice (Table 4). In a comparison between *db*-L and *db*-Hp mice, there were no significant differences in insulin content in the pancreas. Although

Table 3 Effects of diets on expression of genes in the kidney of CT and *db* mice under ad libitum conditions

Diet	CT			*db*		
	L	M	H	L	M	H
n	5	5	5	6	6	5
Agt	1.00 ± 0.12^{a}	1.08 ± 0.17^{ab}	1.39 ± 0.08^{b}	2.59 ± 0.21*	2.27 ± 0.31*	3.05 ± 0.39*
Renin	1.00 ± 0.11^{a}	1.30 ± 0.07^{b}	1.33 ± 0.09^{b}	1.25 ± 0.03	1.24 ± 0.07	1.25 ± 0.08
Ace	1.00 ± 0.31	0.93 ± 0.31	0.96 ± 0.26	0.23 ± 0.05*	0.22 ± 0.05*	0.23 ± 0.06*
Ager	1.00 ± 1.21	1.25 ± 1.31	0.88 ± 0.35	1.07 ± 0.71	2.39 ± 0.76	2.72 ± 2.46
Tgfb1	1.00 ± 0.05	1.39 ± 0.36	1.23 ± 0.22	$0.97 \pm 0.13^{\alpha}$	$1.26 \pm 0.18^{\alpha\beta}$	$1.55 \pm 0.40^{\beta}$
Col1a1	1.00 ± 0.05	1.42 ± 0.62	1.17 ± 0.19	1.16 ± 0.37	1.93 ± 0.39	2.96 ± 3.01
Col4a1	1.00 ± 0.37	1.20 ± 0.38	1.30 ± 0.02	$0.86 \pm 0.28^{\alpha}$	$1.22 \pm 0.15^{\alpha\beta}$	$1.60 \pm 0.46^{\beta}$

Data are the means ± SE. The level of the genes in CT-L mice was set at 1.00. * $P < 0.05$ compared with CT mice fed the respective diet. Values sharing identical superscripts (alphabetical or Greek letters) are not significantly different

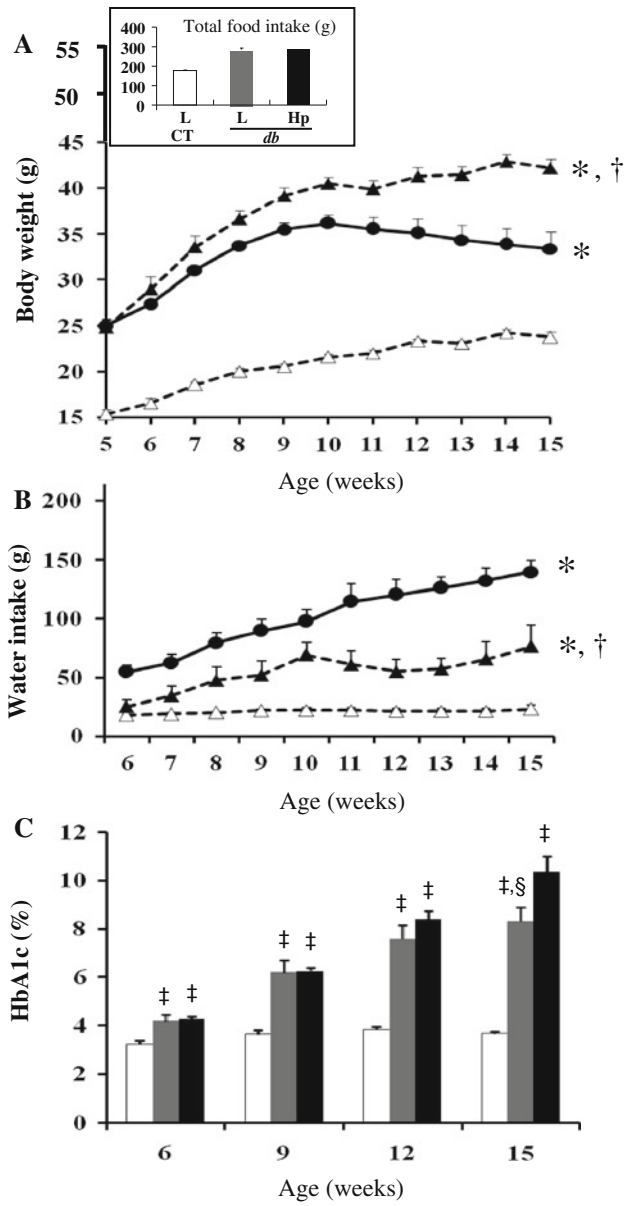


Fig. 4 Phenotypes of *db* and CT mice under pair-feeding conditions. Body weight (**a**) and water intake (**b**) of *db* (*closed symbol*) and CT (*open symbol*) mice fed the different diets for 10 weeks. Diets: L-diet, *triangle* and *long-dotted line*; Hp-diet, *circle* and *thick line*. *db*-Hp: *db* mice supplied with H-diet of equivalent energy to that consumed by *db*-L mice. The total amount of food is shown in the inset of (**a**) *open column*, CT-L; *gray column*, *db*-L; *closed column*, *db*-Hp. The data were analyzed statistically by two-way ANOVA (repeated measurement), and significant differences were determined using Fisher's PSD test for multiple comparisons between *db* and CT mice or between *db* mice fed the diets. $^{*}P < 0.05$ compared with CT mice. $^{\dagger}P < 0.05$ compared with *db*-Hp. $HbA1_c$ (**c**) is shown at 6, 9, 12, and 15 weeks of age. $^{\ddagger}P < 0.05$ compared with CT mice; $^{\S}P < 0.05$ compared with *db*-Hp by unpaired Student's *t* test. The data are presented as the means + SE of 5-6 mice under the respective conditions

db-L and *db*-Hp mice showed a significantly lower *β*-cell distribution rate than CT-L, *db*-L mice showed a significantly higher *β*-cell distribution rate than *db*-Hp mice.

Discussion

The present study revealed the impact of dietary protein content within the range used by humans on food intake, renal manifestations, and glucose levels in *db* mice, an animal model of type 2 diabetes. To the best of our knowledge, this is the first report describing that a low-protein diet within this range improved blood and urinary glucose levels in *db* mice.

Since control mice similarly ate diets composed of different protein content, we could evaluate the effect of dietary protein content on gene expression in the kidney and the pathological findings in the glomerulus. In CT-H mice, *Agt* and *Renin* were more highly expressed than in CT-L mice, suggesting that possible enhancement of the renal RA system is involved in intraglomerular hypertension induced by high-protein intake. These findings are partly consistent with the report by Correa-Rotter et al. [38] who reported a lower expression in *Agt* and higher expression in *Renin* with a low-protein diet (6 %) than high-protein (40 %) diet. The differences from the present study may be due to differences in the species and/or the protein content. Although the mechanism by which dietary protein stimulates *Renin* expression is poorly understood, the following possibility has been suggested. Sodium reabsorption is increased in the ascending segment of the loop of Henle in animals fed a high-protein diet, leading to decreased distal sodium delivery to the macula densa. As a macula densa-mediated effect, it would be expected to stimulate renin release [39]. This possible mechanism, which is consistent with our present data, may be involved in the higher expression of *Renin*, leading to intraglomerular hypertension by high-protein intake. In a rodent study, an additive effect between inhibitors of the RA system and a low-protein diet has been reported [40]. A low-protein diet may have additional beneficial effects through suppressed expression of genes involved in the RA system. In *db*-H mice under ad libitum conditions, the deteriorated renal manifestations, including renal weight, pathological findings in the glomerulus, and the expression of *Tgfb1* and *Col4a1* might have been caused by increased energy intake, rather than increased protein intake.

Under ad libitum conditions in the present study, the high-protein diet led to significantly higher food intake by *db* mice than the low-protein diet, but non-diabetic control mice showed similar food intake, suggesting that higher food intake was due to a diabetic condition. On the other hand, body weight gain was relatively slower in *db*-H than in *db*-L mice (Fig. 1). The growth retardation in *db*-H mice may have been due to loss of glucose in urine. Based on urinary creatinine excreted per day (0.25 mg) according to a published paper [29], the different loss of glucose (3.6 kcal/day) in *db*-H mice than *db*-L mice was

Table 4 Effects of diets on organ weights, blood, and urine parameters in CT and *db* mice under pair-feeding conditions

Diet	CT	*db*	
	L	L	Hp
n	5	6	5
Organ weight			
Kidney (g)	0.144 ± 0.002	0.184 ± 0.004*	0.171 ± 0.004*
Liver (g)	0.94 ± 0.03	1.71 ± 0.10*,†	1.27 ± 0.03*
Fat (g)	0.37 ± 0.06	1.79 ± 0.12*	1.54 ± 0.10*
Heart (g)	0.123 ± 0.005	0.122 ± 0.001†	0.112 ± 0.002
KW/BW (mg/g)	6.09 ± 0.16	4.38 ± 0.20*,†	5.34 ± 0.29
LW/BW (g/g)	0.040 ± 0.002	0.041 ± 0.003	0.038 ± 0.001
FW/BW(g/g)	0.016 ± 0.002	0.042 ± 0.02*	0.046 ± 0.02*
HW/BW (mg/g)	5.18 ± 0.24	2.83 ± 0.03*,†	3.49 ± 0.25*
Blood parameters			
FBG (mg/dl)	148 ± 19	320 ± 51*,†	467 ± 18*
BUN (mg/dl)	8.6 ± 0.8	10.2 ± 1.3†	14.3 ± 0.2*
Cr (mg/dl)	0.16 ± 0.01	0.15 ± 0.02	0.21 ± 0.03
Phosphorus (mg/dl)	5.8 ± 0.4	5.9 ± 0.4	6.3 ± 0.3
Leptin (ng/ml)	2.2 ± 0.7	102.6 ± 9.6*,†	68.0 ± 7.3*
Insulin (ng/ml)	0.46 ± 0.08	4.71 ± 0.64*	3.19 ± 0.77*
Glucagon (pg/ml)	72.0 ± 23.0	198.8 ± 29.5*	126.8 ± 45.5
Corticosterone (ng/ml)	70.6 ± 22.8	314.9 ± 27.5*,†	230.0 ± 21.9*
Urine parameters			
Glucose (mg/mg Cr)	0.54 ± 0.13	4,384 ± 1,222*,†	12,181 ± 2,895*
Albumin (μg/mg Cr)	35 ± 4	320 ± 64*	195 ± 80
Biochemical and pathological findings in pancreas			
Insulin content (U/g)	2.58 ± 0.29	1.51 ± 0.37	1.20 ± 0.25*
β-cell distribution rate (%)	77.0 ± 0.9	58.8 ± 0.6‡,§	46.2 ± 1.6‡

Data are the means ± SE. *KW* kidney weight, *LW* liver weight, *FW* fat weight, *HW* heart weight, *BW* body weight. * $P < 0.05$ compared with CT-L mice; † $P < 0.05$ compared with *db*-H mice by Student's *t* test. ‡ $P < 0.05$ compared with CT-L mice; § $P < 0.05$ compared with *db*-H mice by the Mann-Whitney test

mathematically equivalent to 0.84 g of the food. The higher food intake by *db* mice fed a high-protein diet may be explained by the loss of energy in urine. Similarly, under pair-feeding conditions, *db* mice fed a high-protein diet showed lower body weight than *db*-L mice due to the loss of energy in urine. On the other hand, Teixeira et al. [27] showed a similar amount of food intake by *db* mice fed 12 and 20 % animal protein diets, suggesting that the results reported are consistent with the present study of 12 and 18 % protein diets. That is, urinary loss of glucose in *db* mice fed an 18 % protein diet did not affect food intake significantly, compared with *db* mice fed a 12 % protein diet (Figs. 1b, 2b); therefore, the comparison between 12 and 24 % protein diets is important in the present study.

Regarding glucose levels, Teixeira et al. [27] showed similar blood glucose between *db* mice fed high- and low-protein diets. This finding was consistent with the present study showing no statistical differences in FBG of *db* mice fed the three different diets under ad libitum conditions at 13 weeks of age (Fig. 2a). However, in the present study, *db* fed a high-protein diet (24 %) showed significantly higher blood glucose than *db* fed a low-protein diet (12 %) under pair-feeding conditions (Table 4). Blood glucose under ad libitum conditions might be too high to correspond to the impact of the dietary content; therefore, urinary glucose and glycated hemoglobin in blood should be analyzed, as in the present study. Under pair-feeding conditions, *db* mice fed the low-protein diet showed a significantly lower value of $HbA1_c$, FBG, and urinary glucose than *db* mice fed the high-protein diet, indicating that a low-protein diet improves blood and urinary glucose levels. Additionally, *db* mice have been reported to show exhausted insulin secretion in relatively early life [41, 42]. Consistently, *db* mice under pair-feeding conditions at 15 weeks of age showed lower insulin levels in blood, instead of higher glucose, than *db* mice at 5 weeks of age (Tables 2, 4). Importantly, during the growth of diabetic mice, a low-protein diet seems to delay the exhaustion of insulin secretion because of a higher *β*-cell distribution rate than the value in *db* fed a high-protein diet (Table 4). On the other hand, the renal weight and U-Alb were similar between *db*-L and *db*-Hp mice; however, renal weight per BW in *db*-L was significantly lower than in *db*-Hp mice (4.4 ± 0.2 vs. 5.3 ± 0.3 mg/g BW). U-Alb has been reported to be higher in obesity [43]. Renal manifestations should be evaluated by considering the difference in BW.

The low-protein diet used in the present study contained half the amount of protein of the high-protein diet. Correspondingly, the low-protein diet had a high amount of carbohydrate. In *db* mice, there may be an underlying mechanism that a low-protein diet can suppress the increased blood glucose level compared with a high-protein diet. Theoretically, under the same intake of energy, a low-protein diet could suppress high gluconeogenesis and/or improve low insulin sensitivity. Regarding the underlying mechanism involved in protein content, the induction of anti-insulin hormone secretion, such as glucagon and glucocorticoid, has been suggested [41]; however, in the present study, *db*-L mice showed higher glucagon and corticosterone than *db*-Hp mice at the relevant time point; therefore, these hormones cannot account for amelioration of the blood and urinary glucose levels with low-protein intake. As one possible factor enhancing hepatic gluconeogenesis in high-protein diet, ammonia has been proposed [44, 45]. Ammonia, which is released from proteins through the metabolism, enhances hepatic gluconeogenesis at physiological concentrations [44, 45]. The higher blood and urinary glucose levels in the high-protein diet may be explained by higher hepatic ammonia, corresponding to higher BUN synthesized from ammonia (Tables 2, 4). Further studies are required to reveal the detailed involvement of dietary protein content regulating blood and urinary glucose levels in diabetic mice. Moreover, the conclusions about glucose metabolism remain rather hypothetical at present; therefore, to elucidate the effects of dietary protein on glucose metabolism under diabetic conditions, further experiments related to the glucose tolerance tests, glucose oxidation, and gluconeogenesis are required.

In conclusion, a low-protein diet in the range used in the regular diet of humans improved glucose levels in *db* mice with type 2 diabetes, in addition to relieving renal manifestations in *db* and non-diabetic mice. An appropriate animal model has been established here to examine the underlying mechanism by which dietary protein affects glucose homeostasis and renal manifestations.

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Conflict of interest The authors declare no potential conflicts of interest.

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