

**ORIGINAL****Nonconcordant regulation of mitochondrial respiratory complexes in the kidneys of 5/6 nephrectomized mice**

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**Abstract :** Hyperglycemia induces nonconcordant regulation of renal mitochondrial respiratory complexes, increases oxidative stress, and causes diabetic nephropathy. Hypertension is a complication associated with diabetes and involves glomerular hyperfiltration, the effects of which on mitochondrial respiratory complexes are not well understood. To investigate the effect of glomerular hyperfiltration on renal mitochondrial respiratory complexes, we used the 5/6 nephrectomized BKS.Cg-Dock7<sup>m</sup>+/+Lepr<sup>db</sup>/J, Dock7<sup>m</sup>+/+Lepr<sup>db</sup> mice (db/m-5/6Nx mice) as a model for glomerular hyperfiltration. The BKS.Cg-Dock7<sup>m</sup>+/+Lepr<sup>db</sup>/J, +Lepr<sup>db</sup>/+Lepr<sup>db</sup> mice (db/db mice), a model for type 2 diabetes, was used as the positive control. We investigated the activities and protein levels of the mitochondrial complex, and the mitochondrial DNA and adenosine triphosphate content in the kidneys of these models. Blood chemistry and renal histopathological examination were performed for characterization of the disease. Both models showed expansion of the mesangial matrix of the glomeruli, which is indicative of glomerular hyperfiltration. The activities of complexes I and IV and the protein levels of complexes I and III were nonconcordant in db/m-5/6Nx mice. In conclusion, we demonstrated that nonconcordant regulation of mitochondrial complexes in db/m-5/6Nx mice involved with glomerular hyperfiltration. The progression and/or severity of nephropathy might be affected through a synergistic effect of mitochondrial dysfunction in hyperglycemia and glomerular hyperfiltration. *J. Med. Invest.* 64 : 255-261, August, 2017

**Keywords :** db/m-5/6 nephrectomized mice, db/db mice, glomerular hyperfiltration, mitochondrial respiratory complex

**INTRODUCTION**

The number of patients with chronic kidney disease (CKD) in Japan exceeds 13 million, of which over 300,000 require dialysis. Renal transplantation becomes necessary with the progress of nephropathy (1), and therefore, early detection and treatment is critical. Diabetic nephropathy is the leading cause of CKD, and is caused by microangiopathy and hypertension due to an increase in circulating blood volume. With the progress in diabetes, the glomerular filtration rate (GFR) decreases and albumin is excreted in the urine. In addition, a negative cycle occurs, in which the load on the kidney increases due to a decrease in GFR and renal function deteriorates.

Mitochondria, which supply cellular energy and are involved in metabolism, are important subcellular organelles. Mitochondria have outer and inner membranes composed of phospholipids. The matrix, which is the space within the inner membrane, contains the enzymes required for important metabolic pathways such as the tricarboxylic acid cycle (TCA cycle) and beta-oxidation. Electrons from NADH and FADH<sub>2</sub>, which are derived from glycolysis or the TCA cycle, are transferred to the mitochondrial respiratory chain. These electrons participate in redox reactions in the mitochondrial respiratory complexes I, III, IV or complexes II, III, IV in the inner membrane of the mitochondria. In complex V (ATP synthase),

ADP is converted to ATP by utilizing the energy of transfer of the hydrogen ions accumulated in the intermembrane space. Complexes I and III transfer electrons to CoQ and cytochrome c, respectively, and produce water by transferring electrons to oxygen molecules. Reactive oxygen species (ROS) are generated by the one-electron reduction of oxygen molecules with the electrons from complexes I and III. ROS is reduced to water by the antioxidative activity of glutathione peroxidase and peroxiredoxin. However, if the balance of excessive ROS and antioxidative capacity is disrupted, ROS levels increase, which subsequently causes mitochondrial oxidative stress. Oxidative stress irreversibly damages the mitochondrial constituents and leads to cell death. Therefore, mitochondrial dysfunction has been associated with various diseases. Lee's syndrome is a disease caused by disorders of complex I-V. Also, agrochemical rotenone inhibits complex I. Symptoms of Parkinson's disease are similar to those of rotenone poisoning, and is considered to be caused by complex inhibition. Furthermore, since complex I and III are involved in the production of superoxide, mitochondrial dysfunctions have a high frequency of oxidative stress (2, 3).

It is suggested that mitochondrial dysfunction is involved in the onset and progression of diabetic nephropathy in humans. ROS levels in bovine vascular endothelial cells are related to glucose concentration (4). We reported that thioredoxin 1, which plays an important role in the regulation of intracellular redox balance, markedly suppressed oxidative stress in the kidney of streptozotocin (STZ)-induced diabetic mice and ameliorated characteristic pathophysiological changes of diabetic nephropathy (5). STZ-induced diabetic rats in the early stage of the disease have increased mitochondrial ROS levels and decreased renal complex III activity (6). In

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the kidney of diabetic mice, the activities of complexes I and III increased (7) and the protein levels of complexes I and III increased (8). Furthermore, Zhang *et al.* consider that the nonconcordance of each complex in renal mitochondria of db/db mice is involved in ROS overproduction and oxidative stress in diabetes (8). Hyperglycemia causes hyperosmolarity, which leads to transport of water from within the cell to the extracellular space and increases renal water reabsorption. As a result, circulating blood volume and blood pressure increase. Therefore, in renal pathology of diabetes, it is difficult to distinguish between changes caused by diabetes or hypertension.

Hypertension is generally associated with glomerular hypertension (9). In spontaneously hypertensive rats (SHR), mitochondrial ROS is suggested to increase (10). Hypertension induces decrease of oxidative phosphorylation rate in the kidney of SHR (11). This result shows that oxidative stress due to glomerular hyperfiltration causes dysfunction of the renal mitochondrial respiratory complexes. However, Tapia *et al.* reports that antioxidative enzyme activity in 5/6 nephrectomized rats, a model of glomerular hyperfiltration, decreased, but mitochondrial oxygen consumption does not change (12). Therefore, the influence of glomerular hyperfiltration on mitochondrial function is still unknown.

The effects of hypertension have to be verified to understand the mechanism of development and progression of diabetic nephropathy. In this study, we examined the influence of glomerular hyperfiltration in renal mitochondrial complexes of a 5/6 nephrectomized (5/6Nx) BKS.Cg-Dock7<sup>m</sup>+/+Lepr<sup>db</sup>/J, Dock7<sup>m</sup>+/+Lepr<sup>db</sup> mouse without diabetes. BKS.Cg-Dock7<sup>m</sup>+/+Lepr<sup>db</sup>/J, +Lepr<sup>db</sup>/+Lepr<sup>db</sup> mouse which is murine model of spontaneous type 2 diabetes and found renal oxidative stress (13) was used for the positive control.

## MATERIALS AND METHODS

### Animals

Fifty male 7-week-old BKS.Cg-Dock7<sup>m</sup>+/+Lepr<sup>db</sup>/J, Dock7<sup>m</sup>+/+Lepr<sup>db</sup> mice (db/m mice) and 20 male BKS.Cg-Dock7<sup>m</sup>+/+Lepr<sup>db</sup>/J, +Lepr<sup>db</sup>/+Lepr<sup>db</sup> mice (db/db mice) were purchased from Charles River Laboratories, Japan (Kanagawa, Japan). The mice were individually housed in polycarbonate cages with bedding. The environmental conditions of the animal room were as follows: room temperature, 21.4–24.3°C; relative humidity, 43–72%; air exchange, 6–25 times/h; and a 12 h light/dark cycle (lights on at 7 AM and off at 7 PM). The mice were allowed free access to solid feed (CE-2 gamma irradiated; CLEA Japan, Tokyo, Japan) and tap water.

db/m mice with spontaneous pyelectasia were detected by ultrasonography (Applied XG; Toshiba Medical Systems, Tochigi, Japan) and were excluded from the experiment.

5/6Nx and sham operations were performed using the following procedure. First, two-thirds (upper side, lower side, and outside of the left kidney) were removed from 8-week-old db/m mice while ligating the renal artery, renal vein, and urinary duct. Next, the right kidney was completely excised after 2 weeks (db/m-5/6Nx mice). Sham operation was performed on the left and right sides of the abdomen in db/m mice at 8 and 10 weeks of age, respectively. The same procedures were performed for the sham operation with the exception of ischemia and nephrectomy (db/m-sham mice).

Table 1 shows the experimental design.

Table 2 shows the body weight at necropsy. In db/db mice, the inhibition of body weight gain or decrease of body weight with aging agreed with previously report (14).

The care and use of the animals and experimental protocols used in this study were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited

Table 1. Experimental design

A	16-17 weeks	24-26 weeks	
	of age at necropsy (6-7 weeks after surgery)	of age at necropsy (14-16 weeks after surgery)	
db/m-Sham mice	N = 7	N = 11	
db/m-5/6Nx mice	N = 11	N = 21	
B	13 weeks of age	16-17 weeks of age	21 weeks of age
	at necropsy	at necropsy	at necropsy
db/db mice	N = 6	N = 11	N = 3

Table 2. Body weight at necropsy

A	16-17 weeks of age	24-26 weeks of age	
	db/m-Sham mice	30.0 ± 1.3	32.7 ± 1.9
db/m-5/6Nx mice	28.6 ± 1.8	30.7 ± 1.1	
B	13 weeks of age	16-17 weeks of age	21 weeks of age
	db/db mice	50.4 ± 3.4	41.9 ± 5.1

(Kanagawa, Japan). Animals with poorer performance status were euthanized according to the protocol and not used in this experiment.

### Measurement of blood creatinine/urea nitrogen/glucose levels

Blood was collected from the posterior vena cava under isoflurane anesthesia with a syringe containing heparin sodium. Blood was centrifuged at 7,500 × g for 10 min, and plasma was analyzed using an automated analyzer (LABOSPECT 008; Hitachi High-Technologies Corporation, Tokyo, Japan) and standard reagents (Wako Pure Chemical Industries, Osaka, Japan).

### Histopathology

The left kidney was excised from sacrificed animals after exsanguination. Next, a part of the left kidney was fixed with 10% (v/v) neutral-buffered formalin solution. The remaining kidney was immediately frozen in liquid nitrogen and stored at -80°C until further use. The fixed left kidney was embedded in paraffin, and cut into sections. The sections were stained with hematoxylin and eosin and periodic acid-Schiff (PAS) stain for assessing glomerular changes. The expansion of the mesangial matrix was assessed by analyzing the intensity and distribution of the lesions for each glomerulus.

### Preparation of renal mitochondria

Mitochondria were prepared from the cryopreserved kidneys using a mitochondria isolation kit for tissues (Abcam, Cambridge, United Kingdom). Protein content was measured using the bicinchoninic acid (BCA) protein assay kit (ThermoFisher, Waltham, Massachusetts, United States). The solution was adjusted to 6.0 mg/mL with homogenates containing a protease inhibitor (Sigma-Aldrich, St. Louis, Missouri, United States) and stored at -80°C until further use.

#### Determination of adenosine triphosphate (ATP) content in the kidney

A part of the kidney homogenized for mitochondrial preparation was used for measurement of ATP content using a luminometer (Sirius L Single Tube Luminometer ; Titertek Berthold, Bad Wildbad, Germany) and a reagent kit purchased from Toyo B-net (Tokyo, Japan).

#### Determination of mitochondrial DNA content in the kidney

The quantity of mitochondrial DNA (mtDNA) relative to the amount of nuclear DNA (nDNA) was determined. A QIAamp<sup>®</sup> DNA mini kit (Qiagen, Valencia, California, United States) was used for extraction of mitochondrial DNA and nuclear DNA. DNA content was measured using a NanoDrop ND-8000 (ThermoFisher), and each sample was adjusted to 100 ng/mL and stored at -80°C until further use. A 7600 HT real-time polymerase chain reaction (PCR) instrument (Applied Biosystems, Foster City, California, United States) was used for quantitative PCR with SYBR<sup>®</sup>Green PCR master mix (ThermoFisher). Primers were prepared as previously described and are mentioned below (15, 16) :

mitochondrial D-Loop, forward 5'-AATCTACCATCCTCCGTG-3' and reverse 5'-GACTAATGATTCTTCACCGT-3' ;  
nuclear  $\beta$ -actin, forward 5'-AGCCATGTACGTAGCCATCCA-3' and reverse 5'-TCTCCGGAGTCCATCACAATG-3'.

PCR conditions were as follows : initial denaturation at 95°C for 10 s, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Melting curve analysis was performed at 95°C for 15 s followed by 60°C for 1 min. Samples for calibration curves were extracted and stored in advance from the kidneys of db/m mice using the same method. A calibration curve was prepared for each measurement and the ratio of mtDNA to nDNA was calculated.

#### Assessment of complex activities

The activities of complexes I and IV were measured using a rodent enzyme activity microplate assay kit (Abcam). Each activity level was calculated as a ratio of bovine cardiac mitochondria (Abcam).

#### Assessment of complex protein levels

The solubilized mitochondria and the bovine heart mitochondria (Abcam) with the surfactant were fractionated into each complex using the native page TM bis-tris gel system (BN-PAGE ; Invitrogen, California, United State) with 3–12% bis-tris protein gels, 1.0 mm (Invitrogen). Protein was transferred from the electrophoresis gel onto a PVDF (polyvinylidene fluoride) membrane by electroblotting. Each protein was immunodetected using specific antibodies. An ImageQuant LAS 4000 (GE Healthcare Japan, Tokyo, Japan) was used for measurement, and the value of each sample was calculated as a ratio relative to that of the bovine heart mitochondria. The western blotting detection reagents were purchased from GE Healthcare.

The following primary antibodies were purchased from Abcam : complex I, anti-NDUFA9 (20C11B11B11) ; complex II, anti-SDHA (2E3GC12FB2AE2) ; complex III, anti-UQCRC2 (13G12AF12BB 11) ; complex IV, anti-COX IV (20E8C12) ; complex V, anti-ATP5A (15H4C4). The secondary antibody used was anti mouse IgG-horse radish peroxidase (HRP) (GE Healthcare).

#### Statistical analyses

Measurement data were analyzed as follows. The F-test was first performed for determining the homogeneity of variance between the groups. The Student's t-test was used when the variances were homogeneous, and the Aspin and Welch t-test was performed to

compare the mean in the control group with that in the dosage group when the variances were heterogeneous. The F-test was conducted at the one-tailed significance level of 0.20, and the other tests were conducted at the two-tailed significance levels of 0.05 and 0.01. The SAS system version 8.2 was used for statistical analyses.

## RESULTS

#### Blood creatinine/urea nitrogen/glucose levels

In the db/m-5/6Nx group, the creatinine and urea nitrogen levels were significantly increased at 16-17 and 24-26 weeks of age (Figs. 1A and 1B). To investigate the influence of the onset period of diabetes, in the db/db group, 16-17 and 21 weeks of age were compared to 13 weeks of age animals. The creatinine and urea nitrogen levels of the db/db group did not change (Figs. 1C and 1D). It is likely that creatinine and urea nitrogen did not increase in this study, because reports show that the glucose level in db/db mouse starts to increase by 6 weeks, and blood creatinine and urea nitrogen levels increased after sustained hyperglycemia for 10 weeks (17).

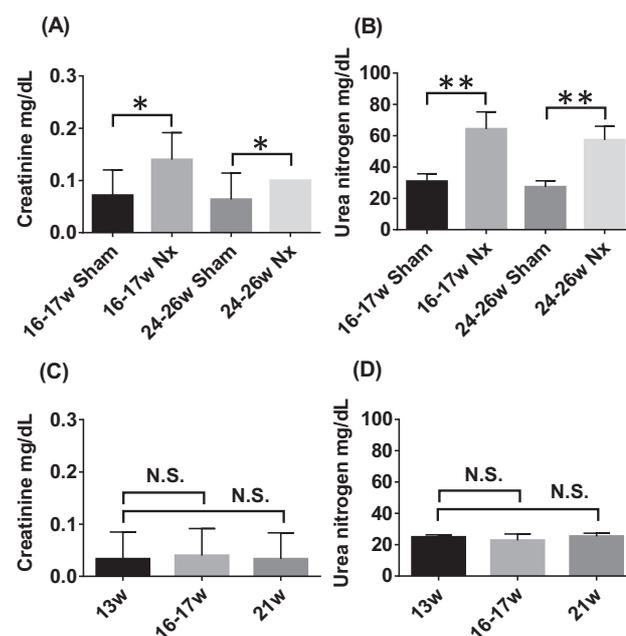


Fig. 1. Blood creatinine and urea nitrogen levels.

Levels of creatinine (A) and urea nitrogen (B) in db/m-5/6Nx group increased compared to those in db/m-sham group at 16-17 weeks of age and 24-26 weeks of age. Creatinine (C) and urea nitrogen (D) in db/db group did not change compared to those at 13 weeks of age. Data are shown as means  $\pm$  standard deviation (SD). N.S., not significant, \*,  $p \leq 0.05$  or \*\*,  $p \leq 0.01$ .

Blood glucose levels were measured for verification of non-diabetic condition ( $224 \pm 18$  vs  $222 \pm 26$  at 16-17 weeks of age,  $231 \pm 27$  vs  $220 \pm 18$  mg/dL at 24-26 weeks of age in db/m-Sham vs db/m-5/6Nx group, respectively). Similarly, glucose levels of the db/db group were  $807 \pm 91$ ,  $740 \pm 144$ , and  $854 \pm 61$  mg/dL at 13, 16-17 weeks and 21 weeks of age, respectively.

### Histopathology

Table 3 shows the histopathological findings of the kidney. Expansion of the mesangial matrix in glomeruli was noted in db/m-5/6 Nx mice at 16-17 and 24-26 weeks of age (Figs. 2B and 2D), and these changes were thought to be associated with nephrectomy. The distribution of the glomerular lesions tended to expand in 24-26 weeks of age compared to that in 16-17 weeks of age. The severities in the changes in individual glomeruli were worse in 24-26 weeks of age than in 16-17 weeks of age. Moreover, slight basophilic renal tubules and dilation of the distal tubules or collecting ducts was observed in the 5/6Nx-db/m groups. No glomerular and tubular lesions were observed in the db/m-sham groups.

Table 3. Histopathological findings of the kidney

	16-17 weeks of age	24-26 weeks of age
m/db 5/6NX (# examined)	11	21
Expansion, mesangial matrix-in kidney	6/1/0	13/4/1
Severity of the glomerular lesion	6/1/0	11/8/0
Dilation, tubule	4/0/0	5/1/0
Tubular basophilia	7/1/0	10/0/0

± / + / ++, ± : Minimal, +: Mild, ++: Moderate

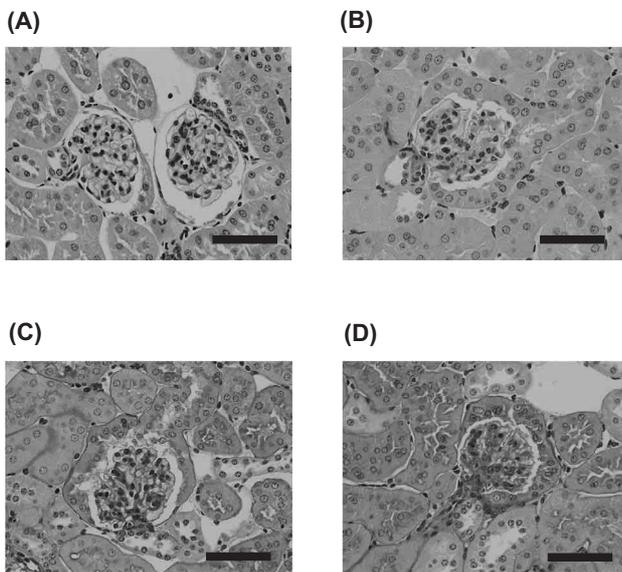


Fig. 2. Representative glomerular changes.

Periodic acid-Schiff staining of glomeruli. db/m-sham mice (A) and db/m-5/6 nephrectomized mice (B) at 16-17 weeks of age. db/m-sham mice (C) and db/m-5/6 nephrectomized mice (D) at 24-26 weeks of age. Expansion of the mesangial matrix is shown in figures 2B and 2D. (Bar = 50 μm)

### ATP content

There was no significant difference in ATP content between the db/m-5/6Nx group and db/m-sham group (Fig. 3A). However, in

the db/db group, there was significant but marginal difference in ATP content between 16-17 and 21, and 13 weeks of age (Fig. 3B).

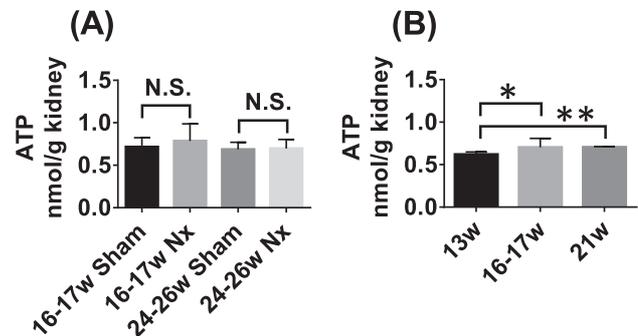


Fig. 3. Renal ATP content.

The concentration of ATP is converted to rate per 1 g of kidney tissue. The ATP content of the db/m-5/6Nx group was not different from that of the db/m-sham group (A). The db/db group showed slight increase in ATP content compared to that of 13-weeks-old mice (B). Data are shown as means ± SD. N.S., not significant, \*,  $p \leq 0.05$  or \*\*,  $p \leq 0.01$ . Data are shown as means ± SD. \*,  $p \leq 0.05$ .

### Mitochondrial DNA content

In the db/m-5/6Nx group, there was no significant difference in the renal mitochondrial content compared to the db/m-sham group (Fig. 4A). Similar result was obtained for the db/db group (Fig. 4B).

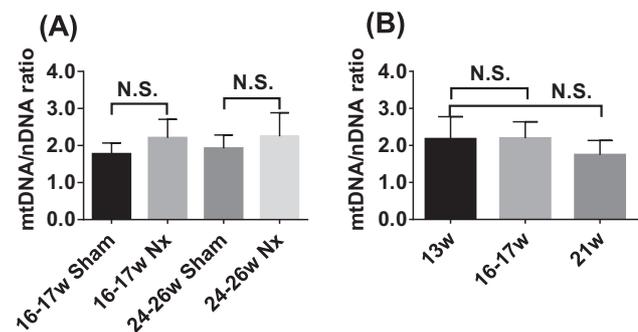


Fig. 4. Mitochondrial DNA contents of the kidney.

The quantity of mitochondrial DNA (mtDNA) relative to the amount of nuclear DNA (nDNA) has been plotted against the age of the animals. mtDNA/nDNA in the db/m-5/6Nx group was not different from that of the db/m-sham group (A). mtDNA/nDNA of the db/db group did not change with the age of the animals (B). N.S., not significant. Data are shown as means ± SD.

### Complex activities

Complex IV activity in the db/m-5/6Nx group animals that were 16-17 weeks of age decreased significantly compared to those in

animals of same age in the db/m-sham group. The activities of complexes I and IV in 24-26 weeks of age decreased significantly compared to those in animals of same age in the db/m-sham group (Fig. 5A and 5B). Furthermore, complex I activity in 16-17-week-old db/db group animals decreased and complex V activity significantly increased compared to corresponding activities in 13 weeks-old animals. The complex activities were similar between 16-17 weeks- and 21 weeks-old animals of the db/db group (Fig. 5C and 5D).

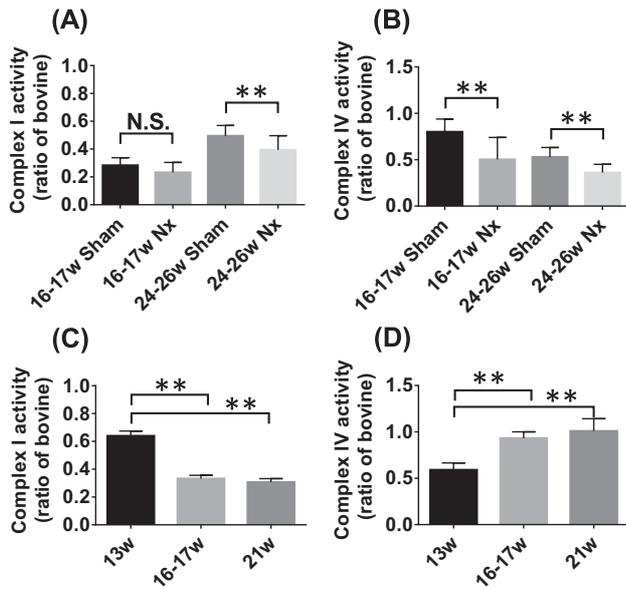


Fig. 5. Activity of renal complexes I and IV (compared to bovine mitochondrial complexes).

For the db/m-5/6Nx group, complex I activity decreased in 24-26 weeks-old animals (A) and complex IV activity decreased in each week for the db/m-5/6Nx group (B) compared to the complex activity of animals of the same age from the db/m-Sham group. For the db/db group, complex I and IV activities decreased (C) and increased (D), respectively compared to those of 13 week-old animals. Data are shown as means  $\pm$  SD. N.S., not significant, \*\*,  $p \leq 0.01$ . Data are shown as means  $\pm$  SD. \*,  $p \leq 0.05$ .

Complex protein levels

Complex I and III protein levels in 24-26 weeks-old animals of the db/m-5/6Nx group decreased significantly compared to those of the db/m-sham group. The protein levels of other complexes were not significantly different at other ages (Fig. 6A). In the db/db group, complex III and IV protein levels at 16-17 weeks and 21 weeks decreased significantly, and these changes were comparable. There was a transient increase in levels of complex I in 16-17-week-old animals compared to their levels in 13 weeks. The levels of complex II decreased significantly only in 21 week-old mice. The levels of complex V did not differ from that of 13 week-old animals at any age (Fig. 6B).

DISCUSSION

The capillary vessels of the glomerulus experience excessive

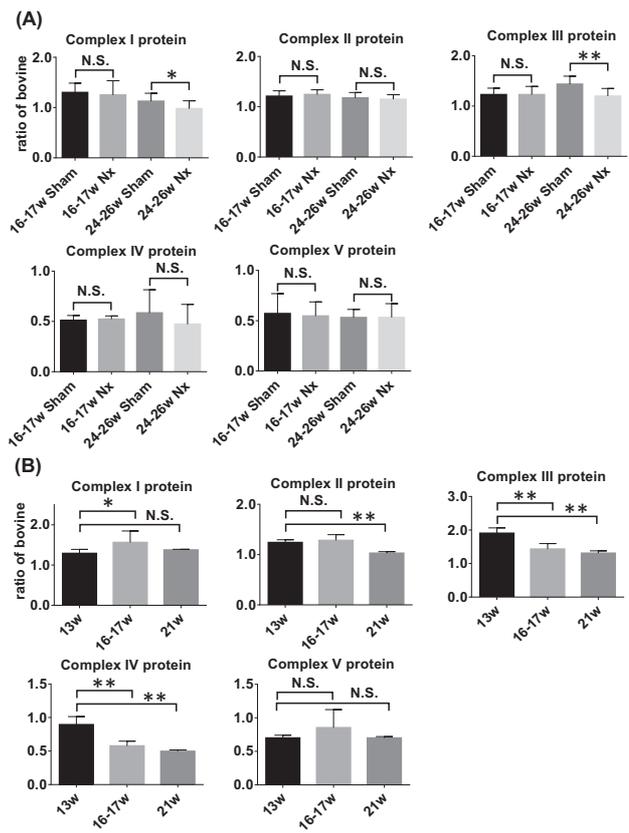


Fig. 6. Complex protein levels in the kidneys (compared to bovine mitochondrial complexes).

Purified mitochondria were electrophoresed by BN-PAGE, analyzed by western blotting, and protein levels were compared to those of animals of the same age. Complex I and III protein levels decreased in 24-26 weeks of age for the db/m-5/6Nx group (A). For the db/db group, the levels of complexes I (16-17 weeks of age), II (21 weeks of age), III, and IV (16-17 and 21 weeks of age) decreased compared to those at 13 weeks of age (B). N.S., not significant, \*,  $p \leq 0.05$  or \*\*,  $p \leq 0.01$ . Data are shown as means  $\pm$  SD. \*,  $p \leq 0.05$ .

pressure in hypertension. Consequently, this causes glomerular hyperfiltration and increases the glomerular mesangial matrix (18). In this study, increase of the glomerular mesangial matrix was observed by histopathological examination of the kidneys of db/m-5/6Nx mouse, which was suggestive of glomerular hyperfiltration. However, db/m-5/6Nx mouse were considered to represent the earliest stage of nephropathy because both ATP and mitochondrial DNA contents in the kidney were similar to those of the db/m-sham group. Increase in blood creatinine and urea nitrogen levels were also observed. However, these changes did not reflect the severity of the nephropathy and might be attributed to the decrease of glomerular filtration rate by 5/6 nephrectomy.

Oxidative stress is believed to be the cause of diabetic nephropathy. ROS accumulating in mitochondria causes mitochondrial respiratory dysfunction, mitochondrial respiratory dysfunction further increases ROS (3). Since mitochondrial ROS is produced in complexes I and III, these complexes are often investigated in studies related to oxidative stress. Reports show that renal complex III activity decreases in diabetic rats (6), whereas the protein levels of renal complexes I and III increase in diabetic mice (8). Our results indicate that the protein level of renal complex I increased transiently in db/db mice, which is in agreement with the results of

other studies. In contrast, activities of complexes I and IV and protein levels of complex I and III decreased in db/m-5/6Nx mice. The decrease in complex IV activity was the earliest detected change in the complexes, and therefore, complex IV was highly susceptible to glomerular hyperfiltration among the mitochondrial complexes. Investigating the activities of complexes II, III, and V in db/m-5/6Nx mice would clarify the relationship between complex IV and glomerular hyperfiltration. The activity of complex IV was low throughout the time period of the study; however, the complex protein levels did not differ from that of the db/m-sham group. The low activity level of complex IV is not due to low protein levels, and the mechanism via which its levels are regulated in diabetic nephropathy is unknown. Cyan, an inhibitor of complex IV, is known to promote ROS production (19). Similar to cyanogenesis, reduction in the activity of complex IV in db/m-5/6Nx mice might be due to be stimulation of ROS production.

In db/m-5/6Nx mice, complex IV activity decreased before the decrease in complex I activity and reduction in complex I and III protein levels. Mitochondrial respiratory chain was nonconcordant in the kidney of the db/m-5/6Nx mouse, which is a model for glomerular hyperfiltration.

## CONCLUSION

In summary, mitochondrial complexes were nonconcordant in the kidney of db/m-5/6Nx mice, a glomerular hyperfiltration model. These results indicate that if glomerular hyperfiltration is coupled with hypertension and diabetes, mitochondrial dysfunction and concomitant nephropathy may augment the severity of the disease in addition to hyperglycemia-mediated mitochondrial dysfunction. The kind of changed complexes were differences between db/m-5/6Nx mice and db/db-mice. At present, we are not understood sure whether this difference is due to hyperglycemia, accumulated anemic toxins or the progression degree of the clinical state. The pathogenesis of glomerular hyperfiltration in the early stages of diabetic nephropathy and hypertensive nephropathy requires further investigation.

## CONFLICT OF INTEREST STATEMENT

None declared.

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