Original Article

Assessment of Mitochondrial DNA Polymorphisms in Salt-Sensitive Hypertension in Dahl Salt-Sensitive Rats

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The Dahl salt-sensitive (DS) rat is the most prevalently used animal model of salt-sensitive hypertension. The purpose of the present study was to test the hypothesis that mitochondrial DNA (mtDNA) polymorphisms influence blood pressure in DS rats. We produced two strains of female F1 rats, one from female DS and male Lewis rats (DL) and the other from Lewis female and DS male rats (LD). These two strains had the same autosomal genetic background, but their mitochondria had different origins. The DL and LD rats had DS and Lewis mitochondria, respectively. A high-salt diet was started at 4 weeks of age. Radiotelemetry devices were implanted into the lower abdominal aorta of these F1 rats at 9 weeks of age. Blood pressure was monitored for 24 h at 11, 12, 13, 14, and 19 weeks of age. No significant differences were observed in blood pressure levels between the strains. Although more than 100 polymorphisms were detected between DS and Lewis rats, it is unlikely that polymorphisms in mtDNA contribute to hypertension in DS rats. Moreover, we found no difference between DS and Lewis rats in the mtDNA copy number in the kidneys, the liver, and the ventricles of the heart before and after salt loading. Thus, it is unlikely that mitochondrial dysfunction due to high blood pressure exacerbated target organ damage. Intriguingly, the time course of body weight gain differed significantly between DL and LD F1 rats, suggesting the influence of mitochondrial polymorphisms on body composition. (*Hypertens Res* 2008; 31: 107–115)

***Key Words*: mitochondrial DNA, hypertension, Dahl salt-sensitive rat**

Introduction

The Dahl salt-sensitive (DS) rat is the most prevalently used animal model of salt-sensitive hypertension. When started on an 8.0% NaCl diet, DS rats rapidly develop low-renin hypertension and renal failure resembling hypertensive nephrosclerosis (*1*, *2*). Although more than 16 genomic regions have been reported to be responsible for this salt-sensitive hypertension, only a few genes have been identified as likely causative ones (*3*–*7*).

Accumulating evidence suggests that mitochondrial dysfunction is associated with the metabolic syndrome (*8*–*11*). Statistical tests developed to assess the involvement of mitochondrial DNA (mtDNA) mutation in a disease demonstrated the contributions of mtDNA mutations in hypertension and diabetes (*10*). Furthermore, a mutation in the mitochondrial transfer RNA has been reported to cause a syndrome including hypertension, hypercholesterolemia, and hypomagnesemia (*11*). Maternal transmission of mutant mtDNA resulted in the death of most mice within 200 days of birth due to renal failure (*12*). In that study, blood pressure was not monitored. It can be speculated that the renal failure induced by a high-salt diet in DS rats is exacerbated by some defects

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Table 1. Sequence Analyses of mtDNAs of DS and Lewis Rats

No.	Map position	Location	Nucleotide change		Amino acid change
			DS	Lewis	
1	935	12S rRNA	A	G	—
2	942	12S rRNA	C	T	—
3	1130	16S rRNA	Del	CCCCCC	—
4	1131	16S rRNA	A	C	—
5	1137	16S rRNA	A	C	—
6	1209	16S rRNA	T	A	—
7	1223	16S rRNA	A	G	—
8	1248	16S rRNA	C	T	—
9	1521	16S rRNA	G	A	—
10	1585	16S rRNA	T	C	—
11	1653–4	16S rRNA	AC	Del	—
12	1716	16S rRNA	T	C	—
13	1832	16S rRNA	G	A	—
14	2170	16S rRNA	T	C	—
15	2836	ND1	T	C	syn
16	2928	ND1	T	C	syn
17	2955	ND1	T	C	syn
18	3000	ND1	T	C	syn
19	3378	ND1	C	T	syn
20	3429	ND1	C	T	syn
21	3435	ND1	T	C	syn
22	3444	ND1	C	T	syn
23	4352	ND2	A	G	Asn→Ser
24	4446	ND2	C	T	syn
25	4554	ND2	T	C	syn
26	4696	ND2	G	A	Ala→Thr
27	4785	ND2	T	A	syn
28	4814	ND2	C	T	Thr→Met
29	4848–50	ND2	Del	CCA	Del→His
30	5200	tRNA-Cys	A	G	—
31	5202	tRNA-Cys	A	G	—
32	5237	tRNA-Cys	T	A	—
33	5269	tRNA-Tyr	G	C	—
34	5559	CO I	A	G	syn
35	5709	CO I	T	C	syn
36	5742	CO I	G	A	syn
37	6012	CO I	T	C	syn
38	6174	CO I	A	G	syn
39	6312	CO I	A	G	syn
40	6438	CO I	C	T	syn
41	6600	CO I	T	C	syn
42	6624	CO I	A	G	syn
43	6663	CO I	T	C	syn
44	6768	CO I	G	A	syn
45	6786	CO I	T	C	syn
46	6825	CO I	T	C	syn
47	6852	CO I	T	C	syn
48	6978	tRNA-Asp	G	A	—
49	7206	CO II	T	C	syn
50	7227	CO II	T	C	syn
51	7401	CO II	A	G	syn

Table 1. Continued

No.	Map position	Location	Nucleotide change		Amino acid change
			DS	Lewis	
52	7498	CO II	A	G	Ile→Val
53	7557	CO II	C	G	syn
54	7578	CO II	A	G	syn
55	7886	ATP 8	C	T	syn
56	7981	ATP 6	G	A	syn
57	8021	ATP 6	G	A	Glu→Arg
58	8150	ATP 6	T	G	Phe→Val
59	8155	ATP 6	C	T	syn
60	8452	ATP 6	C	T	syn
61	8844	CO III	A	G	syn
62	8961	CO III	C	T	syn
63	9120	CO III	T	G	syn
64	9534	ND 3	C	T	syn
65	9669	ND 3	T	C	syn
66	9702	ND 3	C	T	syn
67	9916	ND 4L	T	G	Leu→Arg
68	10051	ND 4L	T	A	Ile→Asn
69	10053	ND 4L	A	T	Thr→Ser
70	10227	ND 4	T	C	Ile→Thr
71	10372	ND 4	A	G	syn
72	10696	ND 4	T	C	syn
73	11128	ND 4	A	C	syn
74	11170	ND 4	T	C	syn
75	11194	ND 4	T	C	syn
76	11389	ND 4	G	A	syn
77	11415	ND 4	T	C	syn
78	11799	ND 5	C	T	syn
79	11831	ND 5	C	T	syn
80	11844	ND 5	A	G	Thr→Ala
81	11918	ND 5	C	T	syn
82	12068	ND 5	C	T	syn
83	12272	ND 5	C	T	syn
84	12350	ND 5	C	T	syn
85	12468	ND 5	C	T	syn
86	12575	ND 5	T	C	syn
87	12635	ND 5	C	T	syn
88	12818	ND 5	A	G	syn
89	13478	ND 5	A	G	syn
90	13647	ND 6	T	C	Ile→Val
91	13693	ND 6	T	C	syn
92	13822	ND 6	T	C	syn
93	14324	Cyt b	C	T	syn
94	14483	Cyt b	G	A	syn
95	14489	Cyt b	C	T	syn
96	14775	Cyt b	G	A	Asp→Asn
97	15005	Cyt b	G	A	syn
98	15062	Cyt b	C	T	syn
99	15188	Cyt b	T	C	syn
100	15209	Cyt b	T	C	syn
101	15333	tRNA-Thr	G	A	—
102	15549	D-loop	C	T	—

Table 1. Continued

No.	Map position	Location	Nucleotide change		Amino acid change
			DS	Lewis	
103	15589	D-loop	A	G	—
104	16313	D-loop	A	G	—

The map position numbers are based on the sequence of Norway rat published on GenBank (AC_000022). ND 1, ND 2, ND 3, ND 4, ND 4L, ND 5, and ND 6 are subunits 1–6 of complex I (NADH dehydrogenase); CO I, CO II, and CO III are subunits 1–3 of complex IV (cytochrome *c* oxidase); ATP 8 and ATP 6 are subunits of complex V (ATP synthase); and Cyt b is a subunit of complex III (ubiquinol: cytochrome *c* oxidoreductase). mtDNA, mitochondrial DNA; syn, synonymous; Del, deletion.

in the mitochondrial system, and that mutations in mtDNA in DS rats may contribute to salt-sensitive hypertension. The purpose of the present study was to assess this hypothesis. Mitochondria are major energy–producing organelles in eukaryotic cells and have their own DNA (mtDNA) (*13*, *14*). Mitochondrial DNA is entirely maternally inherited in mammals (*15*). Therefore, we produced two strains of female F1 rats: one from female DS and male Lewis rats (DL) and the other from female Lewis and male DS rats (LD). Although female F1 rats of these two strains had the same autosomal genetic backgrounds, DL and LD rats had DS and Lewis mitochondria, respectively. Thus, phenotypic differences between the two strains may be ascribed to the differences in mitochondrial polymorphisms. We measured the blood pressure levels of these two strains and assessed the importance of genetic variations of mtDNA in salt-sensitive hypertension.

Methods

Production of F1 Rats and Blood Pressure Measurement

DS and Lewis rats of both sexes were purchased from Sunplanet (Tokyo, Japan) and Charles River Japan (Yokohama, Japan), respectively. Rats were housed in a temperature-controlled room with the lights on from 7:00 AM to 7:00 PM (daytime) and fed normal rat chow (0.5% NaCl; Clea Japan, Tokyo, Japan) and tap water ad libitum. Female DS rats were mated with male Lewis rats to produce DS F1 female rats. Female Lewis rats were mated with male DS rats to produce LD F1 female rats. F1 rats were started on an 8% NaCl diet (Oriental Yeast, Tokyo, Japan) at 4 weeks of age according to the protocol described by Garrett *et al*. (*2*). A radiotelemetry device (Data Sciences International, North St. Paul, USA) were implanted into the lower abdominal aorta of F1 female rats at 9 weeks of age ($n=7$) using sodium pentobarbital as an anesthetic agent (25 mg/kg i.p.). Blood pressure was monitored for 24 h at 11, 12, 13, 14, and 19 weeks of age after 7, 8, 9, 10, and 15 weeks of salt loading. The present study was conducted in accordance with current guidelines for the care and use of experimental animals of the National Cardiovascular Center.

Mitochondrial Sequence and Copy Number Analysis

Male DS and Lewis rats at 4 weeks of age were given either an 8% NaCl diet ($n=4$ for each strain) or normal rat chow containing 0.5% NaCl ($n=4$ for each strain) for 8 weeks and then sacrificed at 12 weeks of age for the measurement of body and organ weights, and extraction of genomic DNA from the livers, kidneys, and ventricles of the heart using a kit (Blood & Cell Culture DNA Midi Kit, Qiagen, Hilden, Germany). Genomic DNA was also isolated from male DS and Lewis rats aged 4 weeks without salt loading ($n=4$ for each strain).

Mitochondrial DNA was amplified by polymerase chain reaction (PCR), and the PCR products were directly sequenced. The copy number of mitochondrion was assessed by competitive PCR according to the protocols described by Vartanian *et al*. (*16*) and Gilliland *et al*. (*17*) with modification. A 9,930-bp PCR product of mtDNA was amplified using the following primer set: forward (5′-CATGGGCTATGT ACTCCCATGAGGAC-3′) and reverse (5′-GGGAAG AAGCCCTAGAAGGTTGGTTGAGCC-3′). Copy number differences were normalized by using a 436-bp 18S rRNA PCR product (forward primer: 5′-GGAATTGACGGA AGGGCACCACCAGGAG-3′, reverse primer: 5′-CGCACT TACTGGGAATTCCTCGTTC-3′). The PCR was carried out in a final reaction volume of 20 μL containing 2 mmol/L Tris-HCL (pH 8.0), 10 mmol/L KCl, 10 μmol/L EDTA, 100 μmol/L DTT, 0.05% Tween20, 0.05% Nonidet P-40, 5% glycerol, 500 μmol/L dNTP, 2.5 U of TaKaRa LA Taq (Takara Bio, Shiga, Japan), 0.5 μmol/L of each primer of mtDNA and 2.5 μmol/L of each primer of 18S rRNA, and 20 ng of total DNA. The reaction was started with 1 min of initial denaturation at 94°C followed by 25 cycles at 94°C for 30 s, 62°C for 30 s, 72°C for 11 min, and the final extension of 72°C for 10 min. Aliquots (5 μL) of the PCR product were electrophoresed on 0.8% agarose gel in TBE buffer at 5 V/cm.

Biochemical Analysis

Blood samples were collected from the abdominal aorta of F1 rats for biochemical analysis. Plasma albumin,

cholesterol, and triglyceride levels were determined by SRL (Tokyo, Japan).

Statistical Analysis

Statistical analyses were performed by using the statistical package JMP6 (SAS Institute, Cary, USA). Possible influences of mitochondrial type on changes in blood pressure and body weight over time were analyzed by the MANOVA procedure. Differences in circulating levels of albumin, cholesterol, and triglycerides were compared between the groups by *t*-test. Results are presented as mean±SD.

Results

Sequence Analyses of mtDNAs of DS and Lewis Rats

Sequence analyses of mtDNAs from male DS and Lewis rats aged 12 weeks revealed 101 single nucleotide and 3 deletion/insertion polymorphisms between the two strains (Table 1). Within the protein-coding regions, 14 polymorphisms resulted in an amino acid change.

Comparisons of Body and Organ Weight and Copy Number Levels between DS and Lewis Rats

Weights of body, kidney, and heart in male DS and Lewis rats were determined at 12 weeks of age after 8 weeks of salt loading (Fig. 1a). No significant differences in body weight between DS and Lewis rats were observed ($p=0.9873$). In contrast, the kidney and heart weights were significantly heavier in DS than in Lewis rats ($p=0.0032$ for kidney and $p=0.0081$ for heart), possibly due to the damage of these organs in DS rats under the high-salt diet.

The copy number of mtDNA was determined in DS and Lewis rats at 4 weeks of age and at 12 weeks of age after 8 weeks of salt loading (Fig. 1b). There were no significant differences between DS and Lewis rats in the copy number of DNA samples from the liver ($p=0.3882$ at 4 weeks, 0.3057 at 12 weeks), the kidneys ($p=0.6269$ at 4 weeks, 0.5255 at 12 weeks), and the ventricles of the heart ($p=0.1147$ at 4 weeks, 0.3860 at 12 weeks). A least-squares fit model was used to examine whether or not strain (DS or Lewis rats), tissue (heart, liver or kidney), or age (4 weeks of age without salt loading or 12 weeks of age after 8 weeks of salt loading) was a significant predictive variable for mtDNA copy number. None of the variables was found to be a predictor of mtDNA copy number; $p=0.6057$, 0.9975, and 0.3975 for strain, tissue, and age, respectively.

Assessment of Blood Pressure in F1 Rats

Blood pressure levels of F1 rats were measured for 24 h at 11, 12, 13, 14, and 19 weeks of age after 7, 8, 9, 10, and 15 weeks

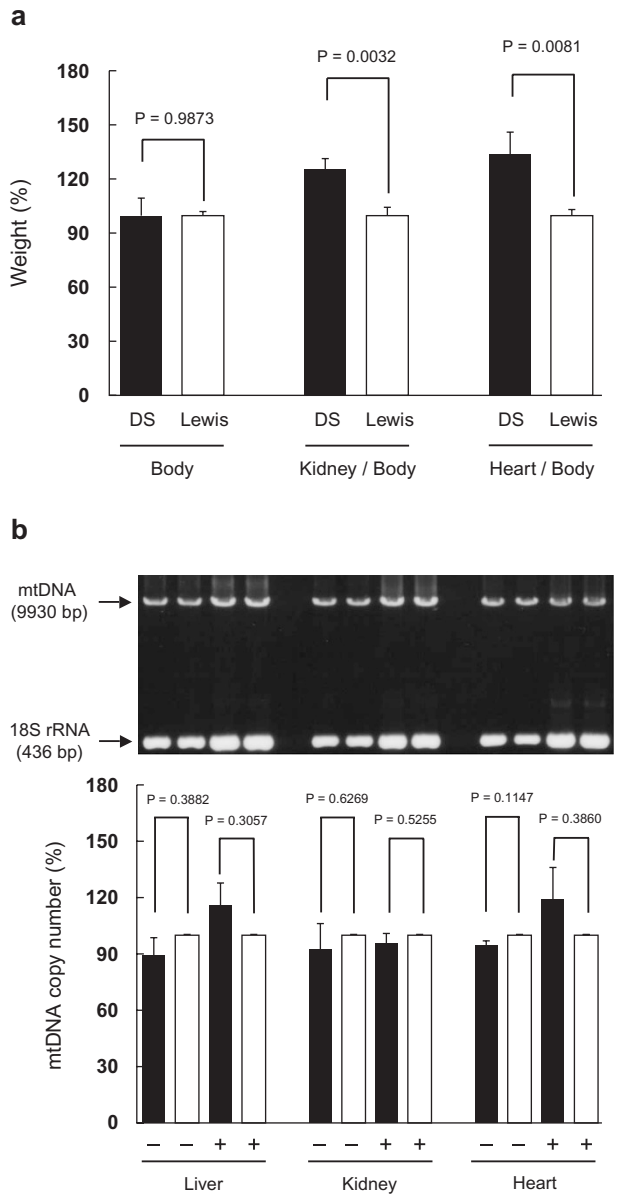

Fig. 1. *a: Body, kidney/body, and heart/body weights were compared between DS (black bar, n=4) and Lewis (white bar, n=4) rats. Kidney and heart weights are shown as percentages of body weight. These data were based on male DS and Lewis rats at 12 weeks of age after 8 weeks of salt loading. Data are presented as mean±SD for each group. Although no significant differences in body weight were found between DS and Lewis rats, the kidney and heart were significantly heavier in DS than in Lewis rats. b: Mitochondrial DNA copy numbers in the liver, kidney, and heart of DS rats (black bar, n=4) are compared with those of Lewis rats (white bar, n=4). Mean±SD are expressed relative to those of Lewis rats set at 100%. The copy numbers were determined by competitive PCR using the DNA samples collected at 4 weeks of age (−) and at 12 weeks of age after 8 weeks of salt loading (+). No significant differences were observed.*

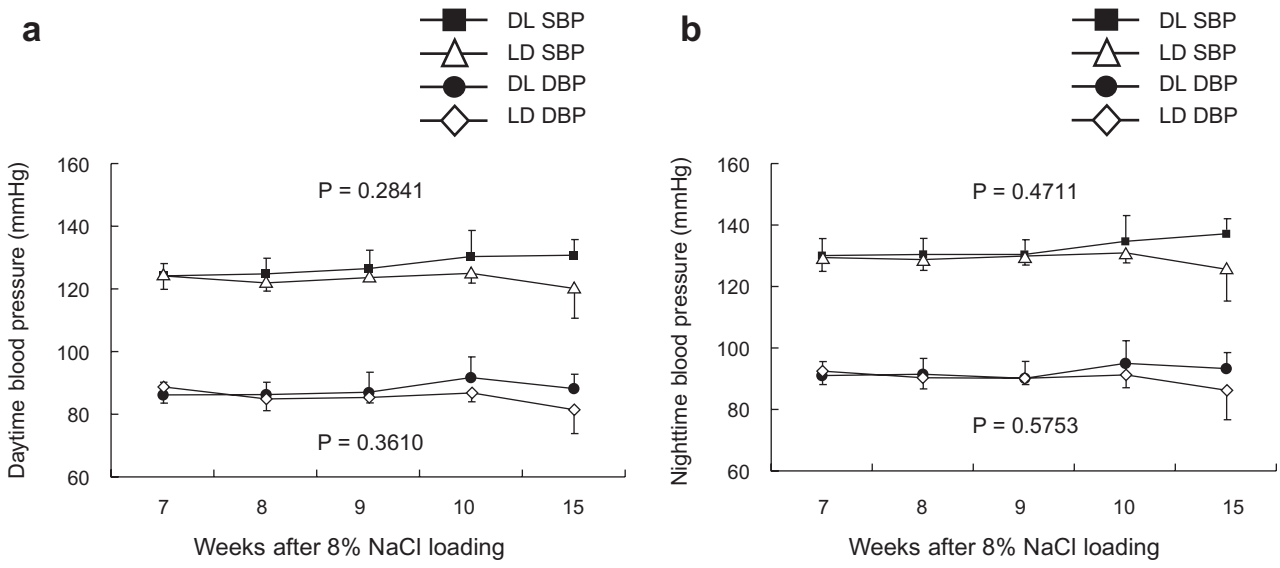

Fig. 2. *Changes in daytime (a) and nighttime blood pressure (b) over time were compared between DL and LD female F1 rats (n = 7 each). Systolic (filled squares) and diastolic (filled circles) blood pressure of DL female F1 rats and systolic (open triangles) and diastolic (open diamonds) blood pressure of LD female F1 rats are shown. Each point represents a group mean, with a vertical bar indicating SD. Blood pressure was monitored by radiotelemetry for 24 h at 11, 12, 13, 14, and 19 weeks of age after 7, 8, 9, 10, and 15 weeks of salt loading. No significant differences were found.*

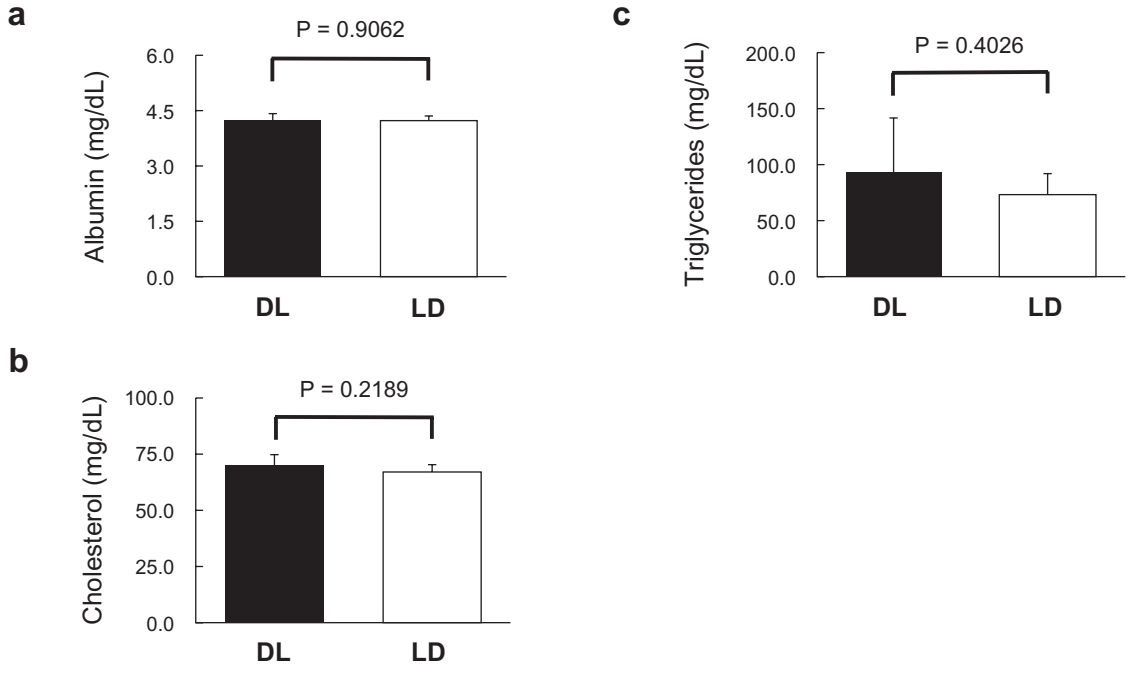

Fig. 3. *Levels of a: albumin, b: cholesterol, and c: triglycerides are compared between DL (black bar, n = 7) and LD (white bar, n = 7) female F1 rats. Data are presented as means ± SD. These data were determined at 19 weeks of age after 15 weeks of salt loading. No significant differences were observed.*

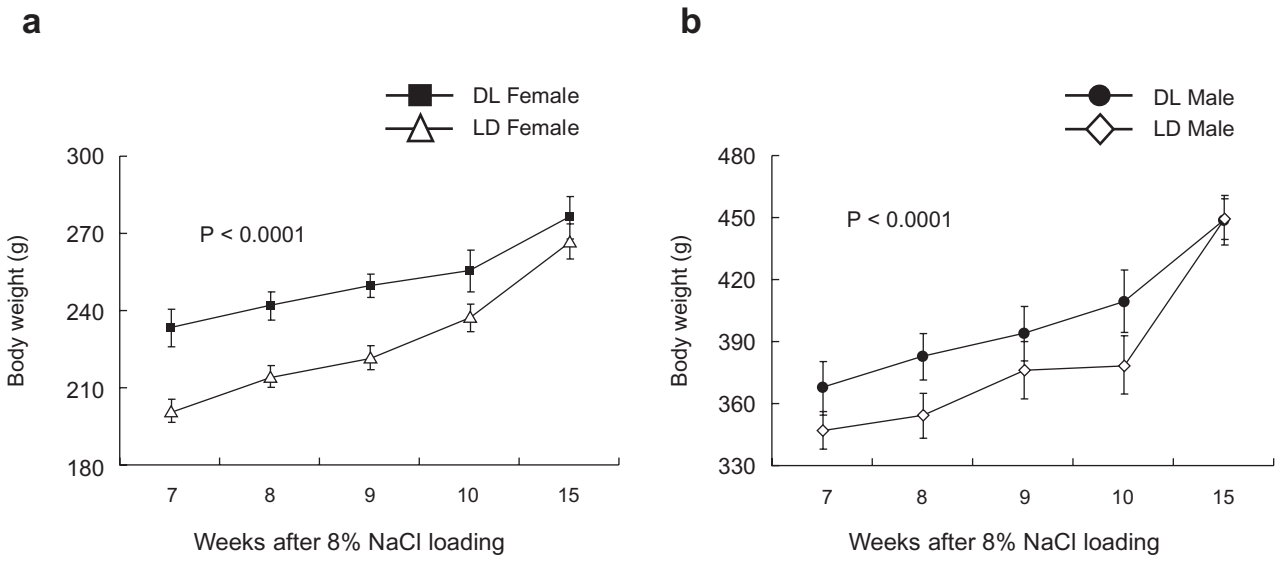

Fig. 4. *The time course of changes in body weight is shown; a: DL female F1 rats (filled squares, n=7) vs. LD female F1 rats (open triangles, n=7) and b: DL male F1 rats (filled circles, n=8) vs. LD male F1 rats (open diamonds, n=6). Data are presented as mean±SD. Body weight was determined at 11, 12, 13, 14, and 19 weeks of age after 7, 8, 9, 10, and 15 weeks of salt loading. Significant differences were found in the time course of body weight gain between DL and LD female or male F1 rats.*

of salt loading (Fig. 2). MANOVA analysis indicated no significant differences were observed between DL and LD female F1 rats in daytime systolic ($p=0.2841$), nighttime systolic ($p=0.4711$), daytime diastolic ($p=0.3610$), and nighttime diastolic ($p=0.5753$) blood pressure.

Assessment of Plasma Albumin, Cholesterol, and Triglyceride Levels in F1 Rats

Plasma albumin, cholesterol, and triglycerides levels were measured at 19 weeks of age after 15 weeks of salt loading in F1 rats (Fig. 3). No significant differences were observed between the DL and LD female F1 rats in plasma albumin ($p=0.9062$), cholesterol ($p=0.2189$), and triglyceride ($p=0.4026$) levels.

Assessment of Body Weight in F1 Rats

Body weight of F1 rats was measured at 11, 12, 13, 14, and 19 weeks of age after 7, 8, 9, 10, and 15 weeks of salt loading (Fig. 4). As expected, the body weight of each F1 rat increased with advancing age. The time course of the changes in body weight differed significantly between the DL and LD female F1 rats; LD female rats weighed less than DL female rats ($p<0.0001$). Since mean body weight measured at 19 weeks of age after 15 weeks of salt loading was not significantly different between female F1 LD and DL rats, changes in body weight from 7 to 15 weeks of age were compared. There was a statistically significant difference in body weight changes under a high-salt diet between the groups, with female F1 LD rats gaining more weight than female F1 DL rats (65.7±13.0 g *vs*. 42.9±11.5 g, $p=0.0046$). LD rats' tendency to weigh less than DL rats throughout the study period was also observed in male F1 rats (Fig. 4b). Similarly, body weight gain was greater in male F1 LD rats than in DL rats (102.5±17.8 g *vs*. 81.3±12.5 g, $p=0.0218$). The litter size for F1 rats in the present study ranged from 8 to 10. Therefore, litter size is not likely a factor in the body weight difference between the DL and LD groups of male and female rats.

Discussion

The present study was conducted to test the hypothesis that mitochondrial polymorphisms might contribute to salt-sensitive hypertension. This hypothesis appeared plausible based on the previous observation that a mutation in mitochondrial transfer RNA has been proven to cause a syndrome including hypertension in humans (*11*). Compared with nonmaternal lineage, maternal lineage was associated with an increase in both systolic and diastolic blood pressure even after adjustment for confounding factors such as age, sex, and body mass index. Moreover, renal failure observed in mice with mtDNA mutations (*12*) resembles hypertension-induced kidney damage in DS rats, suggesting the possible role of mitochondrial polymorphisms in the exacerbation of renal failure associated with salt-sensitive hypertension.

However, our results did not support the hypothesis. We detected no statistically significant differences in blood pressure (Fig. 2). Similarly, no differences in albumin, cholesterol, and triglycerides levels were found between the DL and

LD female F1 rats, both of which possessed the same autosomal genetic background but different mitochondrial genomes (Fig. 3). Since mitochondrial biogenesis is known to be controlled by the nuclear genome (*18*), mtDNA copy number would not vary considerably between DL and LD rats and therefore we can conclude that mtDNA polymorphisms do not contribute to salt-sensitive hypertension in DS rats.

Despite this conclusion, we cannot rule out the possibility that mtDNA may become damaged as a secondary result of salt-sensitive hypertension. We assessed the effect of salt loading on mtDNA copy number in DS rats. Our results showed no significant differences in mtDNA copy number between DS and Lewis rats before and after salt loading despite the pronounced renal and cardiac hypertrophy observed in DS rats exposed to an 8-week high-salt diet (Fig. 1). Furthermore, sequencing analysis revealed a nonsignificant difference in the prevalence of mtDNA point mutations between DS rats given a high-salt diet and DS rats fed a normal salt diet (data not shown). The analysis of point mutation frequencies in mtDNA involved amplification of the D-loop region using kidney and heart DNA samples from DS rats aged 11 weeks old with or without treatment with 8% NaCl, subcloning of the PCR products into the plasmid vector, isolation and purification of plasmid DNA from individual colonies, and sequencing of the plasmid DNAs. The prevalence of mtDNA point mutations in both kidney and heart samples did not differ between DS rats given a high-salt diet and those with a normal salt diet. Although some of the mutations might be due to polymerization errors, salt loading did not seem to cause an increase in mtDNA point mutations in DS rats.

A recent study based on mitochondrial superoxide dismutase (SOD2) knockout mice (*19*) has suggested that hypertension-induced organ damage is worsened by mitochondrial dysfunction. However, our results indicated that mitochondrial dysfunction is unlikely to exacerbate heart failure or renal dysfunction in salt-loaded DS rats.

It should be noted that a major limitation of the present study was that the DL and LD female F1 rats were not salt-sensitive. The systolic blood pressure levels of these F1 rats were under 140 mmHg even after 8 weeks of salt loading (Fig. 2). Establishment of a strain having Lewis-type mitochondria on the DS generic background by back-cross will be necessary for final conclusions.

Intriguingly, the time course of body weight gain appeared to differ significantly between the DL and LD female F1 rats, although the size of litters was not significantly different. Considering the established importance of mitochondria in energy metabolism, it is likely that mitochondrial polymorphisms influence body composition. The mean body weight of the mothers of DL F1 rats measured at 9 weeks of age was 208.2±5.2 g, slightly heavier than mothers of LD F1 rats (188.3±5.9 g, $p=0.0704$). This suggested a possible strain effect on the body weight of the offspring. It is not clear whether the observed difference in time-course changes in body weight between DL and LD F1 rats of either sex is due to differences in uterine size, epigenetic influences, or mitochondrial polymorphisms. The possible involvement of these mtDNA polymorphisms in body weight control and/or adiposity awaits further investigation.

In conclusion, although more than 100 polymorphisms were detected between DS and Lewis rats, these were unlikely to influence blood pressure levels in DS rats.

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