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ORIGINAL ARTICLE

GENERATION AND CHARACTERIZATION OF COUPLING FACTOR 6-OVEREXPRESSING TRANSGENIC MICE

Toshihiro Ashitate[1)], Tomohiro Osanai[1)], Makoto Tanaka[1)], Koji Magota[2)], Takashi Echizen[1)], Hirofumi Tomita[1)] and Ken Okumura[1)]

Abstract We showed that endogenous prostacyclin inhibitor coupling factor 6 (CF6) is released from vascular endothelial cells and its release is stimulated by tumor necrosis factor-α, which is related to congestive heart failure (CHF). We also showed that CF6 increases the gene expression related to CHF. To investigate the role of CF6 in the genesis of CHF, we generated CF6-overexpressing transgenic (TG) mice, and characterized the phenotype. DNA fragment consisting of human elongation factor 1α promoter, and human calcitonin/CF6 fused gene was injected into the embryo of C57BL/6J mouse, and homozygous TG mice were generated. In TG mice, CF6 gene was overexpressed by two fold in overall tissues compared with wild type (WT) mice. Under normal salt diet, blood pressure, heart rate, and the expression of energy metabolism-related genes were similar between TG and WT mice. When the mice were fed with 8% -salt diet for 35 weeks, the mortality of TG mice was higher than that of WT mice (survival rate; 50% in TG versus 92% in WT, $p<0.05$ by log-rank test). This preliminary report indicates that further examination, especially analysis of the cardiac function, is needed to clarify the cause of high mortality of TG mice under high salt intake.

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Key words: coupling factor 6; transgenic mice; congestive heart failure; energy metabolism; high salt loading.

原 著

Coupling factor 6 過剰発現マウスの作成と表現型の解析

芦立俊宗[1)] 長内智宏[1)] 田中真実[1)] 孫田浩二[2)]
越前 崇[1)] 富田泰史[1)] 奥村 謙[1)]

抄録 最近我々は，Coupling factor 6 (CF6) がプロスタサイクリン産生を抑制することを明らかにした．CF6 はうっ血性心不全(CHF)の成因に関与する腫瘍壊死因子により血中に放出される．CHF における CF6 の役割を解明するために，CF6 過剰発現 (TG) マウスを作製し，表現型を野生型と比較検討した．Human elongation factor-1 promoter/ カルシトニン N 末端 / ヒト CF6 からなる DNA 断片を C57BL/6J マウス embryo に導入し，ホモ接合体を作製した．CF6 の発現はすべての臓器で，約 2 倍の亢進を認めた．7 週齢における血圧，心拍数，体重，ならびに心臓のエネルギー代謝に 2 群で差は認めなかった．食塩負荷により血圧，心拍数は 2 群で差を認めなかったが，TG マウスで死亡率の有意な上昇が認められた．早期死亡の原因を解明するために，心機能を含めた更なる解析が必要と考えられた．

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[1)] Department of Cardiology, Hirosaki University Graduate School of Medicine, Hirosaki, Japan
[2)] Asubio Pharma Co., Ltd., Biomedical Center, Biopharma Research Department, Biotechnology Group, 2716-1, Kurakake, Akaiwa, Chiyoda-machi, Ohra-gun, Gunma-ken, 370-0503, Japan
Correspondence: T. Osanai
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[1)] 弘前大学大学院医学研究科循環呼吸腎臓内科学
[2)] アスビオファーマ株式会社生物医学研究所
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INTRODUCTION

Mitochondrial adenosine trisphosphate (ATP) synthase consists of 3 domains: the extrinsic and intrinsic membrane domains, F_1 and F_0, respectively, joined by a stalk[1, 2]. Four subunits of the stalk were identified and designated as coupling factor 6 (CF6), oligomycin-sensitivity conferral protein, and subunits b and d[3–5].

We recently identified CF6 as a novel inhibitor of prostracyclin synthesis and vasoconstrictor: CF6 inhibits prostacyclin synthesis via suppression of cytosolic phospholipase A_2 in the vascular endothelial cells[6]. Intravenous administration of CF6 increases the arterial blood pressure in the fashion of a circulating hormone in spontaneously hypertensive rats[7]. Recently, we showed that the effect of CF6 is mediated by intracellular acidosis after binding to the β-subunit of ATP synthase as a receptor for CF6 at the surface of the vascular endothelial cells[8].

CF6 is released from the vascular endothelial cells and its release is stimulated by tumor necrosis factor (TNF)-α, which is related to congestive heart failure (CHF)[9] through activation of NF-κB signaling pathway[10–12]. Additionally, CF6 increases the gene expression of neuregulin 1 and relaxin 1 related to CHF[13, 14]. Neuregulin-1 induces a negative inotropic effect in the isolated rabbit papillary muscles and a rightward shift of the dose-response curve to isoproterenol[11]. In clinical settings, we and others showed that circulating CF6 is elevated in patients with hypertension, acute myocardial infarction, end-stage renal disease, and diabetes[15–18], all of which share the common pathway to the development of CHF. These suggest a possible linkage between CF6 and CHF, but it still remains to be elucidated. To further investigate the role of CF6 in the genesis of CHF, an animal model overexpressing CF6 was considered to be useful. In the present study, we generated CF6-overexpressing transgenic (TG) mice, and characterized the phenotype.

METHODS

Construction of vectors

The introduced gene product was designed to be released outside of the cells by constructing the fusion of the N-terminal portion of human carcitonin (secretion signal peptide) at the upstream of mature CF6: A gene for the N-terminal portion of human calcitonin (Met 1-Arg 84) was fused to a gene for the mature ATP synthase CF6 (Asn 33-Ala 108). The fused gene was subcloned into pNE plasmid, in which globin promoter of pdKCR-dhfr plasmid[19] were replaced by human elongation factor 1α promoter. The resultant recombinant plasmid was digested with PvuII and Aor51HI to generate 2.8kb of DNA fragment consisting of the human elongation factor 1α promoter, human calcitonin/CF6 fused gene and SV40 polyA additional sequence. It was confirmed in the study by using COS cells that the expressed fusion protein was released outside of the cells as the form of mature human CF6 after cleavage.

Generation of transgenic mice

The DNA fragment was microinjected into the pronuclei of single cell fertilized mouse embryos to generate TG mice. Homozygous TG mice were produced from heterozygous male and female TG mice. Homozygous and heterozygous TG mice were identified by the amount of introduced gene. Briefly, genomic DNA was isolated from tail tissues using QIAamp DNA Kit. To quantify the introduced gene of CF6, we performed real-time quantitative polymerase chain reaction (PCR) using TaqMan chemistry (Applied Biosystems, CA, USA). PCR was carried out according to the protocol supplied with the TaqMan Gold RT-PCR kit (Applied Biosystems) on the ABI PRISM 7700 Sequence Detection System (Perkin-Elmer). Oligonucleotide primers and TaqMan probe for CF6 were designed using Primer Express version 1.5 (Applied

Biosystems) and synthesized by Applied Biosystems. The forward and reverse primers were 5'-TCTTCAGAGGCTCTTCAGGTTCTC-3' and 5'-GCCACTGCTGTAACACCAATGT-3', respectively. The TaqMan probe was 5'-TCATTCGGTCAGCCGTCTCAGTCCAT-3'. It was labeled at the 5'-end with a fluorescent reporter dye FAM (6-carboxy-fluorescein) and at the 3'-end with a quencher dye TAMRA (6-carboxy-tetramethylrhodamine). Oligonucleotide primers and TaqMan probe for mouse glyceraldehydes 3-phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems. Values were averaged from duplicate data and normalized with the mouse GAPDH.

All animals were maintained in the same environment, including the same temperature and humidity and free access to food and water. All experiments were conducted in TG mice and wild type (WT) at the age of 7 through 37 weeks.

Determination of endogenous and introduced coupling factor 6 gene expression

Total RNA was prepared from various tissues using the Trizol RNA purification system. cDNA was prepared from mRNA with oligo (dT) primers. Oligonucleotide primers designed against introduced CF6 were forward primer 5'-GAATAGAAATCTAAGCGACAG-3' and reverse primer 5'-TACAACTAATCCGTGACAAAT-3'. Oligonucleotide primers designed against endogenous CF6 were forward primer 5'-GGCTCTCCTCTGTCCTT-3' and reverse primer 5'-TTGAAGGTAGGAAATGT-3'. The relative quantities of cDNA was assessed by the second PCR amplification of GAPDH. All PCR procedures were performed as follows: 25 cycles for CF6 and GAPDH (45 seconds at 94 ^{o}C, 45 second at 52 ^{o}C for CF6 and 62 ^{o}C for GAPDH, and 1 minute at 72 ^{o}C) and final elongation (5 minutes at 72 ^{o}C).

Experimental design

At the age of 7 weeks, blood pressure, heart rate, body weight, heart weight, and cDNA microarray in the heart tissue were compared between TG and WT mice. Systolic blood pressure and heart rate were measured by tail-cuff method (Softron BP-98A; Softron Co., Tokyo, Japan). In part, TG mice and WT mice were fed with 8% high salt diet (Oriental Yeast Co., Ltd) at the age of 7 through 35 weeks, and the effects of salt intake on blood pressure, heart rate end survival rates were examined. Sodium balance was checked using the metabolic cages at 18 weeks after the initiation of high salt diet.

RNA isolation for cDNA microarrays

Four mice in each group were sacrificed at the age of 7 weeks. LV tissue obtained from the apex region was poured into RNlater immediately. RNA was isolated by using RNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany) according to the manufacturer's instructions. The RNA was quantitated by spectrophotometric analysis at 260 nm. Linear amplification of mRNA from total RNA was obtained using the Amino Allyl MessageAmp aRNA kit (Ambion, Austin, Texas, USA) with two consecutive amplification steps. Two replicates of each experiment were carried out using different microarray slides, where the RNA samples from two different sources were labeled with either Cy3- or Cy5-conjugated deoxyribonucleotides (Amersham Biosciences, Germany). The fluorescent dye on probes derived from the experimental aRNA was Cy5, while the dye on control probes was Cy3.

cDNA microarrays

We used a commercially available cDNA microarray, the AceGene-Mouse Oligo Chip 30K 1 Chip Version (Hitachi Software Engineering Co.,Ltd., Kanagawa, Japan), which contained 30000 cDNA named mouse genes, to indentify genes altered in both TG mice and WT mice fed with high salt diet, compared to before salt

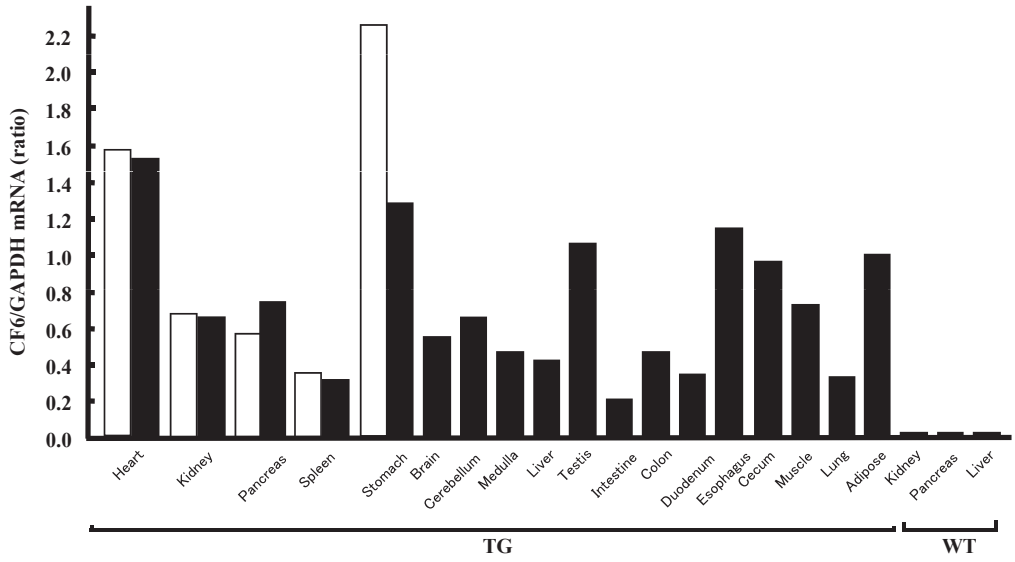

Figure 1 The gene expression of introduced (human) and endogenous (mouse) coupling factor 6 mRNA in transgenic mice.
Abbreviation: WT, wild type.
Closed column: introduced CF6, open column; endogenous CF6.

loading. Labeled probes were mixed with a hybridization solution [5x sodium chloride and sodium citrate (SSC), 0.5% SDS, 4x Denhardt's solution, 20% hybridization solution, 0.1 mg/ml denatured salmon sperm DNA and 10% formamide]. After hybridization for 14h at 46 ℃, the slides were washed in 5x SSC and 0.1% SDS for 2 min at room temperature, 5x SSC and 0.1% SDS for 10 min at 30℃, 0.5x SSC for 2 min at room temperature. Slides were scanned for Cy3 and Cy5 fluorescence with a 428 ARRAY scanner (AFFYMETRIX, Santa Clara, California, USA), and the fluorescence was quantified with DNASIS Array software version 2.6 (Hitachi Software Engineering Co.,Ltd.). The current analysis used intensity-dependent global normalization (mean: 10000). Intensity-dependent normalization is just one technique used to eliminate dye-related artifacts in two-color experiments such as this. The results for each gene were reported as an average obtained from three slides. The data are reported as the normalized ratio of Cy5 for TG mice to Cy3 for WT mice. We used the intensity of 2000 or above.

Statistical analysis and ethical considerations

Results were expressed as mean±SEM. Differences between groups were examined for statistical significance using unpaired *t*-test or analysis of variance (ANOVA) for repeated measure. The level of significance was less than 0.05. All procedures were approved by the ethics committee for animal experimentation of Hirosaki University Graduate School of Medicine.

RESULTS

Characterization of TG mice in the phenotype

As shown in Figure 1, the fused gene of N-terminal portion of human calcitonin and human CF6 was expressed in most organs, especially in the organs where endogenous CF6 was expressed to greater extents. The gene expression of CF6 mRNA in the heart, pancreas, spleen, kidney, and stomach was upregulated by 1.94±0.27 fold in TG mice compared with WT mice.

During development, there was no difference

Table 1 Baseline characteristics of wild type (WT) and coupling factor 6-overexpressing transgenic mice (TG)

	WT (n=4-6)	TG (n=4-6)	P value
Blood pressure (mmHg)	109±8	110±7	NS
Heart rate (bpm)	634±12	642±21	NS
Heart body ratio			
body weight (g)	23.4±0.6	23.1±0.6	NS
heart weight (mg)	111.5±6.7	105.2±2.6	NS
heartweight/body weight ratio (mg/g)	4.76±0.05	4.55±0.04	NS

NS, not significant

Table 2 Gene expression in TG compared with to WT by cDNA microarray analysis

Group	Gene name	Ratio	P value
redox	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	0.91±0.17	NS
	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	0.94±0.21	NS
	cytochrome c oxidase subunit IV isoform 1	0.98±0.09	NS
	cytochrome c oxidase, subunit VIc	1.02±0.04	NS
	cytochrome c oxidase, subunit VIIa 1	1.16±0.02	NS
	cytochrome c oxidase, subunit Va	1.02±0.04	NS
	cytochrome c oxidase subunit VIIc	0.97±0.06	NS
	ubiquinol-cytochrome c reductase, complex III subunit VII	0.92±0.03	NS
	malate dehydrogenase 1, NAD (soluble)	0.95±0.06	NS
glycolysis	aldolase 1, A isoform	1.03±0.08	NS
	phosphoglycerate kinase 2	0.95±0.17	NS
	enolase 3, beta muscle	1.18±0.10	NS
	lactate dehydrogenase C	0.96±0.08	NS
	pyruvate kinase, muscle	0.95±0.15	NS
	glucose-6-phosphatase, catalytic	0.98±0.06	NS
β oxidation	acyl-Coenzyme A dehydrogenase, very long chain	0.93±0.07	NS
	acyl-Coenzyme A dehydrogenase, short chain	1.23±0.14	NS
	enoyl coenzyme A hydratase 1, peroxisomal	1.23±0.07	NS
	acetyl-Coenzyme A acyltransferase 1A	0.97±0.13	NS
	hydroxyacyl-Coenzyme A dehydrogenase	0.96±0.17	NS

TG, coupling factor 6 transgenic mice : WT, wild type mice.
Ratio, TG/WT ratios of each gene expression (n=4).
ATP, adenosine triphosphate : NAD, nicotinamide adenine dinucleotide.
NS, not significant.

in the growth rate (body weight: 23.4±0.6 g in WT mice vs 23.1±0.6 g in TG mice at the age of 7 weeks) and food intake between WT mice and TG mice under normal diet. As shown in Table 1, systolic blood pressure and heart rate at baseline were similar between TG mice and WT mice at the age of 7 weeks.

cDNA microarray of the heart

Table 2 shows the genes related to redox, glycolysis and β-oxidation in the TG heart compared with the WT one at the age of 7 weeks. The genes related to mitochondrial respiratory complexes such as ATP synthase, cytochrome C, and ubiquinol-cytochrome c reductase, and the genes related to the malate-aspartate shuttle such as lactate dehydrogenase and malate dehydrogenase were all similar between TG and WT mice. The genes related to glycolysis and β-oxidation were also similar between the TG heart and the WT heart.

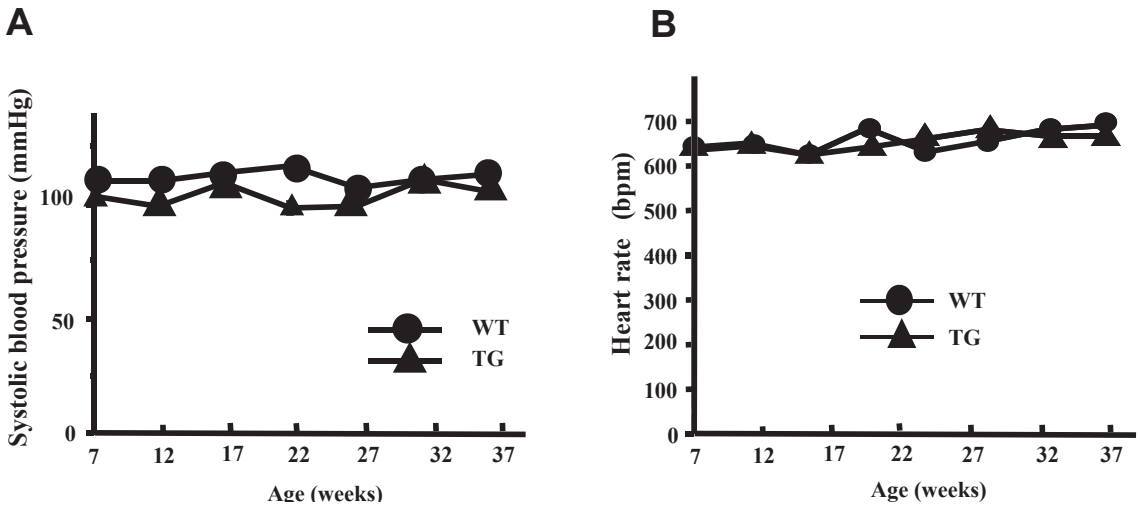

Figure 2 Physical data in transgenic (TG) mice and wild type (WT) mice after high salt intake.
A, Trend of blood pressure in TG mice and WT mice under high salt intake (n=6).
B, Trend of heart rate in TG mice and WT mice under high salt intake (n=6).

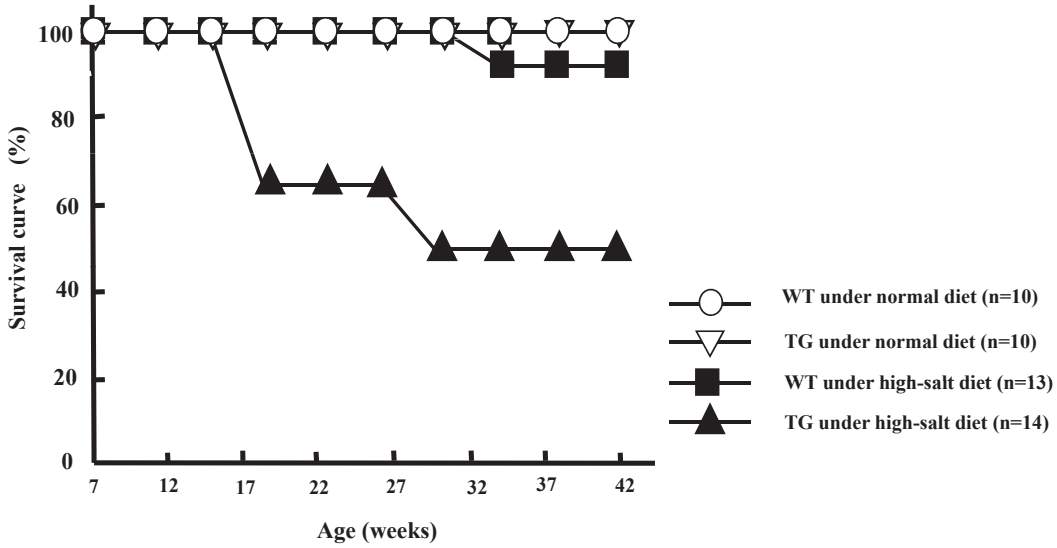

Figure 3 Survival rate in transgenic (TG) mice and wild type (WT) mice after high salt intake.
$p<0.05$ by non-parametric, extended log-rank test.

Effect of salt loading on physical data, sodium balance and survival rates

As shown in Figure 2A and B, either systolic blood pressure or heart rate did not differ between TG mice and WT mice after high salt intake. Body weight and heart weight were similar (body weight: 25.0±0.3 vs 25.6±0.2 g at 4 weeks, 30.0±0.4 vs 30.7±2.5 g at 20 weeks; heart weight: 123.0±2.6 vs 112.6±5.7 mg at 4 weeks, 171.8±6.6 vs 169.0±9.9 mg at 20 weeks) between TG and WT mice. The urinary excretion ratios of sodium/creatinine (51±2 vs 54±4 mEq/g creatinine) and potassium/creatinine (9.8±0.3 vs 9.8±0.5 mEq/g creatinine) were similar between TG mice and WT mice. The sodium retention was also similar between TG mice and WT mice (164±17 mg vs 117±22 mg, $p=0.10$).

Figure 3 shows a Kaplan-Mayer analysis courve of the survival of TG and WT, Seven

out of 14 TG mice (50%) and 1 out of 13 WT mice (8%) were dead during the 35-week period (p=0.014 by log-rank test), despite the fact that none of TG mice and WT mice was dead under normal salt diet.

DISCUSSION

In the present TG mice, the gene expression of CF6 mRNA was upregulated about two-fold in overall tissues, not being associated with impaired energy matabolism-related genes in the heart. Although high salt intake did not affect blood pressure and heart rate in TG mice compared with WT, it resulted in the increased mortality in TG mice.

Generation of TG mice

CF6 is a component of mitochondrial ATP synthase, and functions as an energy transducer. We recently showed that CF6 exerts novel functions outside of the cells[6, 7]. Therefore, the introduced gene product was designed to be released outside of the cells by constructing the fusion of the N-terminal portion of human carcitonin (Met1-Arg84) at the upstream of mature CF6. The N-terminal portion of human carcitonin functions as a secretion signal peptide. We further chose human elongation factor 1α as the promoter of the N-terminal portion of human carcitonin/CF6 fused gene in order to make CF6 gene express in overall tissues including the cardiovascular system. As expected, it was confirmed that the gene expression of CF6 is upregulated about two-fold in overall tissues in TG mice.

Energy metabolism in TG mice heart

In cDNA microarray analysis, the genes related to mitochondrial oxidative phosphorylation, such as cytochrome c oxidase, ubiquinol-cytochrome c reductase and ATP synthase, those related to the malate-aspartate shuttle[20], such as cytosolic malate dehydrogenase and lactate dehydrogenase, those to glycolysis, such as aldolase, enolase and glucose-6-phosphatase, and those to β-oxidation, such as acyl-Coenzyme A dehydrogenase, enoyl coenzyme A hydratase and acetyl-Coenzyme A acyltransferase, were all similar between TG and WT mice. These suggest that ATP generation is not impaired in the heart of the present TG mice under normal diet. It was reported that altered myocardial energy metabolism was seen in diabetic cardiomyopathy and systolic and diastolic dysfunction was displaced in the LV. Diabetic heart is characterized by the reduced glucose and lactate metabolism and the enhanced fatty acid metabolism[21, 22]. Although the metabolic profile of TG heart was not consistent with that of diabetic heart under normal diet, the effect of high salt intake remains to be elucidated.

Effect of high salt intake

High salt intake is one of the most important factors in the initiation and exacerbation of CHF by increasing volume loading and ROS generation. High salt intake also increases the plasma level of CF6[15], thereby leading to further increase in ROS generation. In the present study, we investigated the effect of high salt intake on heart rate, blood pressure and survival rates in TG mice. Salt loading resulted in the increased mortality in TG mice, but it did not affect blood pressure and heart rate. Dahl salt-sensitive rats exhibit overt diastolic CHF at the age of 20 weeks with severe hypertension, when fed with high salt diet since the age of 7 weeks[23]. In the other hand, several recent studies suggest that high salt intake increases oxidative stress in normotensive rats without affecting blood pressure: When Sprague-Dawley rats were fed a low or high salt diet, arteriolar and venular ROS production was increased but hypertension did not develop in the high salt-fed group[24]. To elucidate the effect of high salt intake on cardiac function and ROS generation, further studies should be needed.

In conclusion, we established CF6-overexpressing TG mice in which the gene expression of CF6 was upregulated about two-fold in overall tissues, and the expression of energy metabolism-related genes in the heart was not altered. When the mice were fed with 8% high salt diet, although neither BP nor HR was different between TG and WT, the mortality of TG was higher than that of WT. This preliminary report indicates that the further analysis of the cardiac function and ROS generation is needed to clarify the cause of high mortality of CF6-overexpressing TG mice in the presence of high salt intake.

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