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ORIGINAL ARTICLE

Estrogen attenuates coupling factor 6-induced salt-sensitive hypertension and cardiac systolic dysfunction in mice

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In male coupling factor 6 (CF6)-overexpressing transgenic (TG) mice, a high-salt diet induces hypertension and cardiac systolic dysfunction with excessive reactive oxygen species generation. However, the role of gender in CF6-mediated pathophysiology is unknown. We investigated the effects of ovariectomy and estrogen replacement on hypertension, cardiac dysfunction and Rac1 activity, which activates radical generation and the mineralocorticoid receptor, in female TG mice. Fifteen-week-old male and female TG and wild-type (WT) mice were fed a normal- or high-salt diet for 60 weeks. Systolic and diastolic blood pressures were higher in the TG mice fed a high-salt diet than in those fed a normal-salt diet at 20–60 weeks in males but only at 60 weeks in females. The blood pressure elevation under high-salt diet conditions was concomitant with a decrease in left ventricular fractional shortening. In the WT mice, neither blood pressure nor cardiac systolic function was influenced by a high-salt diet. In the female TG mice, bilateral ovariectomy induced hypertension with cardiac systolic dysfunction 8 weeks after the initiation of a high-salt diet. The ratios of Rac1 bound to guanosine triphosphate (Rac1-GTP) to total Rac1 in the heart and kidneys were increased in the ovariectomized TG mice, and estrogen replacement abolished the CF6-mediated pathophysiology induced under the high-salt diet conditions. The overexpression of CF6 induced salt-sensitive hypertension, complicated by systolic cardiac dysfunction, but its onset was delayed in females. Estrogen has an important role in the regulation of CF6-mediated pathophysiology, presumably via the downregulation of Rac1.
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INTRODUCTION

Premenopausal women have a lower risk and incidence of hypertension and cardiovascular disease than age-matched men. However, this gender advantage gradually disappears after menopause. Although blood pressure is lower in premenopausal women than in men, after menopause, it increases to levels similar to or higher than those of age-matched men.[1,2] The recent Nurse's Health Study[3] and WISE Study,[4] as well as others,[5] have demonstrated that compared with women with normal endogenous estrogen levels, young women undergoing early menopause owing to ovarian dysfunction or bilateral ovariectomy have an increased risk of cardiovascular disease. In animal models of cardiovascular disease, females exhibited lower mortality, less vascular injury, better preservation of cardiovascular function, and slower progression to decompensated heart failure than did males, although such differences were abolished after ovariectomy or induction of a deficiency in endogenous estrogen.[6,7–9] These findings clearly suggest that sex hormones have a cardioprotective role in women. Although randomized, prospective, primary or secondary prevention trials failed to confirm that hormone replacement therapy affords cardioprotection,[10,11] recent Women's Health Initiative studies restricted to younger postmenopausal women showed that the initiation of hormone replacement therapy closer to menopause reduced the risk of cardiovascular disease.[12,13]

Mitochondrial adenosine triphosphate synthase consists of three domains: the extrinsic and intrinsic membrane domains, F_1 and F_o, respectively, joined by a stalk.[14,15] Coupling factor 6 (CF6), a subunit of the stalk, was recently identified to have several novel functions outside the cell. CF6 suppresses the synthesis of prostacyclin in vascular endothelial cells via the inhibition of cytosolic phospholipase A_2, and injection of CF6 induces an increase in arterial blood pressure in rats.[16,17] These functions are mediated by the binding of CF6 to the plasma membrane-bound adenosine triphosphate synthase, ecto-F_1F_o complex, resulting in proton import and intracellular acidosis.[18] The plasma concentration of CF6 in patients with essential hypertension is elevated compared with that of normotensive subjects, and the change in blood pressure due to a high salt intake is positively correlated with

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the change in the plasma concentration of CF6.[19] In CF6-overexpressing transgenic (TG) mice, we have shown that a high salt intake induces cardiac systolic dysfunction and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase upregulation.[20] Rac1, a member of the Rho family of guanosine triphosphate (GTP)ases, has a pivotal role in the potent activation of mineralocorticoid receptor signal transduction[21] and the assembly and activation of the NADPH oxidase enzymatic system.[22,23] In this study, we investigated the effects of ovariectomy and estrogen replacement on CF6-induced salt-sensitive hypertension and cardiac dysfunction in TG mice. Here, we report that estrogen antagonizes CF6-induced salt-sensitive hypertension and cardiac systolic dysfunction by attenuating Rac1 activation under conditions of high salt intake.

METHODS

Animals

The introduced gene product was designed to be released outside the cells by fusing the N-terminal portion of human calcitonin upstream of mature CF6. The gene region encoding the N-terminal portion of human calcitonin (Met 1-Arg 84) was fused to the gene encoding mature adenosine triphosphate synthase CF6 (Asn 33-Ala 108). The fused gene was subcloned into the pNE plasmid, in which the globin promoter of the pdKCR-dhfr plasmid had been replaced by the human elongation factor 1a promoter. The resultant recombinant plasmid was digested with PvuII and Aor51HI to generate a 2.8-kb DNA fragment consisting of the human elongation factor 1a promoter, human calcitonin/CF6 fused gene, and SV40 polyA addition sequence. The DNA fragment was then microinjected into the pronuclei of single-cell fertilized C57BL/6J mouse embryos to generate CF6-overexpressing TG mice. In this study, with COS cells, it was confirmed that the expressed fusion protein was released outside the cells as a form of mature human CF6 (Asn33-Ala108) after cleavage. Two lines of homozygous TG mice were produced. TG mice and wild-type (WT) littermate mice were maintained with standard rat food and free access to water. During development, there was no difference in the growth rate, body weight, or food intake between the two groups. Characterization of the TG mice is described elsewhere.[24] Briefly, the gene expression of CF6 mRNA was upregulated by 1.94 ± 0.27 times in body tissues, such as the heart, pancreas, spleen, kidneys and stomach, in TG mice compared with WT mice. The plasma level of total (human plus mouse) CF6 was twice as high in TG mice as in WT mice. The receptor for CF6, the β subunit of the plasma membrane F_1F_o complex, was expressed in the heart, aorta, kidneys, skeletal muscle and liver, but not in adipose tissue, and was of similar abundance in TG and WT mice. Consistent with the distribution of the ecto-F_1F_o complex, proton uptake was increased in the heart, aorta, kidneys, skeletal muscle and liver of TG mice compared with WT mice, and the intracellular pH value, measured by ^{31}P-magnetic resonance spectroscopy, was decreased by 0.1 unit in the skeletal muscle and liver of TG mice.

Experimental design

All procedures were approved by the ethics committee for animal experimentation of Hirosaki University Graduate School of Medicine. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Male and female TG and WT mice were fed a normal-salt diet or an 8% high-salt diet (Oriental Yeast Co., Ltd., Tokyo, Japan) at the age of 15–75 weeks. Arterial blood pressure, heart rate and cardiac function were measured every 20 weeks after the initiation of a normal- or high-salt diet. In female WT and TG mice at the age of 15–35 weeks and female TG mice at the age of 55–75 weeks that were fed a normal-salt diet, the serum levels of estradiol and Rac 1 activity in the heart and kidneys were measured using a electrochemiluminescence immunoassay (ECLIA) kit (Roche Diagnostics K.K., Tokyo, Japan), and a pull-down assay, respectively. In another experiment, 15-week-old female TG mice underwent bilateral ovariectomy or a sham operation under anesthesia with ketamine ($80\,mg\,kg^{-1}$ i.p.) and xylazine ($8\,mg\,kg^{-1}$ i.p.). One week after the ovariectomy, a 60-day release pellet containing 0.18 mg estradiol or placebo was implanted subcutaneously, and then the mice were fed an 8% high-salt diet for 7 weeks. Blood pressures and heart rates were measured every week, and cardiac function was analyzed every 2 weeks. Rac1 activities in the heart and kidneys were measured using a pull-down assay. The serum level of estradiol was measured in ovariectomized TG mice with and without estradiol replacement.

Blood pressure measurement

Using the Softron BP-98A system (Softron Co., Tokyo, Japan), the systolic and diastolic arterial blood pressures and heart rate were measured by the tail-cuff method in conscious mice. Blood pressures were measured 10 times per day for 4 consecutive days, and a mean value was generated for each individual mouse.

Echocardiography

Cardiac function was analyzed by echocardiography. Transthoracic M-mode images were obtained from the short axis of the left ventricle (LV) in anesthetized mice (ketamine $50\,mg\,kg^{-1}$+xylazine $5\,mg\,kg^{-1}$) using Philips HD11 XE and a 15-Hz linear probe. The LV internal dimensions at the end of systole (LVESD) and end of diastole (LVEDD), LV posterior wall thickness and interventricular septal thickness in diastole were measured and averaged from at least three cardiac cycles. The LV fractional shortening (LVFS) was calculated as $((LVEDD-LVESD)/LVEDD)\times100$.

Rac1 GTPase activation assay

Rac1-GTP levels were determined using a pull-down assay to isolate Rac1-GTP by binding it to the PBD (p21-binding domain) of PAK1 (p21/Cdc42/Rac1-activated kinase 1). The TG and WT mice were homogenized in the assay lysis buffer (25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid, 1% glycerol, 1% NP-40; pH 7.5) and then centrifuged at 14 000 *g* for 10 min at 4 °C. Rac1-GTP was separately concentrated by incubating the lysate with PAK-PBD agarose beads at 4 °C for 1 h, following the protocol of the kit (STA-405, Cell Biolabs, Inc., San Diego, CA, USA). The pull-down supernatant (Rac1-GTP) and the tissue homogenate (total Rac1) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). After incubation with an anti-Rac1 antibody, the Rac1-GTP and total Rac1 contents in these samples were detected using the enhanced chemiluminescence plus detection system (Amersham Pharmacia Biotech, NJ, USA). Rac1-GTP was normalized by total Rac1 in the same sample.

Statistical analysis

All results are expressed as the mean ± s.d. The differences between groups were examined for statistical significance using an analysis of variance followed by Tukey's test. The level of significance was set at $P<0.05$.

RESULTS

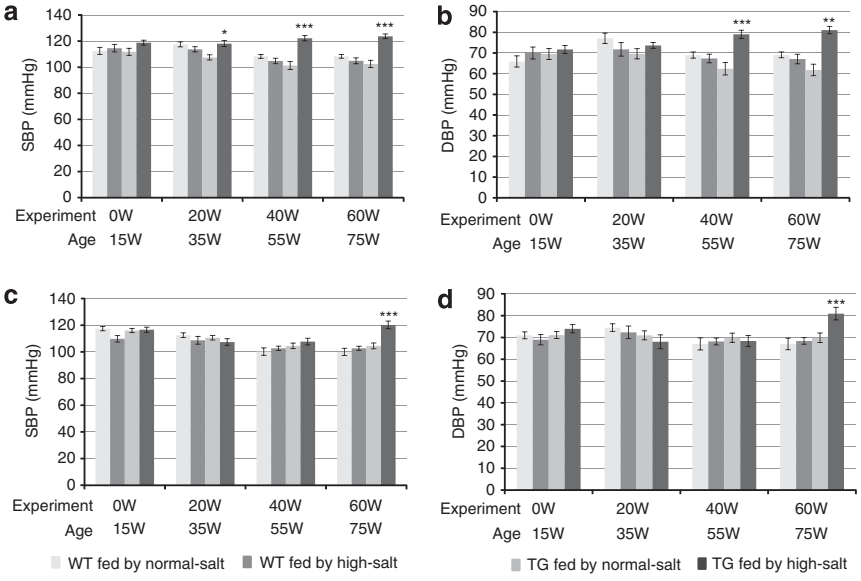
Blood pressure and cardiac function during the 60-week period

As shown in Table 1 and in experiment week 0 in Figures 1 and 2, the baseline arterial blood pressures, heart rate and echocardiographic data were similar in male and female TG and WT mice; only the body weight was lower in females. Figure 1 shows the time course of the systolic and diastolic blood pressures in males (panels a and b) and females (panels c and d) fed a normal- or high-salt diet. The systolic blood pressures were higher in male TG mice fed a high-salt diet than in those fed a normal-salt diet (107 ± 2 *vs.* 118 ± 2 mm Hg, $P<0.05$) at 20 weeks (35 weeks of age) after the initiation of the diets (Figure 1a). At 40 and 60 weeks after the initiation of the diets, the systolic blood pressures were higher in TG mice fed a high-salt diet than in the other three groups fed either a normal- or high-salt diet. Similarly, the diastolic blood pressures were higher in TG mice fed a high-salt diet than in the other three groups fed either a normal- or high-salt diet at 40 weeks and the other two groups at 60 weeks after the initiation of salt diets (Figure 1b). In contrast, it was not until 60 weeks (75 weeks of age) after the initiation of salt diets that the systolic and diastolic blood pressures became higher in female TG mice (Figures 1c and d)

Table 1 Baseline characteristics in male and female mice

	Male mice				*Female mice*			
	WT normal-salt (n=5)	*WT high-salt (n=5)*	*TG normal-salt (n=6)*	*TG high-salt (n=6)*	*WT normal-salt (n=5)*	*WT high-salt (n=5)*	*TG normal-salt (n=5)*	*TG high-salt (n=6)*
BW (g)	31.1 ± 0.8	30.4 ± 1.8	30.4 ± 1.6	30.1 ± 1.6	22.2 ± 0.2*	22.4 ± 0.8*	22.2 ± 1.0*	23.2 ± 1.4*
Heart rate (b.p.m.)	629 ± 107	554 ± 89	571 ± 126	630 ± 75	653 ± 51	652 ± 56	619 ± 94	665 ± 77
Echocardiogram								
LVEDD (mm)	3.78 ± 0.15	4.14 ± 0.25	3.63 ± 0.22	3.67 ± 0.26	3.41 ± 0.88	3.60 ± 0.47	3.14 ± 0.45	3.65 ± 0.31
IVST (mm)	0.72 ± 0.09	0.71 ± 0.14	0.75 ± 0.12	0.75 ± 0.14	0.81 ± 0.12	0.75 ± 0.11	0.69 ± 0.09	0.75 ± 0.13
PWT (mm)	0.59 ± 0.05	0.56 ± 0.07	0.68 ± 0.09	0.73 ± 0.16	0.72 ± 0.12	0.62 ± 0.02	0.70 ± 0.08	0.66 ± 0.09
Averaged WT (mm)	0.65 ± 0.10	0.63 ± 0.16	0.71 ± 0.10	0.74 ± 0.14	0.76 ± 0.12	0.69 ± 0.10	0.70 ± 0.11	0.70 ± 0.13

Abbreviations: BW, body weight; IVST, interventricularseptal thickness in diastole; LVEDD, left ventriculer end-diastolic diameter; PWT, posterior wall thickness in diastole; TG, CF6-overexpressing transgenic mice; WT, Wild type mice.
Averaged WT=(IVST+PWT)/2.
*$P<0.05$ *vs.* TG male.



SBP=systolic blood pressure; DBP=diastolic blood pressure; W=week.
* p<0.05 vs TG normal-salt
** p<0.05 vs TG normal-salt and WT high-salt
*** p<0.05 vs TG normal salt, WT high-salt and WT normal-salt

Figure 1 Time course of systolic and diastolic blood pressures (SBP, DBP) in CF6-overexpressing TG and WT mice during a 60-week period. (**a**) Time course of SBP in male mice. (**b**) Time course of DBP in male mice; *n*=5 for male WT mice fed a normal-salt diet, *n*=5 for male WT mice fed a high-salt diet, *n*=6 for male TG mice fed a normal-salt diet and *n*=6 for male TG mice fed a high-salt diet. (**c**) Time course of SBP in female mice. (**d**) Time course of DBP in female mice; *n*=5 for female WT mice fed a normal-salt diet, *n*=5 for female WT mice fed a high-salt diet, *n*=5 for female TG mice fed a normal-salt diet and *n*=6 for female TG mice fed a high-salt diet.

fed a high-salt diet than in the other three groups fed either a normal- or high-salt diet (105 ± 2 *vs.* 120 ± 3 mm Hg, $P<0.05$).

Figure 2 shows LVFS in males (upper panel a) and females (lower panel b) fed a normal- or high-salt diet. In parallel with the changes in blood pressure, LVFS was lower in male TG mice fed a high-salt diet than in the other 3 groups fed either a normal- or high-salt diet at 20–60 weeks (Figure 2a) (TG at 20 weeks: 32.9 ± 1.0 *vs.* 28.7 ± 1.2%, $P<0.05$). In contrast, LVFS was lower in female TG mice fed a high-salt diet than in the other three groups fed either a normal- or high-salt diet at only 60 weeks (Figure 2b) (TG at 60 weeks: 34.2 ± 1.7 *vs.* 26.9 ± 0.8%, $P<0.05$).

Body weight, heart rate, LVDD, interventricular septal thickness, posterior wall thickness and averaged wall thickness remained similar among the four groups in both males and females during the 60 weeks.

Blood pressure and cardiac function by ovariectomy and/or estrogen replacement

The baseline body weight, heart rate, arterial blood pressure and echocardiographic data were all similar among the three groups: female TG mice that underwent a sham operation or bilateral ovariectomy with or without estrogen replacement therapy. As

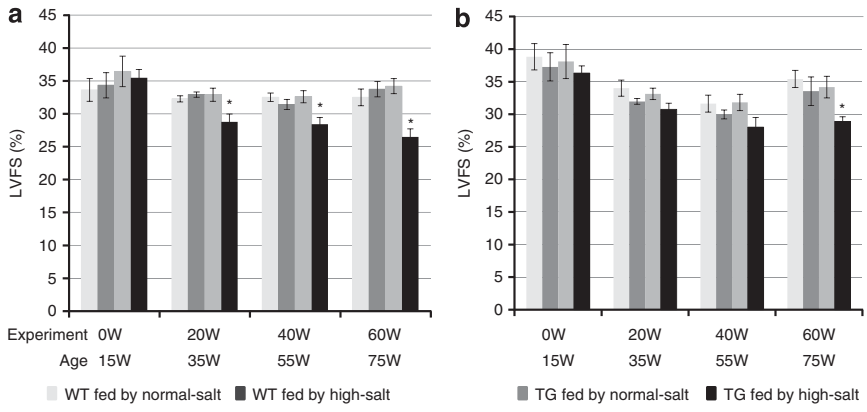


Figure 2 Time course of left ventricular fractional shortening (LVFS) in CF6-overexpressing TG and WT mice during a 60-week period. (**a**) Time course of LVFS in male mice; *n*=5 for male WT mice fed a normal-salt diet, *n*=5 for male WT mice fed a high-salt diet, *n*=6 for male TG mice fed a normal-salt diet and *n*=6 for male TG mice fed a high-salt diet. (**b**) Time course of LVFS in female mice; *n*=5 for female WT mice fed a normal-salt diet, *n*=5 for female WT mice fed a high-salt diet, *n*=5 for female TG mice fed a normal-salt diet and *n*=6 for female TG mice fed a high-salt diet.

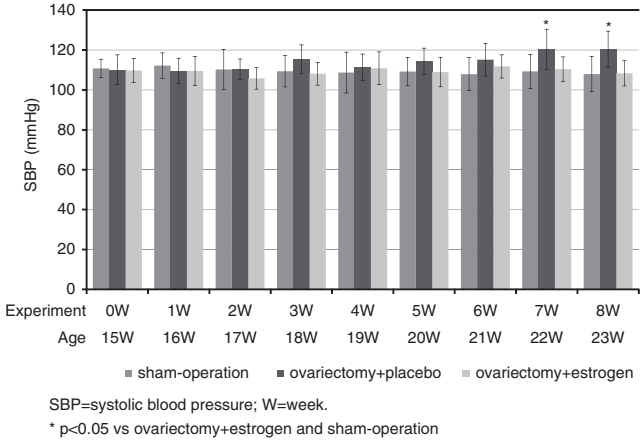


Figure 3 Time course of SBP in female CF6-overexpressing TG mice that underwent a sham operation or bilateral ovariectomy with or without estrogen replacement therapy; *n*=5 for female sham-operated TG mice (sham operation), *n*=6 for female ovariectomized TG mice with a placebo (ovariectomy+placebo) and *n*=5 for female ovariectomized TG mice with estrogen replacement (ovariectomy+estrogen).

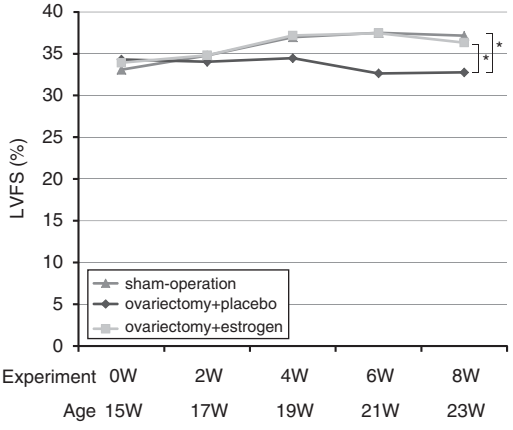


Figure 4 Time course of LVFS in female CF6-overexpressing TG mice that underwent a sham operation or bilateral ovariectomy with or without estrogen replacement therapy; *n*=5 for female sham-operated TG mice (sham operation), *n*=6 for female ovariectomized TG mice with a placebo (ovariectomy+placebo) and *n*=5 for female ovariectomized TG mice with estrogen replacement (ovariectomy+estrogen).

shown in Figure 3, the systolic blood pressures in ovariectomized TG mice were increased compared with those in mice that underwent a sham operation at 7–8 weeks (22–23 weeks of age) under a high-salt diet. In ovariectomized TG mice with estrogen replacement, neither systolic nor diastolic blood pressure was increased by a high salt intake.

Figure 4 shows the time course of LVFS in female TG mice that underwent either a sham operation or bilateral ovariectomy with or without estrogen replacement therapy. Consistent with the changes in blood pressure, under a high-salt diet, LVFS in ovariectomized TG mice was decreased compared with that in mice that underwent a sham operation. In ovariectomized TG mice with estrogen replacement, LVFS was unchanged under a high-salt diet. Body weight, heart rate, LVDD, interventricular septal thickness, posterior wall thickness and averaged wall thickness remained similar among the three groups during the 8 weeks.

Serum level of estradiol in female TG mice

The serum levels of estradiol under a normal-salt diet were 16.3 ± 2.5, 25.3 ± 9.1 and $< 10\,\mathrm{pg\,ml^{-1}}$ in female WT and TG mice at the age of 15–35 weeks and female TG mice at the age of 55–75 weeks (all *n*=3), respectively. In the ovariectomized TG mice with and without estradiol replacement, at the age of 23 weeks (*n*=4 and 3), the serum levels of estradiol were 23.3 ± 4.6 and $< 10\,\mathrm{pg\,ml^{-1}}$, respectively.

Rac-1 activity in the kidneys and heart

Figure 5 shows the Rac1 activities in the kidneys and heart of female WT and TG mice at the age of 15–35 weeks and female TG mice at the

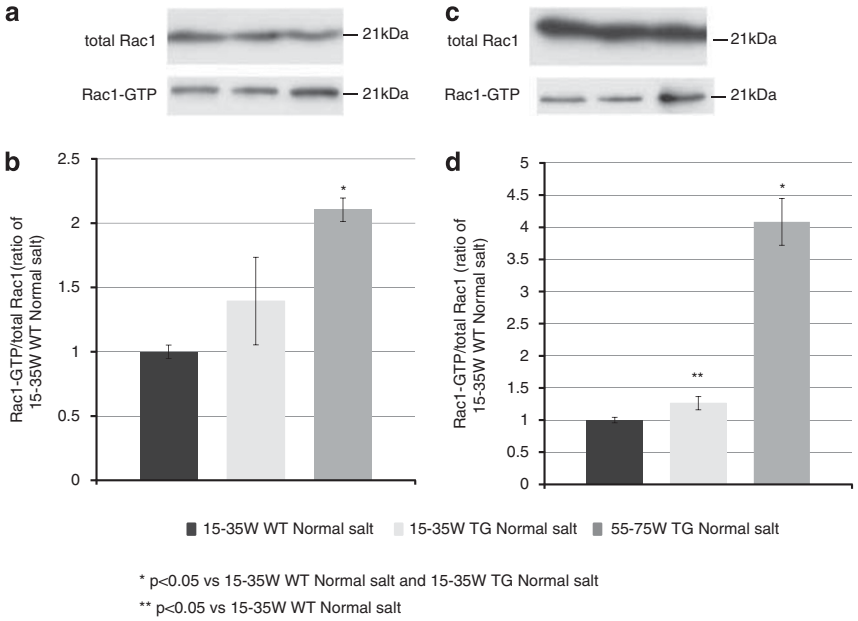


Figure 5 Ratio of Rac1-GTP to total Rac1 in the heart and kidneys of female CF6-overexpressing TG and wild type (WT) mice under a normal-salt diet; n=3 for female WT mice at the age of 15–35 weeks, n=3 for female TG mice at the age of 15–35 weeks and n=3 for female TG mice at the age of 55–75 weeks. (**a**) Representative western blots of total Rac1 and Rac1-GTP in the kidneys. (**b**) Ratio of Rac1-GTP to total Rac1 in the kidneys. (**c**) Representative western blots of total Rac1 and Rac 1-GTP in the heart. (**d**) Ratio of Rac1-GTP to total Rac1 in the heart.

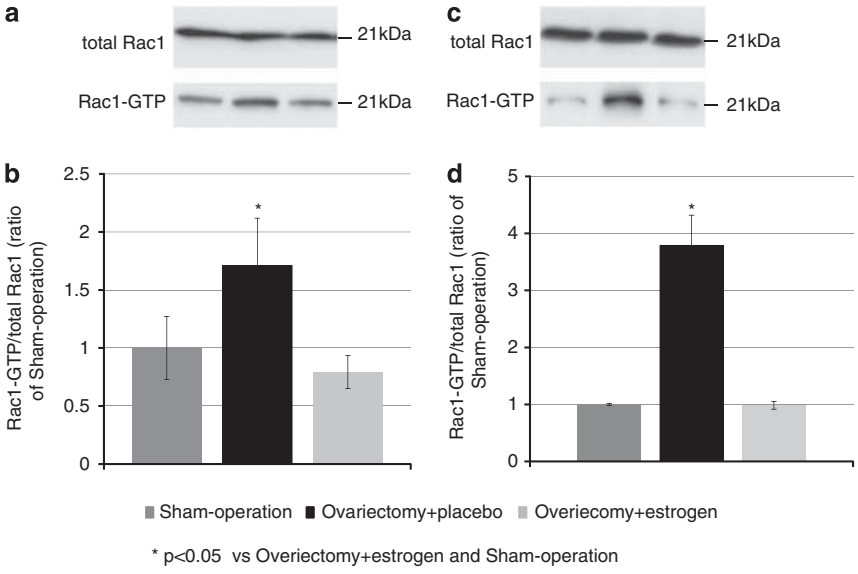


Figure 6 Ratio of Rac1 bound to guanosine triphosphate (Rac1-GTP) to total Rac1 in the heart and kidneys of female CF6-overexpressing TG mice that underwent a sham operation or bilateral ovariectomy with or without estrogen replacement therapy; n=6 for female ovariectomized TG mice with a placebo (ovariectomy+placebo), n=5 for female ovariectomized TG mice with estrogen replacement (ovariectomy+estrogen) and n=5 for female sham-operated TG mice (sham operation). (**a**) Representative western blots of total Rac1 and Rac1-GTP in the kidneys. (**b**) Ratio of Rac1-GTP to total Rac1 in the kidneys. (**c**) Representative western blots of total Rac1 and Rac 1-GTP in the heart. (**d**) Ratio of Rac1-GTP to total Rac1 in the heart.

age of 55–75 weeks (all n=3) under a normal-salt diet. As shown in panel a, the representative bands for Rac1-GTP in the kidneys were increased in female TG mice at the age of 55–75 weeks. The ratio of Rac1-GTP to total Rac1 was greater in female TG mice at the age of 55–75 weeks than in female WT and TG mice at the age of 15–35 weeks (panel b). In the heart (panels c and d), the ratio of Rac1-GTP to total Rac1 was greater in female TG mice than in female WT mice at the age of 15–35 weeks and was greater in female TG mice at the age of 55–75 weeks than in female WT and TG mice at the age of 15–35 weeks.

Figure 6 shows Rac1 activities in the kidneys and heart in female TG mice that underwent a sham operation or bilateral ovariectomy with or without estrogen replacement therapy. As shown in panel a, the representative bands for Rac1-GTP in the kidneys were increased in the ovariectomized TG mice without estrogen replacement but reversed to the level of the sham control by estrogen replacement

therapy. The ratio of Rac1-GTP to total Rac1 was greater in the ovariectomized TG mice without estrogen replacement than in those that underwent ovariectomy with estrogen replacement or in those that underwent a sham operation (panel b). In the heart (panels c and d), the ratio of Rac1-GTP to total Rac1 was also greater in the ovariectomized TG mice without estrogen replacement than in those with estrogen replacement or in those that underwent a sham operation.

DISCUSSION

The major findings of the present study are as follows. Overexpression of CF6 induced salt-sensitive hypertension and cardiac systolic dysfunction at 20 weeks (35 weeks of age) after the initiation of a high-salt diet in male CF6-overexpressing TG mice and at 60 weeks (75 weeks of age) in female TG mice. Bilateral ovariectomy induced an increase in the systolic and diastolic blood pressures and cardiac systolic dysfunction in female TG mice (22–23 weeks of age) at 7–8 weeks after the initiation of a high-salt diet, both of which were abolished by estrogen replacement therapy. The ratio of Rac1-GTP to total Rac1 increased in the heart and kidneys in TG mice that underwent bilateral ovariectomy; however, this was attenuated by estrogen replacement.

The incidence and prevalence of heart and renal disease are lower in premenopausal women than in age-matched men.[2,25–27] However, this difference gradually disappears after menopause, and cardiovascular risk becomes significantly higher in older women than in age-matched men.[28,29] To investigate the difference between genders in CF6-induced cardiovascular disorders, we first examined the long-term effect of a high-salt diet on arterial blood pressure in male and female TG mice. The result showed that overexpression of CF6 induced an increase in blood pressure at 20 weeks after the initiation of a high-salt diet in male TG mice (35 weeks of age) and at 60 weeks in females (75 weeks of age). It is noted that WT mice showed no increase in blood pressure under a high-salt diet, suggesting that overexpression of CF6 has an important role in the genesis of salt-sensitive hypertension in mice. This seems consistent with the clinical findings that the plasma concentrations of CF6 in patients with essential hypertension were elevated compared with those in normotensive subjects, and the change in blood pressure after a 7-day high salt intake was positively correlated with the change in the plasma concentration of CF6.[19] In parallel with the onset of hypertension, cardiac systolic function was impaired in TG mice receiving a high-salt diet. It should be emphasized that the onset of CF6-induced pathophysiology was delayed in females.

The long-term effects of estrogen are mediated by both estrogen receptor α and estrogen receptor β,[30] whereas the rapid nongenomic effect of estrogen is involved in the calcium-mediated activation of endothelial nitric oxide synthase,[31] cGMP and intracellular signal transduction pathways.[32,33] Estrogen acts via the G-protein-coupled estrogen receptor GPR30, and the transactivation of epidermal growth factor receptors, such as phosphatidylinositol 3-kinase (PI3K), induces rapid signal transduction.[34] In the heart, activation of GPR30 with the specific agonist G1 reduces ischemia/reperfusion injury and preserves cardiac function, acting through PI3K-dependent Akt pathways,[35] suggesting a cardioprotective role of GPR30. To investigate the role of estrogen in cardioprotection, we examined the effects of bilateral ovariectomy and estrogen replacement on the CF6 overexpression-related increases in blood pressure and cardiac systolic dysfunction under a high-salt diet. The results showed that bilateral ovariectomy induced an increase in systolic and diastolic blood pressures and cardiac systolic dysfunction within 8 weeks (23 weeks of age) after the initiation of a high-salt diet, and estrogen replacement attenuated CF6 overexpression-mediated salt-sensitive hypertension and cardiac systolic dysfunction. With regard to cardioprotection, estrogen was shown to increase the expression of superoxide dismutase and inhibit NADPH oxidase activity.[8,32,36] Estrogen increases protein S-nitrosylation, a common posttranslational protein modification,[37] and reduces inflammatory markers[32,38] and afterload- or agonist-induced cardiac hypertrophy via the inhibition of calcineurin hypertrophic transcription factor and mitogen-activated protein kinase signaling pathways.[39] Estrogen also improves endothelial and myocardial function after ischemia by an antiapoptotic and pro-survival effect on cardiomyocytes,[40,41] endothelial progenitor cell mobilization[42] and mesenchymal stem cell-mediated vascular endothelial growth factor release.[43,44] These rapid effects of estrogen on the action of CF6 were not examined in this study. However, CF6 attenuates prostacyclin generation via the inhibition of cytosolic phospholipase A_2,[16] nitric oxide generation via the upregulation of asymmetric dimethylarginine or the inhibition of endothelial nitric oxide synthase phosphorylation[45,46] and the PI3K/Akt cascade.[24] Therefore, the rapid effects of estrogen, such as the increase in nitric oxide and the induction of the PI3K-Akt cascade, may antagonize the action of CF6. This issue remains to be clarified in a future study.

Recently, it was shown that Rac1, a member of the Rho family of GTPases, has a pivotal role in the potent activation of mineralocorticoid receptor signal transduction[21] and the assembly and activation of the NADPH oxidase enzymatic system.[22,23] We thus investigated the molecular mechanisms by which estrogen attenuated CF6 overexpression-mediated salt-sensitive hypertension and cardiac systolic dysfunction. The results showed that in female TG mice fed a high-salt diet, the ratios of Rac1-GTP to total Rac1 in the kidneys and heart were increased by ovariectomy and attenuated by estrogen replacement, suggesting that Rac1 may be involved in the suppressive effects of estrogen on CF6-induced salt-sensitive hypertension and cardiac systolic dysfunction. In another study, we showed that in female TG mice at the age of 55–75 weeks under a normal-salt diet, Rac1 activities in the kidneys and heart were increased, whereas the serum level of estradiol was decreased to $<10\,\mathrm{pg\,ml^{-1}}$. This suggests that a negative relationship between estrogen and Rac1 activity may be present in TG mice.

Rac1 belongs to the small (21 kDa) Rho GTPase family, which binds to and hydrolyzes GTP. In an active GTP-bound state, Rac1 is involved in cellular functions in the vessels, heart and kidneys. In the cardiovascular system, activation of Rac1 is essential for the release of reactive oxygen species in the vessel walls and heart,[22,47,48] which impairs endothelial and cardiac functions and accelerates the progression of atherosclerotic lesions and heart failure.[49–52] The NADPH oxidase complex is regarded as an important source of reactive oxygen species in the vessel walls and heart.[53] Rac1 GTPase has a pivotal role in the assembly and activation of the NADPH enzymatic system, which is composed of several subunits, including p22phox, the flavoprotein p91phox and the cytoplasmic subunits p47phox and p67phox.[22,23] We previously showed that overexpression of CF6 induced activation of NADPH oxidase in the heart under a high-salt diet, resulting in cardiac systolic dysfunction in male mice. In the present study, we further showed that estrogen attenuated CF6-induced cardiac systolic dysfunction, presumably via Rac1 inactivation in females, although the activity of NADPH oxidase was not measured by analysis of radical generation. Another role for Rac1 is the activation of mineralocorticoid receptor signal transduction.[21] Renal Rac1, which is activated by a high-salt diet in Dahl salt-sensitive rats, is associated with massive proteinuria.[21] According to the dual role of

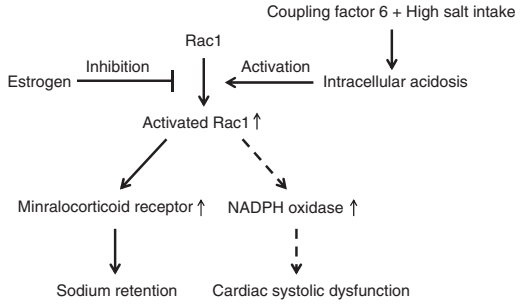


Figure 7 Summary of the proposed mechanism of the interaction of CF6 and estrogen.

Rac1 in the heart and kidneys, the estrogen-induced decrease in the ratio of Rac1-GTP to total Rac1 seems to explain not only a mechanistic linkage of CF6-mediated salt-sensitive hypertension to systolic cardiac dysfunction under a high-salt diet but also the disparity of gender in the onset of salt-sensitive hypertension and systolic cardiac dysfunction in female TG mice.

The present study showed that estrogen replacement led to the delayed onset of CF6-induced salt-sensitive hypertension and rescue from cardiac systolic dysfunction, presumably via the inactivation of the Rac1 GTPase in the kidneys and the heart (Figure 7). According to experimental and population-based studies, female gender and estrogen protect the cardiovascular system against radical- and aldosterone-induced injury. Thus, the present findings seem to provide new insight into our understanding of gender-related medicine in cases of pathological conditions with CF6 overexpression.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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