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Exercise training upregulates nitric oxide synthases in the kidney of rats with chronic heart failure

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SUMMARY

1. There is an interaction between heart and kidney diseases, which is a condition termed cardiorenal syndrome. Exercise training has cardioprotective effects, involving upregulation of endothelial (e) nitric oxide synthase (NOS) in the cardiovascular system. However, the effects of exercise training on NOS in the kidney with heart disease are unknown.

2. The aim of the present study was to investigate whether exercise training upregulates NOS in the kidney, left ventricle and aorta of rats with chronic heart failure (CHF).

3. Male Sprague-Dawley rats underwent left coronary artery ligation (LCAL) to induce CHF and were randomly assigned to sedentary or treadmill exercise groups 4 weeks after LCAL. Three days after exercising for 4 weeks, urine samples were collected for 24 h and blood samples were collected following decapitation. Nitric oxide synthase activity and protein expression were examined.

4. Significant interactions between CHF and exercise training were observed on parameters of cardiac and renal function. Exercise training improved cardiac function, decreased plasma B-type natriuretic peptide levels, decreased urinary albumin excretion and increased creatinine clearance in CHF rats. Nitric oxide synthase activity, eNOS expression and neuronal (n) NOS expression were significantly decreased in the left ventricle and kidney of CHF rats. Exercise training significantly increased NOS activity and eNOS and nNOS expression.

5. Upregulation of NOS in the kidney and left ventricle may contribute, in part, to the renal and cardiac protective effects of exercise training in cardiorenal syndrome in CHF rats.

Key words: chronic heart failure, exercise, kidney, nitric oxide synthase.

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INTRODUCTION

The heart and kidney play important interacting roles in the regulation of the systemic circulation. Chronic heart disease and chronic kidney disease frequently coexist, a condition termed cardiorenal syndrome.[1] Approximately 60–80% of patients with chronic heart failure (CHF) exhibit renal dysfunction.[2] Chronic heart failure can cause progressive renal injury, a condition termed Type 2 cardiorenal syndrome.[1] Furthermore, CHF decreases cardiac output and renal blood flow, and the sympathetic nervous system and renin–angiotensin–aldosterone system (RAAS) are activated as neurohumoral regulators to preserve the circulation.[3]

Exercise training (exercise) is known to have cardioprotective effects in animal models and humans with heart disease[4] and is recommended as cardiac rehabilitation for patients with CHF.[5] In addition, exercise can produce antihypertensive and renoprotective effects in rats exhibiting chronic renal failure (CRF) with 5/6 nephrectomy.[6] Exercise improves blunted renal excretory responses to acute volume expansion with normalization of renal sympathetic nerve activity (RSNA) in CHF rats.[7] However, the factors responsible for the beneficial effects of exercise on the kidney in CHF are not fully understood.

Nitric oxide (NO) is a vasodilatory factor synthesized by three isoforms of NO synthase (NOS), namely endothelial (e), neuronal (n) and inducible (i) NOS. Nitric oxide plays an important role in protecting cardiac function against myocardial ischaemia.[4] The expression of eNOS protein was reported to be downregulated in the left ventricle (LV) of rats after experimental myocardial infarction (MI), whereas the eNOS enhancer AVE9488 improved LV remodelling.[8] Interestingly, exercise can restore LV function and prevent fibrosis and apoptosis of the myocardium in $eNOS^{+/+}$ mice with MI, whereas the cardioprotective effects of exercise are attenuated in $eNOS^{+/-}$ and $eNOS^{-/-}$ mice.[9] In addition to eNOS expression in the cardiovascular system, exercise restores decreased nNOS expression in the paraventricular nucleus of CHF rats and contributes to the restoration of increased RSNA.[10]

Although previous studies have demonstrated that the cardioprotective effects of exercise are mediated by upregulation of eNOS in the cardiovascular system,[4] the effects of exercise on NO in the kidney in CHF remain unclear. Nitric oxide has various renal effects, including regulation of renal haemodynamics, renin secretion and inhibition of tubular Na reabsorption, tubuloglomerular feedback (TGF) and RSNA.[11,12] Chronic NOS inhibition induces renal damage, proteinuria and glomerular sclerotic

injury in rats,[13] whereas eNOS and nNOS expression is downregulated in the kidney of rats with CRF.[14] Recent studies have reported that exercise has antihypertensive and renoprotective effects with upregulation of renal eNOS[15,16] and nNOS[15] expression in spontaneously hypertensive rats (SHR).

The localization and involvement of renal NOS in the renal vessels and tubules have been studied extensively. The renal vesa recta, glomeruli and afferent arterioles contain large amounts of constitutive NOS isoforms (eNOS and nNOS),[17] whereas nNOS expression in the macula densa of the cortex contributes to the regulation of TGF and renin secretion.[12,18] In each segment of the rat kidney, eNOS and nNOS protein levels are different, being low in the cortex, high in the outer medulla and particularly high in the inner medulla.[19] Furthermore, NO has been reported to contribute to improvements in medullary blood flow in Dahl salt-sensitive rats,[20] suggesting that NO and NOS play differing roles in each segment of the kidney.

Therefore, in the present study we tested the hypothesis that NOS expression in the kidney and cardiovascular system is downregulated in CHF and that exercise restores cardiac function with upregulation of NOS expression in those tissues. To this end, we examined whether NOS expression was downregulated and exercise could upregulate NOS expression in various regions of the kidney (i.e. cortex, outer medulla and inner medulla) and cardiovascular system in CHF rats.

METHODS

Animals

Twenty-five male Sprague-Dawley rats (8 weeks old; weighing 250–270 g) were obtained from SLC (Shizuoka, Japan). Rats were housed in the animal care facility at Tohoku University School of Medicine, with free access to standard laboratory chow and water, a controlled temperature (24°C) and under a 12 h light–dark cycle. All protocols involving rats were reviewed by and received prior approval from the Animal Welfare Committee of Tohoku University.

Surgical procedures

Rats underwent either left coronary artery ligation (LCAL; $n = 15$) to induce MI or a sham operation ($n = 10$), as described previously.[21] Briefly, rats were anaesthetized with sodium pentobarbitone (50 mg/kg, i.p.) and buprenorphine (0.01 mg/kg, s.c.) was given before surgery. The trachea was then cannulated to facilitate ventilation (room air; 60 strokes/min; tidal volume 3 mL). In the LCAL group, following left thoracotomy, the heart was exteriorized and a 6–0 nylon suture was ligated between the pulmonary artery outflow tract and left atrium. The sham rats underwent thoracotomy and manipulation of the heart but without LCAL. In the LCAL group, two rats died during surgery or within 1 week after surgery.

Echocardiographic measurement

To evaluate cardiac function, echocardiographic measurements were performed before and after the exercise protocol using an echocardiographic system equipped with a 10 MHz transducer (SonoHeart Elite; SonoSite, Bothell, WA, USA). Rats were anaesthetized with 2% isoflurane mixed with oxygen. Parasternal long and short axis views of the LV were obtained, ensuring that the mitral and aortic valves and apex were well visualized. Area fraction and wall area were determined by planimetry of end-diastolic and end-systolic volumes in the parasternal short axis. Measurements of LV end-diastolic and end-systolic dimensions were obtained in the M-mode at the mid-papillary level from more than three beats and fractional shortening (FS) was calculated as follows:

$$\mathrm{FS}(\%) = (\mathrm{LVIDd} - \mathrm{LVIDs})/\mathrm{LVIDd}) \times 100$$

where LVIDs and LVIDd are LV internal diameter at systole and diastole, respectively. All measurements were averaged over three consecutive cardiac cycles. Chronic heart failure was characterized by an LV ejection fraction (EF) < 40%, or LVFS < 15% (10 of 13 LCAL rats were identified as having CHF).

Experimental groups and exercise training protocol

Four weeks after surgery, rats were randomized into groups as follows ($n = 5$ in each group): Sed-Sham, sedentary sham group; Ex-Sham, exercised sham group; Sed-CHF, sedentary CHF group; and Ex-CHF, exercised CHF group. Rats in the Ex-Sham and Ex-CHF groups were exercised on a treadmill (KN-73; Natsume Industries, Tokyo, Japan) for 10 min/day at an initial treadmill speed of 10 m/min on a 0% grade. Treadmill speed was increased gradually over a period of 1 week to 25 m/min and the duration of exercise was increased to 60 min/day. Oxygen consumption (VO_2) when rats were running at a speed of 25 m/min corresponded to 60–80% of peak VO_2. The treadmill exercise (10–60 min/day, 5 days/week) was performed for 4 weeks.

Exercise endurance ability

Peak VO_2 and total running distance were measured using an oxygen–carbon dioxide metabolism measuring system with a sealed chamber treadmill (Model MK-5000; Muromachikikai, Tokyo, Japan), as described previously,[22,23] on the final day of the exercise protocol. Briefly, rats were submitted to stepwise increasing exercise by 5 m/min for 3 min each on a motor treadmill at an initial speed of 15 m/min. The exercise was stopped when rats were unable to continue running because of exhaustion despite contact with a shock bar located at the rear of treadmill belt.

Blood pressure and plasma and urine parameters

Systolic blood pressure (SBP) was measured after the exercise protocol in conscious rats using an indirect tail-cuff method (Model UR-5000; Ueda, Tokyo, Japan), as described previously.[24] After the exercise protocol, all rats were housed in individual metabolic cages (Model ST; Sugiyama-General, Tokyo, Japan) for 3 days to acclimatize to the conditions. Urine samples were collected on ice over a period of 24 h. Four days after the last exercise session, all rats were anaesthetized with sodium pentobarbitone (50 mg/kg, i.p.) and blood samples were collected

after rats had been decapitated. The blood samples were centrifuged for 5 min at 1500 *g* and the supernatant was collected and stored at −80°C. Creatinine, blood urea nitrogen, total cholesterol and triglyceride plasma levels and urinary creatinine, protein and sodium were determined using standard autoanalytical techniques (BML, Tokyo, Japan). Plasma B-type natriuretic peptide (BNP) levels were measured by SRL (Tokyo, Japan). Urinary albumin concentrations were determined using commercially available assay kits (AKRAL-120; Shibayagi, Shibukawa, Japan), as described previously.[25]

Renal NOS activity measurements

After rats had been decapitated, their hearts, thoracic aorta and kidneys were quickly removed and the non-infarcted zone of the LV was isolated from the heart. The kidney was hemisected and sectioned into the cortex, the inner stripe of the outer medulla and the inner medulla. These tissues were homogenized in 100 mmol/L potassium buffer (pH 7.25) containing 30% glycerol, 1 mmol/L dithiothreitol and 0.1 mmol/L phenylmethylsulphonyl fluoride. The samples were snap frozen in liquid nitrogen and stored at −80°C. Protein concentrations in the samples were determined using the Bradford method[26] with bovine γ-globulin as the standard (Bio-Rad Laboratories, Hercules, CA, USA).

For determination of NOS activity, the *in vitro* formation of nitrate/nitrite (NO_x) by each tissue was evaluated using commercially available kits (Oxford Biomedical Research, Rochester Hills, MI, USA), as described previously.[15]

Western blot analysis

Nitric oxide synthase protein expression was examined by western blot analysis, as described previously.[15,19] Briefly, protein samples (50 μg) were separated by electrophoresis on a 5.8% sodium dodecyl sulphate polyacrylamide gel and blotted onto nitrocellulose membranes. Proteins were revealed with specific antibodies raised against eNOS, nNOS and iNOS (BD Transduction Laboratories, San Diego, CA, USA) and were developed using an enhanced chemiluminescence kit (Super Signal; Thermo Fisher Scientific, Waltham, MA, USA). The intensities of the bands for each NOS protein were normalized against those for β-actin, used as an internal standard. The intensity of the bands in the Sed-Sham group was assigned a value of 1.

Statistical analysis

For comparisons of biochemical parameters and all NOS data, two-way ANOVA was used to test for differences between sedentary and exercised rats under conditions of sham operation and CHF. For echocardiographic data, repeated-measures ANOVA with conditions × treatment × time interaction was used. If the ANOVA showed a significant effect, further post hoc analysis was performed using Tukey's method for comparisons between four groups and the Bonferroni test was used for comparisons over time and between the four groups for echocardiographic data. In the Bonferroni test, *P*-values were adjusted by dividing them by four to account for the comparisons between 4 and 8 weeks in each of the four groups. All statistical tests were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). Two-tailed $P < 0.05$ was considered significant. Data are presented as the mean ± SEM.

RESULTS

Effects of exercise on biochemical parameters

Table 1 summarizes the characteristics of the four groups at the end of the experiment. Significant differences were observed in

Table 1 Effects of exercise training on biochemical parameters in sedentary and exercised sham-operated and chronic heart failure rats

	Sed-Sham	Ex-Sham	Sed-CHF	Ex–CHF	Interaction (*P* value)
BW (g)	458 ± 13	426 ± 14	399 ± 25	398 ± 16	0.393
Heart weight/BW (g/kg)	2.58 ± 0.09	3.13 ± 0.24	4.45 ± 0.50**	4.02 ± 0.23*	0.129
Lung weight/BW (g/kg)	4.24 ± 0.17	4.09 ± 0.08	11.09 ± 1.46**	6.46 ± 1.26†	0.034
Kidney weight/BW (g/kg)	5.56 ± 0.10	6.09 ± 0.29	6.44 ± 0.53	6.11 ± 0.08	0.179
SBP (mmHg)	114 ± 4	109 ± 1	99 ± 7	103 ± 8	0.412
Plasma					
Cr (mg/dL)	0.248 ± 0.020	0.248 ± 0.016	0.256 ± 0.017	0.214 ± 0.009	0.215
BUN (mg/dL)	18.7 ± 0.9	17.4 ± 1.2	16.9 ± 0.8	15.1 ± 0.4*	0.082
TC (mg/dL)	65.6 ± 3.8	74.8 ± 4.3	58.3 ± 4.0	54.2 ± 2.2	0.091
TG (mg/dL)	119 ± 15	59 ± 13*	57 ± 16*	53 ± 6*	0.047
BNP (pg/mL)	124 ± 5	114 ± 2	253 ± 45**	138 ± 15††	0.021
Urine					
Protein (mg/day)	24.9 ± 2.1	14.0 ± 1.6	15.6 ± 4.9	19.7 ± 2.7	0.029
Sodium (mmol/day)	1.93 ± 0.07	1.40 ± 0.12	1.48 ± 0.26	1.70 ± 0.12	0.032
ACR (mg/g Cr per day)	203 ± 18	163 ± 22	562 ± 76**	256 ± 58††	0.017
Cr clearance/BW (mL/min per 100 g)	1.08 ± 0.08	0.88 ± 0.10	0.99 ± 0.04	1.39 ± 0.13†	0.006

Data are mean ± SEM ($n = 5$ rats per group). *$P < 0.05$, **$P < 0.01$ compared with the sedentary sham-operated (Sed-Sham) group; †$P < 0.05$, ††$P < 0.01$ compared with the sedentary chronic heart failure (Sed-CHF) group (two-way ANOVA with conditions × treatment interaction followed by Tukey's post hoc test).

Ex-Sham, exercised sham-operated rats; Ex-CHF, exercised chronic heart failure rats; BW, bodyweight; SBP, systolic blood pressure; Cr, creatinine; BUN, blood urea nitrogen; TC, total cholesterol; TG, triglycerides; BNP, B-type natriuretic peptide; ACR, albumin : creatinine ratio.

the ratio of lung weight : bodyweight, plasma BNP levels, urinary albumin-to-creatinine ratio (ACR) per day and the ratio of creatinine clearance : bodyweight between the effects of CHF and exercise. Although there was no significant difference in bodyweight between the groups, the heart weight : bodyweight and lung weight : bodyweight ratios were significantly higher in the Sed-CHF group than in the Sed-Sham group. Furthermore, the lung weight : bodyweight ratio was significantly lower in the Ex-CHF compared with Sed-CHF group. There were no differences in the kidney weight : bodyweight ratio or in SBP among the groups. Plasma creatinine levels did not differ significantly between the groups. Plasma BNP levels were significantly higher in the Sed-CHF group than in the Sed-Sham group, but were significantly lower in the Ex-CHF compared with Sed-CHF group. Urinary ACR per day was significantly greater in the Sed-CHF than Sed-Sham group, whereas the ACR per day was significantly reduced in the Ex-CHF compared with Sed-CHF group. The creatinine clearance : bodyweight ratio was significantly higher in the Ex-CHF compared with Sed-CHF group.

Effects of exercise on cardiac function

Table 2 summarizes the echocardiographic data 4 and 8 weeks after surgery. For all echocardiographic data, significant interactions between the effects of CHF and exercise were observed. Four weeks after surgery, cardiac function was impaired in CHF compared with sham rats, with a significant reduction in EF and FS in both CHF groups compared with both sham groups. There was also a significant increase in LVIDs and LVIDd in both CHF groups compared with both sham groups. Eight weeks after surgery, cardiac function was further impaired in the Sed-CHF group, with a trend for a further decrease in EF and FS and a significant increase in LVIDs and LVIDd compared with 4 weeks after surgery. In contrast with the Sed-CHF group, cardiac function was improved in the Ex-CHF group 8 weeks after surgery, with a significant increase in EF and FS compared with 4 weeks after surgery. These echocardiographic data indicate that LCAL-induced cardiac dysfunction was improved by exercise for 4 weeks.

Effects of exercise on exercise endurance

Table 3 gives peak VO_2 and total running distance for the four groups. Two-way ANOVA with conditions × treatment interaction showed no significant effect on peak VO_2 and total running distance. Peak VO_2 was significantly higher in the sham groups than in the CHF groups ($P < 0.005$) and significantly higher in the Ex groups than in the Sed groups ($P < 0.001$). Similarly, total running distance was significantly longer in the sham groups than in the CHF groups ($P < 0.001$) and significantly longer in the Ex groups than in the Sed groups ($P < 0.001$).

Effects of exercise on NOS activity

Figure 1 shows NOS activity in the four groups. A significant interaction was observed by two-way ANOVA in the LV, aorta and all renal tissues ($P < 0.01$ for all). Nitric oxide synthase activity was significantly lower in the LV and all renal tissues (cortex, outer medulla and inner medulla) of the Sed-CHF group than the Sed-Sham group, with no differences in the aorta. Nitric oxide synthase activity was significantly higher in the LV, aorta and all renal tissues in the Ex-CHF group compared with the Sed-CHF group. In addition, NOS activity was significantly higher in the aorta and renal inner medulla of the Ex-Sham group compared with the Sed-Sham group, but was not significantly different in the LV, renal cortex and outer medulla.

Effects of exercise on NOS expression

Figure 2 shows immunoblots comparing levels of eNOS protein in the four groups. A significant interaction was observed by two-way ANOVA in the LV, aorta and all renal tissues ($P < 0.01$ for all). Levels of eNOS protein were significantly lower in the LV and all renal tissues of the Sed-CHF compared with

Table 2 Effects of exercise training on echocardiographic data obtained 4 and 8 weeks after left coronary artery ligation (or sham operation) in sedentary and exercised sham-operated and chronic heart failure rats

	Sed-Sham	Ex-Sham	Sed-CHF	Ex–CHF	Interaction (*P*)
Ejection fraction (%)					
4 weeks	68.8 ± 3.9	79.1 ± 1.1	34.1 ± 2.7**	35.0 ± 1.5**	< 0.001
8 weeks	73.0 ± 1.0	72.6 ± 1.9	28.9 ± 2.4**	57.7 ± 3.8**††§	
Fractional shortening (%)					
4 weeks	32.7 ± 3.0	38.1 ± 1.1	13.1 ± 1.2**	13.7 ± 0.9**	< 0.001
8 weeks	35.4 ± 0.8	35.3 ± 1.5	10.8 ± 1.0**	24.5 ± 2.6**††§	
LV internal diameter at systole (mm)					
4 weeks	5.95 ± 0.37	4.79 ± 0.30	9.28 ± 0.23**	8.66 ± 0.34**	< 0.001
8 weeks	5.87 ± 0.13	6.02 ± 0.11§	10.67 ± 0.15**§	7.93 ± 0.28**††	
LV internal diameter at diastole (mm)					
4 weeks	8.83 ± 0.21	7.76 ± 0.40	10.67 ± 0.16**	10.02 ± 0.31	< 0.005
8 weeks	9.09 ± 0.16	9.31 ± 0.15§	11.97 ± 0.08**§	10.46 ± 0.12**††	

Data are mean ± SEM ($n = 5$ rats per group). **$P < 0.01$ compared with the sedentary sham-operated (Sed-Sham) group; ††$P < 0.01$ compared with the sedentary chronic heart failure (Sed-CHF) group; §$P < 0.01$ compared with values obtained at 4 weeks in the same group. Data were analysed by repeated-measure ANOVA with conditions × treatment × time interaction followed by the Bonferroni post hoc test. *P* values were adjusted by dividing them by four to account for comparisons between 4 and 8 weeks in each of the four groups .

Ex-Sham, exercised sham-operated rats; Ex-CHF, exercised chronic heart failure rats; LV, left ventricle.

Table 3 Effects of exercise training on peak oxygen consumption and total running distance in sedentary and exercised sham-operated and chronic heart failure rats

	Sed-Sham	Ex-Sham	Sed-CHF	Ex-CHF	*P* values		
					Interaction	Sham *vs* CHF	Sed *vs* Ex
Oxygen uptake (mL/min per kg)	63.1 ± 4.4	82.3 ± 3.0	44.2 ± 7.5	66.9 ± 4.8	0.736	< 0.005	< 0.001
Distance (m)	279 ± 16	822 ± 15	128 ± 22	611 ± 34	0.208	< 0.001	< 0.001

Data are mean ± SEM (n = 5 rats per group). Data were analysed by two-way ANOVA with conditions × treatment interaction.

All rats were submitted to stepwise increasing exercise on a motor treadmill. The exercise was stopped when rats were unable to continue running because of exhaustion.

Sed-Sham, sedentary sham-operated rats; Ex-Sham, exercised sham-operated rats; Sed-CHF, sedentary chronic heart failure rats; Ex-CHF, exercised chronic heart failure rats; Sham, sham operation; CHF, chronic heart failure; Sed, sedentary; EX, exercise.

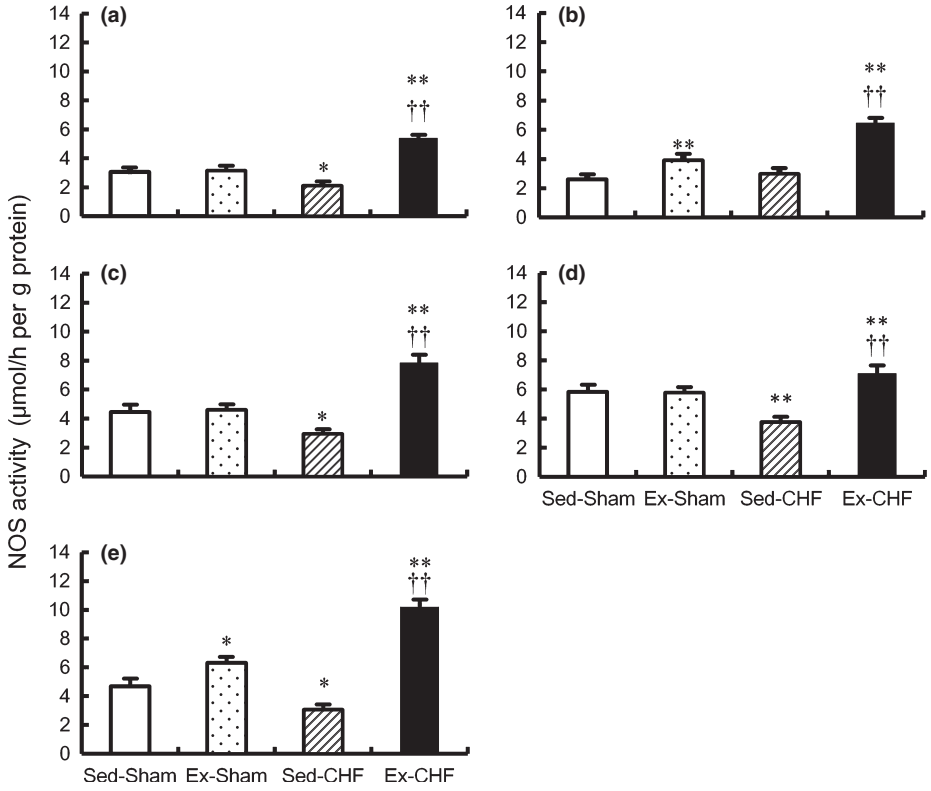

Fig. 1 Effects of exercise training on nitric oxide synthase (NOS) activity in the (a) left ventricle, (b) thoracic aorta, (c) renal cortex, (d) outer medulla and (e) inner medulla of sedentary sham-operated (Sed-Sham), exercised sham-operated (Ex-Sham), sedentary chronic heart failure (Sed-CHF) and exercised chronic heart failure (Ex-CHF) rats. Data are the mean ± SEM (n = 5 rats per group). *P < 0.05, **P < 0.01 compared with the Sed-Sham group; $^{\dagger\dagger}P$ < 0.01 compared with the Sed-CHF group (two-way ANOVA with conditions × treatment interaction followed by Tukey's post hoc test).

Sed-Sham group, but were not significantly different in the aorta. Levels of eNOS protein were significantly higher in the LV, aorta and all renal tissues of the Ex-CHF group compared with the Sed-CHF group. In addition, eNOS protein levels were significantly higher in the aorta and renal inner medulla of the Ex-Sham group compared with the Sed-Sham group, but were not significantly different in the LV, renal cortex and outer medulla.

Figure 3 shows immunoblots comparing nNOS protein levels in the four groups. A significant interaction was observed by two-way ANOVA in the LV and all renal tissues (P < 0.01 for all). Levels of nNOS protein were significantly lower in the LV and all renal tissues of the Sed-CHF compared with Sed-Sham group. Levels of nNOS protein were significantly higher in the LV and all renal tissues of the Ex-CHF compared with the Sed-CHF group. In addition, nNOS protein levels were significantly higher in the LV and renal inner medulla of the Ex-Sham compared with Sed-Sham group, but were not significantly different in the renal cortex and outer medulla. Bands for nNOS in the aorta were not detected in the Sed-Sham and Sed-CHF groups, but were detected in the Ex-Sham and Ex-CHF groups. Levels of iNOS protein in the LV, aorta and all renal tissues did not differ significantly among the groups (data not shown).

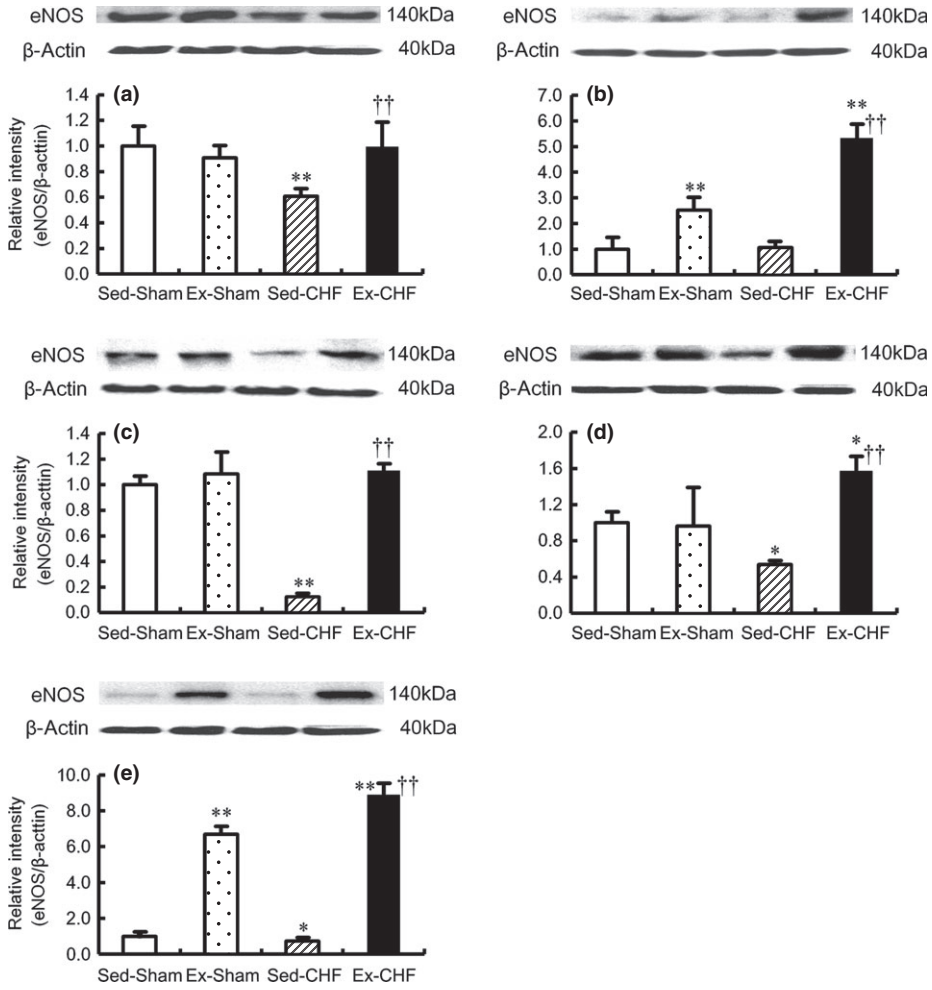

Fig. 2 Effects of exercise training on endothelial nitric oxide synthase (eNOS) protein expression in the (a) left ventricle, (b) thoracic aorta, (c) renal cortex, (d) outer medulla and (e) inner medulla of sedentary sham-operated (Sed-Sham), exercised sham-operated (Ex-Sham), sedentary chronic heart failure (Sed-CHF) and exercised chronic heart failure (Ex-CHF) rats. Top panels show representative immunoblots from the different groups. The intensities of the eNOS bands (140 kDa) for each protein were normalized against that of β-actin (40 kDa) and the intensity of the band in the Sed-Sham group was assigned a value of 1. Data are the mean ± SEM (n = 5 rats per group). *$P < 0.05$, **$P < 0.01$ compared with the Sed-Sham group; $^{\dagger\dagger}P < 0.01$ compared with the Sed-CHF group (two-way ANOVA with conditions × treatment interaction followed by Tukey's post hoc test).

DISCUSSION

The present study showed that exercise upregulated NOS activity and eNOS and nNOS expression in the kidney and cardiovascular system in CHF rats, which was accompanied by improvements in cardiac function and albuminuria. Furthermore, a significant interaction between effect of CHF and effect of exercise was observed on the parameters of cardiac and renal function.

More severe LV dysfunction and remodelling after MI develops in eNOS-deficient compared with wild-type mice,[27] whereas endothelial overexpression of eNOS attenuates LV dysfunction in mice after MI.[28] In addition, more severe LV dysfunction and remodelling after MI develop in nNOS-knockout mice.[29] Therefore, by increasing eNOS and nNOS expression, exercise may improve LV dysfunction and remodelling after MI.

To the best of our knowledge, the present study is the first to demonstrate that eNOS and nNOS expression is reduced in the kidney of CHF rats. In agreement with the present study, regulation of renal O_2 consumption is impaired in dogs with CHF,[30] suggesting that renal NO may be downregulated in CHF. In addition, eNOS and nNOS expression has been reported to be downregulated in the kidney of rats with CRF,[14] and chronic NOS inhibition induces renal damage, proteinuria and glomerular sclerotic injury.[13] In the present study, urinary albumin excretion was increased in CHF rats. Therefore, the decreased eNOS and nNOS expression in the kidney may indicate the development of renal dysfunction in CHF. Decreased shear stress via decreased renal blood flow may be one of the mechanisms underlying the downregulation of NOS activity and NOS expression in the kidneys in CHF. In addition, decreased renal O_2 consumption[30] may contribute to the downregulation of these renal NOS factors in CHF.

A previous study reported that acute exercise downregulated eNOS expression in the rat kidney, which was considered to occur in response to a reduction in renal blood flow and shear stress during exercise.[31] In the present study, because rats were killed 4 days after the last exercise session, the effects of exercise were not those of acute exercise, but the effects of chronic exercise for 4 weeks. Exercise may initially improve cardiac output and then gradually lead to restoration of renal blood flow in

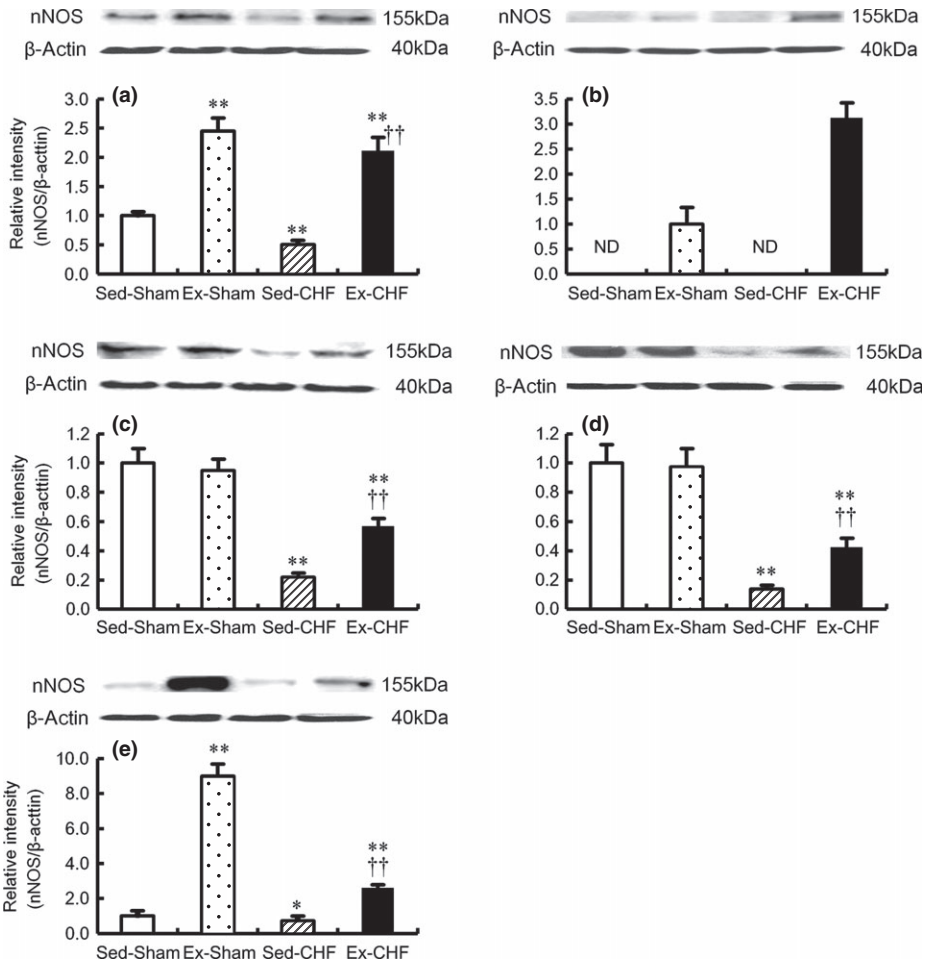

Fig. 3 Effects of exercise training on neuronal nitric oxide synthase (nNOS) protein expression in the (a) left ventricle, (b) thoracic aorta, (c) renal cortex, (d) outer medulla and (e) inner medulla of sedentary sham-operated (Sed-Sham), exercised sham-operated (Ex-Sham), sedentary chronic heart failure (Sed-CHF) and exercised chronic heart failure (Ex-CHF) rats. Top panels show representative immunoblots from the different groups. The intensities of the nNOS bands (155 kDa) for each protein were normalized against those for β-actin (40 kDa) and the intensity of the band in the Sed-Sham group was assigned a value of 1. Data are the mean ± SEM (n = 5 rats per group). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with the Sed-Sham group; $^{\dagger\dagger}P < 0.01$ compared with the Sed-CHF group (two-way ANOVA with conditions × treatment interaction followed by Tukey's post hoc test). ND, not detected.

CHF. Restoration of renal blood flow may be responsible for the increase in eNOS expression in the kidney.

The present study showed that exercise induced nNOS expression in the kidney and cardiovascular system. In CHF rabbits, exercise attenuates enhanced RSNA and normalizes changes in the expression of nNOS protein, angiotensin (Ang) II AT_1 receptor protein and AngII in the carotid body.[32] Therefore, induction of nNOS expression in the cardiovascular system and kidney by exercise may contribute, in part, to the beneficial effects of exercise through inhibition of the sympathetic nervous system and RAAS. In addition, the induction of nNOS expression in the renal cortex by exercise suggests that increased nNOS in the macula densa may contribute to the regulation of TGF and the RAAS.[12,18]

In the present study, NOS activity in the LV, aorta and all renal tissues in the Ex-CHF group was higher than in the other groups. In addition, eNOS expression in the aorta, renal outer medulla and inner medulla in the Ex-CHF group was higher than in the other groups. However, nNOS expression in all renal tissues was not completely restored to normal levels in the Ex-CHF group. These findings suggest that eNOS has a greater effect on increased NOS activity than nNOS. Shear stress by blood flow may be the main factor affecting eNOS expression. Sensitivity of eNOS expression to shear stress may be high under conditions of low blood flow in CHF. This possibility may support our finding that eNOS and NOS activities in the Ex-CHF group were higher than in the other groups.

A dilated LV cavity was observed under normal conditions in Ex-Sham rats. Exercise causes sport-specific LV remodelling, enlarges LV end-diastolic diameters and increases LV wall thickness.[33] In our model, chronic exercise may have caused the eccentric LV enlargement. In the kidney, exercise had no effect on ACR or creatinine clearance in sham normal rats, but upregulated eNOS and nNOS expression in the inner medulla. However in CHF, exercise improved cardiac and renal function and increased NOS activity and eNOS and nNOS expression in the LV and kidney. Therefore, elevation of these NO factors by exercise may contribute, at least in part, to the recovery of cardiac and renal function in CHF rats. Under normal conditions, elevated nNOS in the LV may contribute to a dilated LV cavity,

leading to an increase in exercise endurance ability. However, elevated NOS activity and eNOS and nNOS expression in the renal inner medulla may have no effect on renal function. Previously, we reported that exercise upregulates renal NADPH oxidase activity in Wistar-Kyoto rats but has no effect on renal function.[15] In the present study, exercise may have had an effect on renal oxidative stress in normal rats. Further studies are necessary to identify the different mechanisms operating under normal and CHF conditions.

The increased NOS factors in the LV may play some role in the improvement in exercise endurance, whereas the increased NOS factors in the kidney may not have a direct effect. However, increased NOS activity in the kidney may contribute indirectly to improvements in cardiac function by suppressing the RAAS and sympathetic nerve activity, or other neurohumoral factors. Furthermore, increased NOS in the kidney may be transported to skeletal muscle and have beneficial effects there, such as vasodilation, leading to improvements in exercise endurance.

In our study model, CHF causes progressive renal injury, resulting in a condition termed Type 2 cardiorenal syndrome.[1] In the present study, exercise may have initially improved cardiac function and secondarily improved renal function, thereby inducing a beneficial interaction between the heart and kidney. Indeed, CHF rats were able to run on a treadmill at 25 m/min, as were sham rats, in 1 week, suggesting that exercise may improve cardiac function at an early stage. In a previous study, we found that 4 weeks of exercise had no effect on renal NOS expression and function in SHR, but 8 weeks exercise affected both parameters.[15] Furthermore, 8 weeks exercise had no effect on renal NOS expression and function in rats exhibiting CRF with 5/6 nephrectomy, but 12 weeks exercise affected both parameters (D Ito, unpubl. data, 2012), suggesting that exercise takes a long time to affect renal function. Further studies are needed to identify the mechanism underlying the cardiorenal protection afforded by exercise.

In conclusion, we found that downregulation of NOS activity and eNOS and nNOS expression in the cardiovascular system and kidney of CHF rats was improved by exercise, resulting in improved cardiac function, albuminuria and increased creatinine clearance. These findings suggest that upregulation of renal eNOS and nNOS may be a novel mechanism involved in the beneficial effects of exercise in CHF and that exercise may be a therapeutic approach for patients with cardiorenal syndromes.

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DISCLOSURE

The authors declare no conflicts of interest.

REFERENCES

1. Ronco C, McCullough P, Anker SD *et al.* Cardio–renal syndromes: Report from the consensus conference of the acute dialysis quality initiative. *Eur. Heart J.* 2010; **31**: 703–11.
2. Fonarow GC, Heywood JT. The confounding issue of comorbid renal insufficiency. *Am. J. Med.* 2006; **119**(Suppl.): S17–25.
3. Schrier RW, Abraham WT. Hormones and hemodynamics in heart failure. *N. Engl. J. Med.* 1999; **341**: 577–85.
4. Calvert JW. Cardioprotective effects of nitrite during exercise. *Cardiovasc. Res.* 2011; **89**: 499–506.
5. Anderson JL, Adams CD, Antman EM *et al.* ACC/AHA 2007 guidelines for the management of patients with unstable angina/non ST-elevation myocardial infarction: A report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Writing Committee to Revise the 2002 Guidelines for the Management of Patients With Unstable Angina/Non ST-Elevation Myocardial Infarction): Developed in collaboration with the American College of Emergency Physicians, the Society for Cardiovascular Angiography and Interventions, and the Society of Thoracic Surgeons: Endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation and the Society for Academic Emergency Medicine. *Circulation* 2007; **116**: e148–304.
6. Kohzuki M, Kamimoto M, Wu XM *et al.* Renal protective effects of chronic exercise and antihypertensive therapy in hypertensive rats with chronic renal failure. *J. Hypertens.* 2001; **19**: 1877–82.
7. Zheng H, Li YF, Zucker IH, Patel KP. Exercise training improves renal excretory responses to acute volume expansion in rats with heart failure. *Am. J. Physiol. Renal Physiol.* 2006; **291**: F1148–56.
8. Fraccarollo D, Widder JD, Galuppo P *et al.* Improvement in left ventricular remodeling by the endothelial nitric oxide synthase enhancer AVE9488 after experimental myocardial infarction. *Circulation* 2008; **118**: 818–27.
9. de Waard MC, van Haperen R, Soullie T, Tempel D, de Crom R, Duncker DJ. Beneficial effects of exercise training after myocardial infarction require full enos expression. *J. Mol. Cell. Cardiol.* 2010; **48**: 1041–9.
10. Zheng H, Li YF, Cornish KG, Zucker IH, Patel KP. Exercise training improves endogenous nitric oxide mechanisms within the paraventricular nucleus in rats with heart failure. *Am. J. Physiol. Heart Circ. Physiol.* 2005; **288**: H2332–41.
11. Garvin JL, Herrera M, Ortiz PA. Regulation of renal NaCl transport by nitric oxide, endothelin, and ATP: Clinical implications. *Annu. Rev. Physiol.* 2011; **73**: 359–76.
12. Mount PF, Power DA. Nitric oxide in the kidney: Functions and regulation of synthesis. *Acta Physiol.* 2006; **187**: 433–46.
13. Baylis C, Mitruka B, Deng A. Chronic blockade of nitric oxide synthesis in the rat produces systemic hypertension and glomerular damage. *J. Clin. Invest.* 1992; **90**: 278–81.
14. Fujihara CK, Mattar AL, Vieira JM Jr *et al.* Evidence for the existence of two distinct functions for the inducible NO synthase in the rat kidney: Effect of aminoguanidine in rats with 5/6 ablation. *J. Am. Soc. Nephrol.* 2002; **13**: 2278–87.
15. Ito D, Ito O, Cao P *et al.* Effects of exercise training on nitric oxide synthase in the kidney of spontaneously hypertensive rats. *Clin. Exp. Pharmacol. Physiol.* 2013; **40**: 74–82.
16. Agarwal D, Elks CM, Reed SD, Mariappan N, Majid DS, Francis J. Chronic exercise preserves renal structure and hemodynamics in spontaneously hypertensive rats. *Antioxid. Redox Signal.* 2012; **16**: 139–52.
17. Mattson DL, Wu F. Nitric oxide synthase activity and isoforms in rat renal vasculature. *Hypertension* 2000; **35**: 337–41.
18. Tojo A, Onozato ML, Fujita T. Role of macula densa neuronal nitric oxide synthase in renal diseases. *Med. Mol. Morphol.* 2006; **39**: 2–7.
19. Ito D, Ito O, Mori N *et al.* Atorvastatin upregulates nitric oxide synthases with Rho-kinase inhibition and Akt activation in the kidney of spontaneously hypertensive rats. *J. Hypertens.* 2010; **28**: 2278–88.
20. Mori T, O'Connor PM, Abe M, Cowley AW Jr. Enhanced superoxide production in renal outer medulla of Dahl salt-sensitive rats reduces nitric oxide tubular–vascular cross-talk. *Hypertension* 2007; **49**: 1336–41.

21. Marumo R, Kaizuma S, Nogae S *et al.* Differential upregulation of rat Na–K–Cl cotransporter, rBSC1, mRNA in the thick ascending limb of Henle in different pathological conditions. *Kidney Int.* 1998; **54**: 877–88.
22. Tufescu A, Kanazawa M, Ishida A *et al.* Combination of exercise and losartan enhances renoprotective and peripheral effects in spontaneously type 2 diabetes mellitus rats with nephropathy. *J. Hypertens.* 2008; **26**: 312–21.
23. Ohki-Hamazaki H, Watase K, Yamamoto K *et al.* Mice lacking bombesin receptor subtype-3 develop metabolic defects and obesity. *Nature* 1997; **390**: 165–9.
24. Ikeda K, Nara Y, Yamori Y. Indirect systolic and mean blood pressure determination by a new tail cuff method in spontaneously hypertensive rats. *Lab. Anim.* 1991; **25**: 26–9.
25. Rafiq K, Nakano D, Ihara G *et al.* Effects of mineralocorticoid receptor blockade on glucocorticoid-induced renal injury in adrenalectomized rats. *J. Hypertens.* 2011; **29**: 290–8.
26. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 1976; **72**: 248–54.
27. Scherrer-Crosbie M, Ullrich R, Bloch KD *et al.* Endothelial nitric oxide synthase limits left ventricular remodeling after myocardial infarction in mice. *Circulation* 2001; **104**: 1286–91.
28. Jones SP, Greer JJ, van Haperen R, Duncker DJ, de Crom R, Lefer DJ. Endothelial nitric oxide synthase overexpression attenuates congestive heart failure in mice. *Proc. Natl Acad. Sci. USA* 2003; **100**: 4891–6.
29. Dawson D, Lygate CA, Zhang MH, Hulbert K, Neubauer S, Casadei B. nNOS gene deletion exacerbates pathological left ventricular remodeling and functional deterioration after myocardial infarction. *Circulation* 2005; **112**: 3729–37.
30. Adler S, Huang H, Loke K, Xu X, Laumas A, Hintze TH. Modulation of renal oxygen consumption by nitric oxide is impaired after development of congestive heart failure in dogs. *J. Cardiovasc. Pharmacol.* 2001; **37**: 301–9.
31. Miyauchi T, Maeda S, Iemitsu M *et al.* Exercise causes a tissue-specific change of NO production in the kidney and lung. *J. Appl. Physiol.* 2003; **94**: 60–8.
32. Li YL, Ding Y, Agnew C, Schultz HD. Exercise training improves peripheral chemoreflex function in heart failure rabbits. *J. Appl. Physiol.* 2008; **105**: 782–90.
33. Baggish AL, Wood MJ. Athlete's heart and cardiovascular care of the athlete. Scientific and clinical update. *Circulation* 2011; **123**: 2723–35.