

Cardiac sympathetic hyperinnervation in deoxycorticosterone acetate-salt hypertensive rats

Tsung-Ming LEE*†, Chien-Chang CHEN‡§ and Nen-Chung CHANG†||¶

*Cardiology Section, Department of Medicine, Chi-Mei Medical Center, Tainan, Taiwan, †Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei, Taiwan, ‡Institute of Biomedical Engineering, National Cheng-Kung University, Tainan, Taiwan, §Division of Cardiovascular Surgery, Chia-yi Christian Hospital, Chia-yi City, Taiwan, ||Division of Cardiology, Department of Internal Medicine, Taipei Medical University Hospital, Taipei, Taiwan, and ¶Cardiovascular Research Center, Taipei Medical University Hospital, Taipei, Taiwan

A B S T R A C T

Sympathetic activities are elevated in the central SNSs (sympathetic nervous systems) of hypertensive animals, but it is not known whether sympathetic innervation is also elevated in the heart. Sympathetic hyper-responsiveness in hypertension may result from oxidative stress. The aim of the present study was to investigate sympathetic hyperinnervation in DOCA (deoxycorticosterone acetate)-salt hypertensive rats with established hypertension. At 4 weeks after the start of DOCA-salt treatment and uninephrectomization, male Wistar rats were randomized into three groups for 8 weeks: vehicle, NAC (*N*-acetylcysteine) and triple therapy (hydralazine, hydrochlorothiazide and reserpine). DOCA-salt was associated with increased oxidant release. DOCA-salt produced concentric left ventricular hypertrophy and cardiomyocyte hypertrophy. Sympathetic hyperinnervation was observed in DOCA-salt rats, as assessed by myocardial noradrenaline levels, immunofluorescent analysis of tyrosine hydroxylase, growth-associated factor 43 and neurofilament and Western blotting and real-time quantitative RT-PCR (reverse transcription-PCR) of NGF (nerve growth factor). Arrhythmic scores during programmed stimulation in DOCA-salt rats were significantly higher than those in the control rats. Triple therapy, despite being effective on BP (blood pressure), offered neither attenuated cardiomyocyte hypertrophy nor anti-arrhythmia. The effects of DOCA-salt treatment on NGF expression, sympathetic hyperinnervation and arrhythmias were attenuated by NAC. Furthermore, the effects of NAC on NGF were abolished by administering BSO (*L*-buthionine sulfoximine), an inhibitor of glutamate-cysteine ligase. In conclusion, DOCA-salt treatment contributes to up-regulation of NGF proteins probably through a free radical-dependent pathway in a BP-independent manner. DOCA-salt rats treated with NAC attenuate sympathetic hyperinnervation and thus show a beneficial effect on arrhythmogenic response to programmed electrical stimulation.

Key words: arrhythmia, deoxycorticosterone acetate-salt, hypertension, reactive oxygen species, sympathetic innervation.

Abbreviations: BP, blood pressure; BSO, *L*-buthionine sulfoximine; DHE, dihydroethidium; DOCA, deoxycorticosterone acetate; FS, fractional shortening; GSH, reduced glutathione; IVS, intraventricular septum; LV, left ventricular; LVEDD, LV end-diastolic diameter dimension; LVESD, LV end-systolic diameter dimension; LVPW, LV posterior wall; NAC, *N*-acetylcysteine; NGF, nerve growth factor; PW, posterior wall; ROS, reactive oxygen species; RT-PCR, reverse transcription-PCR; RWT, relative wall thickness; SBP, systolic BP; SNS, sympathetic nervous system; TNF α , tumour necrosis factor α ; Unx, uninephrectomy; VF, ventricular fibrillation; VT, ventricular tachycardia.

Correspondence: Dr Nen-Chung Chang (email ncchang@tmu.edu.tw).

INTRODUCTION

Cardiovascular sympathetic activation is a common complication of hypertension, which results in disabling clinical manifestations and may predispose to arrhythmias. The high incidence of arrhythmias in human hypertension has been well established [1]; however, the mechanisms of arrhythmias are not well defined. Cardiac noradrenaline spill over has shown an increase in patients with major ventricular arrhythmias [2,3], suggesting increased sympathetic activities. The SNS (sympathetic nervous system) constitutes an important putative drug target not only for arresting hypertension but also the progression of common cardiovascular comorbidities [4,5]. Given that hypertension is a powerful independent predisposing risk factor for development of arrhythmias, the development of effective therapeutic strategies to attenuate SNS is clearly warranted.

Increased sympathetic nerve density has been shown to be responsible for the occurrence of lethal arrhythmias and sudden cardiac death in humans [6]. NGF (nerve growth factor) is a prototypic member of the neurotrophin family, members of which are critical for the differentiation, survival and synaptic activity of the peripheral sympathetic and sensory nervous systems [7]. Levels of NGF expression within innervated tissues roughly correspond to innervation density [8]. The treatment of anti-NGF by administering antisera, target ablation, or gene disruption has been shown to prevent nerve sprouting [9]. These results demonstrated a role of NGF in the regulation of sympathetic innervation.

Hypertension induced by the administration of DOCA-salt (deoxycorticosterone acetate plus high-salt intake) in the rat has been extensively studied as an experimental animal model of mineralocorticoid-dependent hypertension. Although increased sympathetic activity in the kidney and muscle has been demonstrated in salt-sensitive hypertension [10,11], the contribution of cardiac sympathetic nervous activity has received very little attention. DOCA-salt hypertension is characterized by an overproduction of the endothelin-1-induced superoxide anion [12] and is associated with decreased myocardial GSH (reduced glutathione) activity [13]. GSH deficiency is thought to contribute to the progression of cardiac remodelling and failure [14]. GSH exerts a potent neuroprotective activity [15]. DOCA-salt has been shown to induce myocardial sensitization to VF (ventricular fibrillation) [16]; however, the involved mechanism remained unclear. Thus the purpose of the present study was: (i) to investigate whether treatment of rats with DOCA-salt can result in heart hyperinnervation through increased expression of NGF, (ii) to assess the role of the superoxide anion in sympathetic innervation and (iii) to evaluate the effects of NAC (*N*-acetylcysteine) and BSO (*L*-buthionine sulfoximine), a specific and transition-state inhibitor of glutamate-

cysteine ligase, on sympathetic innervation and arrhythmias.

MATERIALS AND METHODS

Animals

The animal experiment was approved and conducted in accordance with local institutional guidelines for the care and use of laboratory animals and conformed with 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).

Part 1

Male Wistar rats aged 8 weeks were anaesthetized with ketamine/xylazine (90/9 mg/kg of body weight, intraperitoneally), and the left kidney was removed via a left flank incision. On the following day, these rats were treated twice weekly with DOCA suspended in corn oil and administered subcutaneously (15 mg/kg of body weight), and 1% NaCl was added to their tap water for drinking. At 4 weeks after the start of DOCA-salt treatment, these rats were randomly assigned into either vehicle group, NAC (250 mg/kg of body weight per day; Sigma) or triple drugs (hydralazine + hydrochlorothiazide + reserpine, 30.0 + 10.0 + 0.2 mg/kg of body weight per day) for 8 weeks. The dose of NAC used in this study has been shown to effectively modulate cardiac GSH without significantly changing BP (blood pressure) [17]. Triple drugs were used to determine the effect of BP on sympathetic hyperinnervation. Triple-drug therapy was given in drinking water as described [18]. Control rats underwent left Unx (uninephrectomy) and were supplied with normal tap water. Unx-operated rats served as controls to exclude the possibility that the drugs themselves such as reserpine and hydralazine directly altered sympathetic activities. In each treated group, drugs were withdrawn approximately 24 h before the end of the experiments in order to eliminate their pharmacological actions.

Part 2

To evaluate the importance of GSH in NAC-related NGF expression, we performed an *in vitro* experiment. Twelve weeks after DOCA-salt administration, rat hearts were isolated and subjected to no treatment (vehicle), NAC (60 mM), BSO (200 μ M, Sigma) and NAC + BSO. Each heart was perfused with a non-circulating modified Tyrode's solution as previously described [19]. Drugs were infused for 120 min. The dose of BSO has been shown to cause the depletion of intracellular GSH [20]. NAC-promoted survival of sympathetic neurons occurs at concentrations of 20–60 mM [21]. At the end of the study, all hearts ($n = 10$ per group) were used for Western blotting.

BP measurements in conscious rats

SBP (systolic BP) was measured before the start of DOCA injection and once per 4 weeks until the end of the experiment in conscious, pre-warmed, restrained rats by tail-cuff plethysmography. At least seven determinations were made in every session and the mean of the lowest three values was taken as the SBP level. The tail-cuff method used in this study has been validated and correlated well with intra-arterial method [22]. After the arterial pressure measurement, rats were anaesthetized for echocardiogram and electrophysiological studies. After *in vitro* electrophysiological studies, the atria and the right ventricle were trimmed off, and the left ventricle was rinsed in cold physiological saline, weighed and immediately frozen in liquid nitrogen after obtaining a coronal section of the left ventricle for histological analysis, Western blotting and RT-PCR (reverse transcription-PCR) assessment.

Echocardiography

At the end of the experiment, rats were lightly anaesthetized with an intraperitoneal dose of ketamine/xylazine (90/9 mg/kg of body weight). Echocardiographic measurements were done with a HP Sonos 5500 system with a 15-6L (6–15 MHz, SONOS 5500; Agilent Technologies) probe as previously described [20]. M-mode tracing of the left ventricle was obtained from the parasternal long-axis view to measure LVEDD (left ventricular end-diastolic diameter dimension) and LVESD (left ventricular end-systolic diameter dimension) and FS (fractional shortening; %) was calculated according to the method of the American Society for Echocardiology [23]. For the RWT (relative wall thickness) of the left ventricle, the following formula was used:

$$\text{RWT} = 2 \times \text{PW}(\text{posterior wall})\text{thickness}/\text{LVEDD}$$

For the LV (left ventricular) mass index, the following formula was used:

$$\begin{aligned} \text{LV mass index} &= \text{LV mass}/\text{body weight, considering} \\ \text{LV mass (mg)} &= [(\text{LVEDD} + \text{IVS} + \text{PW})^3 \\ &\quad - \text{LVEDD}^3] \times 1.055 \end{aligned}$$

where 1.055 is the density of the rat myocardium (in mg/mm³). Measurements represented the mean of at least five consecutive cardiac cycles. Analyses were performed by an experienced observer blinded to the treatment groups to which the animals were allocated.

In vitro electrophysiological studies

To assess the potential arrhythmogenic risk of sympathetic innervation, we performed programmed electrical stimulation. DOCA-salt activates key brain sites, such as the paraventricular nucleus [24], that directly drive sympathetic premotor neurons in the rostral ventrolateral medulla [25]. To avoid the confounding effect of central sympathetic activities on pacing-induced

ventricular arrhythmias, we used the Langendorff heart. Each heart was perfused with modified Tyrode solution (117.0 mM NaCl, 23.0 mM NaHCO₃, 4.6 mM KCl, 0.8 mM NaH₂PO₄, 1.0 mM MgCl₂, 2.0 mM CaCl₂ and 5.5 mM glucose) equilibrated at 37°C and oxygenated with a 95% O₂/5% CO₂ gas mixture. The perfusion medium was maintained at a constant temperature of 37°C with a constant flow at 4 ml/min as described previously [26]. Atrial and ventricular epicardial ECGs were continuously recorded. After the perfusion of the isolated hearts was completed, hearts were observed for 10 min to allow stabilization of contraction and rhythm. Programmed electrical stimulation was performed with electrodes sewn to the epicardial surface of the right ventricular outflow tract. Pacing pulses were generated from a Bloom stimulator (Fischer Imaging). Stimulation intensity was twice the threshold, and stimulus length was 5 ms. Induction of ventricular arrhythmias was then attempted by ventricular stimulation at a basic cycle length of 120 ms (S₀) with single (S₁), double (S₂), and triple (S₃) extrastimuli delivered after eight paced beats. The end point of ventricular pacing was induction of ventricular tachyarrhythmia. A preparation was considered non-inducible when pacing produced either no ventricular premature contraction or only self-terminating salvos of <6 beats. Ventricular tachyarrhythmias including VT (ventricular tachycardia) and VF were considered non-sustained when it lasted ≤15 beats and sustained when it lasted >15 beats. An arrhythmia scoring system was modified as described previously [26]: 0, non-inducible preparations; 1, non-sustained tachyarrhythmias induced with three extra stimuli; 2, sustained tachyarrhythmias induced with three extra stimuli; 3, non-sustained tachyarrhythmias induced with two extra stimuli; 4, sustained tachyarrhythmias induced with two extra stimuli; 5, non-sustained tachyarrhythmias induced with one extra stimulus; 6, sustained tachyarrhythmias induced with one extrastimulus; and 7, tachyarrhythmias induced during the eight paced beats. If the heart stopped before the pacing, the arrhythmia score assigned to that heart was 8. When multiple forms of arrhythmias occurred in one heart, the highest score was used. The experimental protocols were typically completed within 10 min.

Morphometric determination of myocyte size and fibrosis

As ventricular remodelling during hypertension is a combination of reactive fibrosis and myocyte hypertrophy, we measured cardiomyocyte sizes in addition to myocardial weight to avoid the confounding influence of non-myocytes on cardiac hypertrophy. LV sections from the free wall were stained with H/E (haematoxylin/eosin). For consistency of results,

myocytes positioned perpendicularly to the plane of the section with a visible nucleus and cell membrane clearly outlined and unbroken were then selected for the cross-sectional area measurements [26]. This area was determined by manually tracing the cell contour on a digitized image acquired on the image-analysis system (Image Pro Plus) as previously described [24]. A total of 100 myocytes were selected in the left ventricle of each heart and analysed by an observer blinded to the experimental treatment.

Additionally, heart sections were stained with Masson's trichrome for assessment of the degree of fibrosis. The percentage of blue staining, indicative of fibrosis, was measured (ten fields randomly selected on each section). The value was expressed as the ratio of trichrome-stained fibrosis area to total area. All sections were evaluated without prior knowledge of which section belonged to which rat.

***In situ* detection of superoxide**

For evaluating myocardial intracellular superoxide production using *in situ* DHE (dihydroethidium; Invitrogen Molecular Probes) fluorescence, OCT-embedded LV tissues were sectioned (10 μm) at -20°C . After fixing, tissues were incubated with DHE in PBS (10 μM) in a dark, humidified container at room temperature (23°C) for 30 min. Generation of superoxide anions by tissue was demonstrated by a red fluorescence, which was detected through a 580-nm long pass filter, using a digital camera mounted on an Olympus fluorescent microscope. The density of the images was reported as arbitrary units/ mm^2 field [27].

Real-time RT-PCR of NGF

Real-time quantitative RT-PCR was performed from samples obtained from the LV-free wall with the TaqMan system (Prism 7700 Sequence Detection System; PE Biosystems) as previously described [28]. For NGF, the primers were 5'-CGTACCCTGACACCAATCT-3' (sense) and 5'-GGCTCCAGAGACAAGAAACG-3' (antisense). For cyclophilin, the primers were 5'-ATGGTCAACCCACCGTGTTCTTCG-3' and 5'-CGTGTGAAGTCACCACCCTGACACA-3'. Cyclophilin mRNA was chosen as the internal standard because it is expressed at a relatively constant level in virtually all tissues. For quantification, NGF expression was normalized to the expressed housekeeping gene cyclophilin. Reaction conditions were programmed on a computer linked to the detector for 40 cycles of the amplification step.

Western blot analysis of NGF

Rabbit polyclonal antibodies to NGF (Chemicon) were used. Western blotting procedures were described previously [28]. Experiments were replicated three times and results expressed as the mean value.

Immunofluorescent studies of tyrosine hydroxylase, growth-associated factor 43 and neurofilament

In order to investigate the spatial distribution and quantification of sympathetic nerve fibres, analysis of immunofluorescent staining was performed on LV-free wall. Paraffin-embedded tissues were sectioned at a thickness of 5 μm . Tissues were incubated with anti-tyrosine hydroxylase (1:200 dilution; Chemicon), anti-growth associated protein 43 (a marker of nerve sprouting, 1:400 dilution; Chemicon), and anti-neurofilament antibodies (a marker of sympathetic nerves, 1:1000 dilution; Chemicon [29]) in 0.5% BSA in PBS overnight at 37°C . Rhodamine-conjugated anti-rabbit IgG from goat was used as secondary antibody for the tyrosine hydroxylase staining and FITC (Sigma) for the growth-associated factor 43 and neurofilament staining. Isotype-identical directly conjugated antibodies served as a negative control.

The slides were coded so that the investigator was blinded to the identification of the rat sections. The nerve density was measured on the tracings by computerized planimetry (Image Pro Plus; Media Cybernetics) as described previously [28]. The density of nerve fibres was qualitatively estimated from 10 randomly selected fields at a magnification of $\times 400$ and expressed as the ratio of labelled nerve fibre area to total area.

Laboratory measurements

GSH activity of homogenized LV-free wall was measured by using a commercially available kit (Cayman Chemical) following the manufacture's instructions. Heart tissue was homogenized in cold 50 mM Mes buffer (pH 6–7, provided with the assay kit) containing 1 mM EDTA per g of tissue and centrifuged at 10 000 g for 15 min at 4°C to obtain supernatant for GSH analysis. The absorbance was read at 405 nm. GSH activity is expressed as $\mu\text{m/g}$ of protein.

Although cardiac innervation was detected by immunofluorescent staining of tyrosine hydroxylase, growth-associated factor 43 and neurofilament, it did not imply that the nerves are functional. Thus to examine the sympathetic nerve function, we measured noradrenaline levels from LV-free wall. Total noradrenaline was measured using a commercial ELISA kit (Noradrenalin ELISA; IBL Immuno-Biological Laboratories).

Histological collagen results were confirmed by the hydroxyproline assay adapted from Stegemann and Stalder [30]. The homogenized LV-free wall was used to measure the hydroxyproline content. The results were calculated as hydroxyproline content/weight of tissue.

Superoxide production by LV-free wall was measured using lucigenin (5 μM bis-*N*-methylacridinium nitrate, Sigma) enhanced chemiluminescence as described previously [27]. The specific chemiluminescence signal was

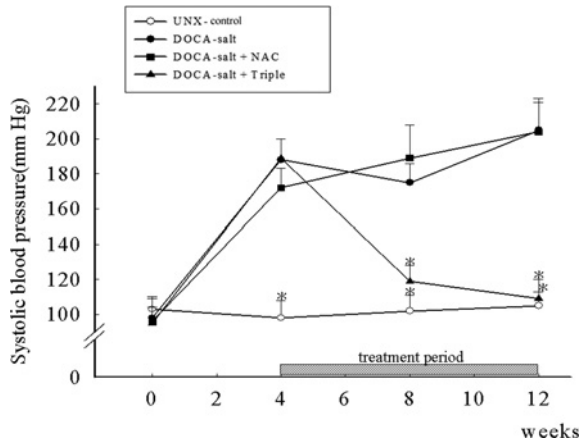


Figure 1 Time course in tail SBP of uninephrectomized control rats and DOCA-salt hypertensive rats treated with vehicle, NAC or triple-treated

Values are the means \pm S.D. ($n = 5$ or 6 per group). * $P < 0.05$ compared with DOCA-salt rats treated with vehicle or NAC at the same age.

calculated after subtraction of background activity and expressed as c.p.m./mg.

Statistical analysis

Results were presented as means \pm S.D. Statistical analysis was performed using the SPSS statistical package (version 11.0). Differences among the groups were tested by a one-way ANOVA, except in Figure 1 where a two-way ANOVA was used. In case of a significant effect, the measurements between the groups were compared with Bonferroni's correction. Electrophysiological data (scoring of programmed electrical stimulation-induced arrhythmias) were compared by a Kruskal–Wallis test, followed by a Mann–Whitney test. The significant level was assumed at value of $P < 0.05$.

RESULTS

SBP and LV weights

As shown in Figure 1 and in Table 1, SBP was progressively elevated by treatment with DOCA-salt. Twelve weeks after the start of DOCA-salt treatment, tail SBP of vehicle-treated rats was 205 ± 18 mmHg, whereas that of the control group was 105 ± 8 mmHg. Daily oral administration of NAC for 8 weeks did not reduce the hypertension induced by DOCA-salt. Triple therapy markedly suppressed the DOCA-salt-induced hypertensive effects, and the level of SBP after 8 weeks was similar to that of the control group.

Relative heart weights corrected by body weight at the end of the experimental period (20 weeks of age) are presented in Table 1. Consistent with a previous study [31], the gain in body weight in vehicle-treated DOCA-salt rats was less than that in the control rats despite no

difference in weight at the start of the study. The vehicle- and triple-treated DOCA-salt rats had a significant increase in LV weight/body weight ratio, compared with control and NAC-treated DOCA-salt rats.

Echocardiographic data

After 12 weeks of treatment, echocardiography showed concentric cardiac hypertrophy in DOCA-salt rats. IVS, LVPW and RWT were increased, and LVEDD and LVESD decreased (Table 2). The LV mass index was significantly higher compared with control. Co-administration of NAC in DOCA-salt rats improved cardiac geometry by restoring IVS and LVPW to values comparable to control.

Compared with control, cardiac function assessed by FS was not altered in all DOCA-salt rats (Table 2). NAC did not improve cardiac function.

Cardiac hypertrophy and fibrosis

To characterize the cardiac hypertrophy on a cellular level, morphometric analyses of LV sections were performed on different treatment groups (Figure 2, top panels). Compared with control, DOCA-salt rats showed structural changes such as increased cardiomyocyte sizes, consistent with LV remodelling. NAC-treated DOCA-salt rats had a significant decrease in cardiomyocyte size compared with vehicle- and triple-treated DOCA-salt rats.

At 12 weeks after DOCA-salt, collagen volume fraction increased 2.89-fold ($P < 0.0001$) in the vehicle-treated group compared with the control group (Figure 2, middle panels). NAC-treated DOCA-salt rats had a significant decrease in fibrosis compared with vehicle- and triple-treated DOCA-salt rats.

Hydroxyproline content was measured (Figure 2, bottom panel). There was a marked increase in collagen content induced by DOCA-salt treatment, and this was significantly attenuated by NAC.

Tissue GSH, superoxide and noradrenaline levels

DOCA-salt was associated with a significant reduction in GSH content compared with controls (9.5 ± 1.7 compared with 14.6 ± 2.5 $\mu\text{mol/g}$ of protein respectively; $P < 0.001$) (Table 1). Treatment with NAC had a significant replenishment effect on GSH depletion.

Myocardial superoxide in vehicle-treated DOCA-salt rats was significantly increased as compared with control ($P = 0.006$). Myocardial superoxide in NAC-treated DOCA-salt rats can be reduced to levels similar to those in the control. On the other hand, triple therapy failed to suppress the superoxide production induced by the DOCA-salt treatment.

To investigate the possible role of cardiac noradrenaline synthesis, we determined the LV noradrenaline levels. LV noradrenaline levels were significantly

Table 1 Cardiac morphology, haemodynamics and tissue GSH, superoxide and noradrenaline concentration at the end of study

Values are means \pm S.D. BW, body weight; NE, noradrenaline; LVW, LV weight. * $P < 0.025$ compared with control; † $P < 0.017$ compared with vehicle- and triple-treated DOCA-salt groups.

Parameter	Unx-control (n = 12)	DOCA		
		Vehicle (n = 12)	NAC (n = 12)	Triple (n = 12)
BW (g)	437 \pm 15	354 \pm 18*	376 \pm 17*	356 \pm 16*
Heart rate (beats/min)	403 \pm 7	398 \pm 10	405 \pm 8	413 \pm 12
SBP (mmHg)	105 \pm 8	205 \pm 18*	204 \pm 17*	109 \pm 11
LVW/BW (mg/g)	2.34 \pm 0.45	3.56 \pm 0.65*	2.65 \pm 0.39†	3.65 \pm 0.44*
GSH (μ mol/g of protein)	14.6 \pm 2.5	9.5 \pm 1.7*	19.4 \pm 2.9*†	10.4 \pm 2.6*
Superoxide (10^3 c.p.m./mg)	1.62 \pm 0.38	2.18 \pm 0.32*	1.59 \pm 0.28†	2.49 \pm 0.38*
NE (μ g/g of protein)	1.28 \pm 0.25	2.26 \pm 0.21*	1.38 \pm 0.31†	2.38 \pm 0.35*

Table 2 Echocardiographic findings at the end of study

Values are means \pm S.D. IVS, interventricular septum; LVMI, LV mass index. * $P < 0.025$ compared with control; † $P < 0.017$ compared with the vehicle- and triple-treated DOCA-salt groups. ‡ $P < 0.017$ compared with vehicle-treated DOCA-salt group.

Parameter	Unx-control	DOCA		
		Vehicle	NAC	Triple
LVEDD (mm)	8.7 \pm 0.9	5.6 \pm 1.1*	8.6 \pm 0.8‡	8.1 \pm 1.2‡
LVESD (mm)	2.5 \pm 0.8	1.5 \pm 0.9*	2.6 \pm 1.1‡	2.6 \pm 0.9‡
IVS (mm)	2.0 \pm 0.5	3.2 \pm 0.4*	2.1 \pm 0.5†	2.8 \pm 0.5*
LVPW (mm)	1.9 \pm 0.4	3.1 \pm 0.3*	2.1 \pm 0.3†	2.7 \pm 0.4*
FS (%)	71 \pm 3	73 \pm 5	70 \pm 5	69 \pm 4
LVMI (mg/g)	3.07 \pm 0.14	4.27 \pm 0.24*	3.89 \pm 0.18*†	5.15 \pm 0.28*‡
RWT	0.44 \pm 0.15	1.09 \pm 0.13*	0.49 \pm 0.19†	0.67 \pm 0.14*‡

up-regulated, 1.8-fold, in the vehicle-treated DOCA-salt rats in comparison with control (2.26 ± 0.21 compared with 1.28 ± 0.25 μ g/g of protein respectively; $P < 0.001$). When compared with vehicle-treated DOCA-salt rats, NAC-treated DOCA-salt rats had significantly lower LV noradrenaline.

Myocardial DHE staining

DHE reacts with superoxide radicals to form ethidium bromide, which in turn intercalates with DNA to provide nuclear fluorescence as a marker of superoxide radical generation. As shown in Figure 3, DOCA-salt markedly enhanced the intensity of the DHE staining in the vehicle-treated rats compared with control. When compared with vehicle-treated DOCA-salt rats, NAC-treated DOCA-salt rats had significantly reduced intensity of the fluorescent signal. Triple therapy failed to suppress the intensity of the fluorescent signal induced by DOCA-salt.

Immunofluorescent analyses of nerve fibres

The tyrosine hydroxylase-immunostained nerve fibres appeared to be oriented in the longitudinal axis of

adjacent myofibres (Figure 4). Tyrosine hydroxylase-positive nerve density was significantly increased in the vehicle-treated DOCA-salt rats compared with the control group. NAC-treated DOCA-salt rats show lower nerve density than vehicle- and triple-treated DOCA-salt rats ($0.12 \pm 0.08\%$ in NAC group compared with $0.25 \pm 0.10\%$ in vehicle and $0.28 \pm 0.05\%$ in triple-treated, both $P < 0.05$). Similar to tyrosine hydroxylase results, densities of growth-associated protein 43 (Figure 5) and neurofilament-positive (results not shown) nerves were significantly attenuated in the NAC-treated DOCA-salt rats compared with those in vehicle- and triple-treated DOCA-salt groups. These morphometric results mirrored those of noradrenaline contents.

NGF protein and mRNA expression

Western blot analysis shows that NGF levels were significantly up-regulated, 5.3-fold, in the vehicle-treated DOCA-salt rats than in control ($P < 0.0001$, Figure 6 upper panel). When compared with vehicle-treated DOCA-salt rats, NAC-treated DOCA-salt rats had significantly lower NGF levels. Triple therapy failed to attenuate NGF levels. To elucidate the role of GSH in modulating NGF, BSO was assessed in an *in vitro*

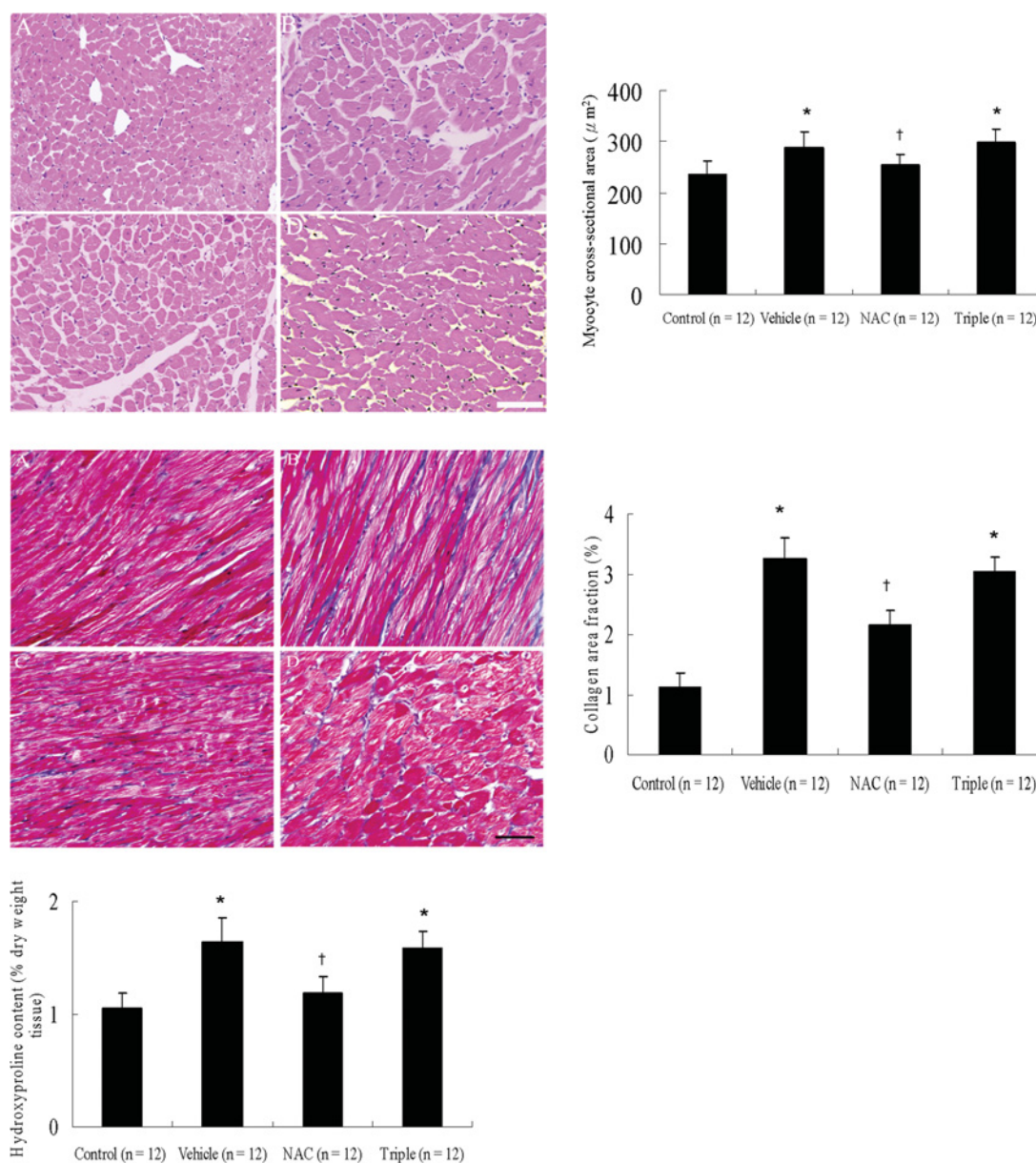


Figure 2 Cardiac hypertrophy and fibrosis

Top panels, quantitative analysis of the cardiomyocyte sizes in different treated groups. Middle panels, representative sections with Masson's trichrome staining (blue; magnification, $\times 400$) 12 weeks after DOCA-salt administration including 8 weeks' treatment. Collagen deposition within left ventricle is reduced after NAC treatment. (A) control; (B) vehicle-treated DOCA-salt rat; (C) NAC-treated DOCA-salt rat; (D) triple-treated DOCA-salt rat. Bottom panel, graphical representation of hydroxyproline content. Values are means \pm S.D. The number of animals in each group is indicated in parentheses. Scale bar, 50 μm . * $P < 0.05$ compared with control; † $P < 0.05$ compared with vehicle- and triple-treated DOCA-salt groups.

model. The lower panel in Figure 6 shows that BSO significantly increased expression of NGF compared with NAC alone, confirming the role of GSH in mediating NGF expression.

PCR amplification of the cDNA revealed that the NGF mRNA levels showed a 5.6-fold up-regulation in the vehicle-treated DOCA-salt rats compared with control ($P < 0.0001$, Figure 7). In NAC-treated DOCA-salt rats, the NGF mRNA levels were significantly

decreased compared with those in the vehicle-treated DOCA-salt rats.

Electrophysiological stimulation

To further elucidate the physiological effect of attenuated sympathetic hyperinnervation, ventricular pacing was performed. There was no inducible VT and VF in the control rats (Figure 8). In contrast, ventricular tachyarrhythmias consisting of VT and VF were inducible

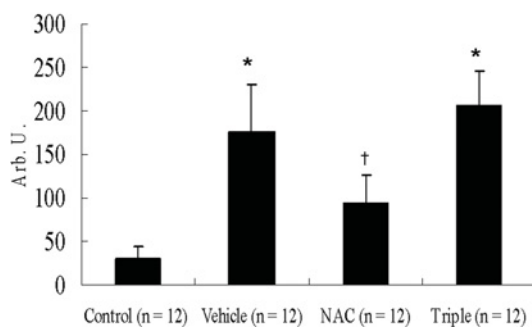
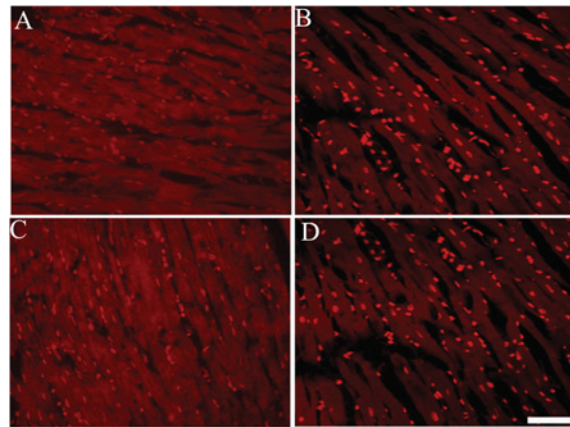


Figure 3 Detection of superoxide in myocardium by DHE staining

Compared with control, the DHE fluorescence intensity in the myocardium of the vehicle-treated DOCA-salt group was significantly increased. (A) Control; (B) vehicle-treated DOCA-salt rat; (C) NAC-treated DOCA-salt rat; (D) triple-treated DOCA-salt rat. In the lower panel, values are given as means \pm S.D. The number of animals in each group is indicated in parentheses. Scale bar, 50 μ m. * P < 0.05 compared with control; † P < 0.05 compared with vehicle- and triple-treated DOCA-salt groups. Magnification, \times 400. Arb. u. arbitrary units.

by programmed stimulation in vehicle-treated DOCA-salt rats. NAC treatment significantly decreased the inducibility of ventricular tachyarrhythmias compared with vehicle treatment ($P=0.04$). Triple therapy significantly increased the arrhythmic scores compared with the NAC treatment ($P=0.03$).

DISCUSSION

In the present study, we report three findings. First, a significant increase of the sympathetic innervation in the DOCA-salt rats occurs in the myocardium with enhanced noradrenaline concentrations. Secondly, free radicals were significantly increased in the DOCA-salt rats. Thirdly, DOCA-rats treated with an antioxidant attenuated sympathetic hyperinnervation and were associated with a decreased inducibility of ventricular arrhythmias independent of BP. These results of DOCA-

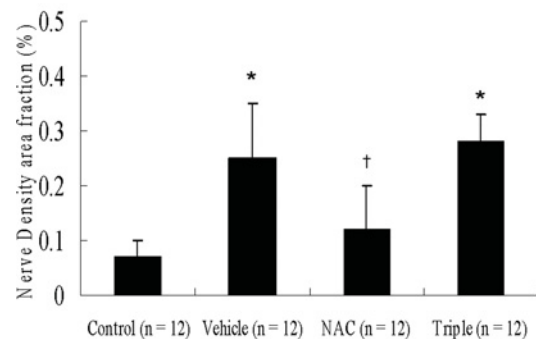
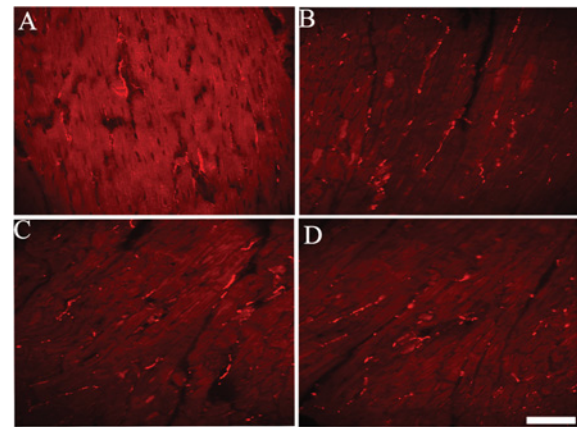


Figure 4 Immunofluorescent (rhodamine) staining for tyrosine hydroxylase

Tyrosine hydroxylase-positive nerve fibres are located between myofibrils and are oriented in the same longitudinal direction as that of the myofibrils. (A) control; (B) vehicle-treated DOCA-salt rat; (C) NAC-treated DOCA-salt rat; (D) triple-treated DOCA-salt rat. In the lower panel, values are given as means \pm S.D. The number of animals in each group is indicated in parentheses. Scale bar, 50 μ m. * P < 0.05 compared with control; † P < 0.05 compared with vehicle- and triple-treated DOCA-salt groups. Magnification, \times 400.

salt hypertensive rats were documented structurally by increase in cardiac nerve sprouting, molecularly by myocardial NGF protein and mRNA levels, biochemically by tissue GSH and noradrenaline levels, pharmacologically by NAC administration, and functionally by pacing-induced fatal ventricular tachyarrhythmias. Thus, in the DOCA-salt hypertension, arrhythmias may originate at least in part from increase in cardiac sympathetic drive resulting from hyperinnervation. Furthermore, the results of the present study were consistent with the findings of Bax et al. [32], showing that cardiac sympathetic hyperinnervation measured by 125 I-metaiodobenzylguanidine scintigraphy resulted in increased inducibility of ventricular arrhythmias during electrophysiology testing.

The effect of DOCA-salt and antioxidant on neural and electrophysiological remodelling was supported by three lines of evidence. (i) Substantial evidence indicates

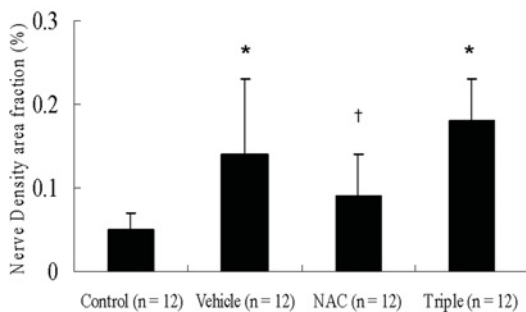
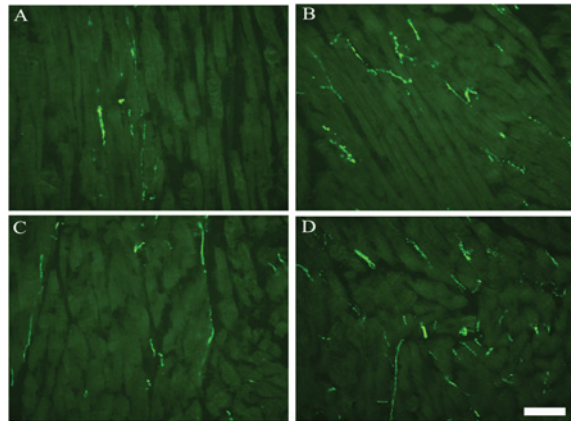


Figure 5 Immunofluorescent (FITC) staining for growth-associated protein 43

Growth-associated protein 43-positive staining was markedly increased in the group treated with vehicle-treated DOCA-salt rats. (A) control; (B) vehicle-treated DOCA-salt rat; (C) NAC-treated DOCA-salt rat; D triple-treated DOCA-salt rat. In the lower panel, values are given as means \pm S.D. The number of animals in each group is indicated in parentheses. Scale bar, 50 μ m. * $P < 0.05$ compared with control; † $P < 0.05$ compared with vehicle- and triple-treated DOCA-salt groups. Magnification, $\times 400$.

that the balance between oxidants and antioxidants is severely disturbed in DOCA-salt hypertension. Our present study showed that oxidative stress as assessed by GSH levels, chemiluminescence-detected superoxide content, and DHE staining is increased in the myocardium, consistent with a recent study showing that oxidative stress is increased and myocardial GSH levels are decreased in DOCA-salt hypertension [13]. (ii) DOCA-salt treatment increased myocardial noradrenaline concentration and NGF expression, which can be attenuated by an antioxidant. The beneficial effects of NAC on attenuated sympathetic innervation might be associated with increased GSH levels. Cardiac sympathetic activity was assessed through measurement of noradrenaline activity, which provides independent assessment of the level of neuronal sympathetic activity. A reduced expression of the NAC-induced NGF in DOCA-salt rats was blocked by BSO. This result suggests that NAC lowers NGF proteins in DOCA-salt

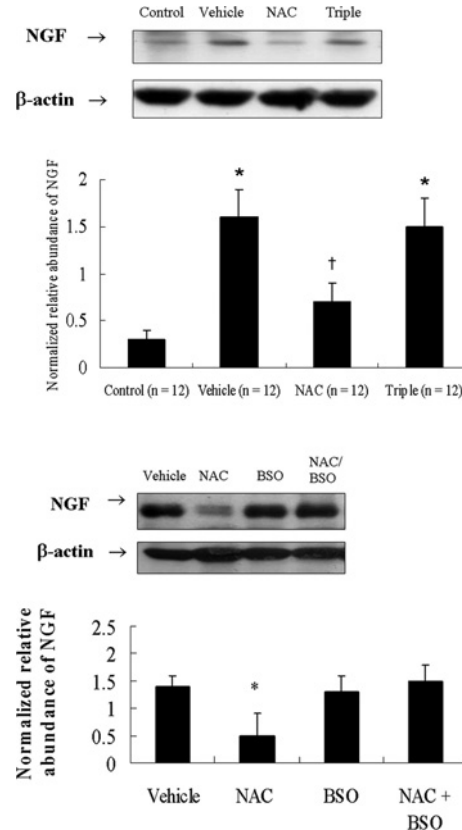


Figure 6 *In vivo* and *in vitro* Western blot analysis of NGF

Upper panels, Western blot analysis of NGF (molecular mass, 13 kDa) in LV homogenates. When compared with vehicle-treated DOCA-salt rats, NAC-treated DOCA-salt rats had significantly lower NGF levels by quantitative analysis. Relative abundance was obtained by normalizing the density of NGF protein against that of β -actin. Results are means \pm S.D. of three independent experiments. * $P < 0.05$ compared with control; † $P < 0.05$ compared with vehicle- and triple-treated DOCA-salt groups. Lower panel, Western blot analysis of NGF to confirm the effect of GSH on NGF in LV homogenates in a rat isolated heart model. A significantly increased NGF is noted in the group treated with a combination of NAC and BSO compared with NAC alone. Densitometric quantification of NGF is expressed as the ratio of the density of β -actin. Results are means \pm S.D. of three independent experiments. * $P < 0.008$ compared with vehicle-, BSO- and NAC + BSO-treated groups.

rats, in part, by replenishing GSH in the myocardium. (iii) DOCA-salt treatment increased the severity of pacing-induced fatal arrhythmias, which can be attenuated by an antioxidant. The severity of pacing-induced fatal arrhythmias was associated with the degree of sympathetic innervation. The finding was consistent with the findings of Cao et al. [6], showing that increased post-injury sympathetic nerve density may be responsible for the occurrence of ventricular arrhythmias and sudden cardiac death in animals and patients.

Although the actions of sympathetic hyperinnervation in blood vessels have been well described, the effects of sympathetic hyperinnervation on the myocardium

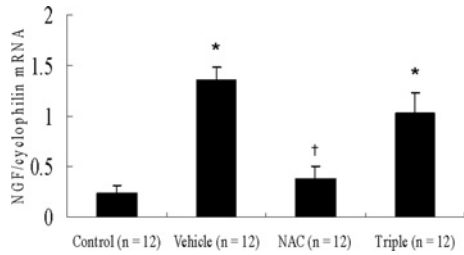


Figure 7 LV NGF mRNA levels in the control, and the vehicle-, NAC- and triple-treated DOCA-salt rats

Each mRNA was corrected for the mRNA level of cyclophilin. The number of animals in each group is indicated in parentheses. Values are means \pm S.D. * P < 0.05 compared with control; † P < 0.05 compared with vehicle- and triple-treated DOCA-salt groups.

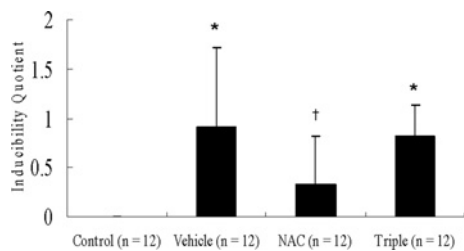


Figure 8 Inducibility quotient of ventricular arrhythmias by programmed electrical stimulation 12 weeks after DOCA-salt administration including 8 weeks of treatment

The number of animals in each group is indicated in parentheses. * P < 0.001 compared with control; † P < 0.05 compared with vehicle- and triple-treated DOCA-salt groups.

are not known, nor is it known how the sympathetic hyperinnervation in the myocardium of hypertension is related to the pathogenesis of arrhythmia. By far the most commonly used method for cardiac sympathetic neuroimaging is scintigraphy or SPECT (single-photon emission computed tomography) after intravenous injection of ^{123}I -metaidobenzylguanidine. Although they provide anatomic depiction of sympathetic innervation, the kinetics of radioactivity concentrations in the tissue cannot be assumed to indicate the fate of endogenous noradrenaline. Furthermore, Herman et al. [33] showed that ^{123}I -metaidobenzylguanidine does not provide an accurate indication of adrenergic integrity. Thus, we used immunohistochemical staining to directly confirm the changes of sympathetic innervation.

The sympathetic hyperactivity in the heart of DOCA-salt rats in the present study appears to be in contrast with other studies in which the lack of cardiac sympathetic activity and in plasma noradrenaline has been observed [34]. This difference with previous studies may be due to the different ages of rats studied with differences in the severity of hypertension. In the present study, adult rats aged 20 weeks at the end of the study,

which developed severe hypertension, were used. In contrast, previous studies used young rats, which developed mild to moderate hypertension. The phase of the hypertension is important because there are different determinants at various times [35]. It is possible, therefore, that in the young rats that developed mild to moderate hypertension, less increased sympathetic neuronal activity may cause the reduced severity of the hypertension. Indeed, our results were consistent with the findings of de Champlain et al. [36], showing that circulating noradrenaline was significantly increased in DOCA-salt rats compared with control.

The present study was undertaken to examine the BP-independent effects of DOCA-salt on myocardial structure and function. We lowered BP by a non-renin-angiotensin II-dependent and a non-endothelin-1 triple-drug therapy [18,37]. Ventricular remodelling assessed by cardiomyocyte hypertrophy and fibrosis, as well as sympathetic hyperinnervation in the triple-treated DOCA-salt group were markedly impaired compared with those in the control group despite the fact that SBP in the two groups was nearly equal. Thus, our results provide direct evidence that DOCA-salt causes cardiac damage independent of BP. The results were consistent with previous findings [38], showing that although BP was markedly ameliorated by triple-drug therapy, it did not prevent target damage.

Other mechanisms

Although the present study suggests that the mechanisms of NAC-induced attenuation of sympathetic innervation and arrhythmias may be related to attenuated GSH-dependent NGF expression, other potential mechanisms need to be studied such as direct antioxidant effect, TNF α (tumour necrosis factor α) and fibrosis. First, oxygen-derived free radicals can directly enhance the liberation of noradrenaline from sympathetic nerves through injury to membranes of the nerve endings [39]. The anti-arrhythmic effect of NAC could be due to its direct antioxidant effect of attenuating noradrenaline release from sympathetic nerves. Secondly, NAC may attenuate arrhythmias by scavenging superoxide in a GSH-independent manner. Superoxide has been shown to modulate the sympathetic excitability by quenching or inactivating NO [40], which is known to exist in the SNS [41]. By causing a reduction in NO levels, superoxide would eliminate this inhibitory effect of NO and this would result in an increased excitability of sympathetic neurons [42]. Thirdly, NAC acting as a TNF α antagonist may directly attenuate ventricular arrhythmias [43]. TNF α prolonged action potential duration and may be associated with susceptibility to lethal ventricular arrhythmias [44]. No data are available regarding local expression of TNF α changes after the administration of NAC in DOCA-salt rats and their functional consequences. Further studies are needed to

elucidate the role of TNF α in the effect of NAC on ventricular arrhythmias. Finally, NAC might prevent fatal arrhythmias by attenuating cardiac fibrosis as shown in this study. Decreasing oxidative stress can reverse tissue fibrosis and may therefore be a promising novel strategy to reduce arrhythmias [45]. Preventing cardiac fibrosis may be an upstream approach to anti-arrhythmic therapy.

Clinical implications

The concept of cardiac remodelling is well established in hypertension; however, the importance of cardiac innervation has only recently received attention. Our results showed that the therapeutic targeting of free radicals represents an attractive strategy to reduce arrhythmias. The finding was not consistent with previous studies showing that antioxidant therapy with vitamins C and E remains ineffective in their therapeutic anti-arrhythmias [46]. Recently, we have demonstrated that there were differences in sympathetic innervation between NAC and vitamins C and E. Although both NAC and vitamin treatments displayed comparable reduction in free radicals, tissue GSH levels were significantly increased after treatment with NAC compared with vitamins. Furthermore, the NGF promoter contains activator protein-1 [47], which is subjected to redox regulation through its conserved cysteine residue [48]. NAC has been shown to react with cysteine residue on the activator protein-1 molecule [49]. It is possible that NAC may attenuate the expression of NGF by inhibiting the activator protein-1. Thus it is not surprising that NAC can reduce sympathetic innervation; however, vitamins C and E did not provide protective effects. Secondly, the inducibility of ventricular tachyarrhythmia by programmed electrical stimulation is a well-established marker of an increased risk of ventricular tachyarrhythmia [50]. The severity of arrhythmia is proportional to the quantities of noradrenaline overflow [51]. The *in situ* perfused heart model allows us to study the role of activation of cardiac sympathetic nerves alone and minimize confounding factors such systemic neurohormonal changes seen with DOCA-salt. Thus, this anti-arrhythmic effect of NAC may have important clinical implications regardless of the underlying mechanisms. Although caution should be taken in extrapolating to humans results obtained with experimental animals, the attenuation of free radicals is central to myocardial arrhythmogenicity in the diseased state and a therapeutic strategy may be to correct the process of ventricular remodelling. Thirdly, the treatment was started in established hypertension when some degree of cardiac damage was already present. Thus inhibition of free radical signalling with NAC may regress established cardiac hypertrophy induced by pressure overload.

Study limitations

There are some limitations in the present study that have to be acknowledged. First, tissue hypertrophy alone cannot account for the difference seen in myocardial sympathetic innervation density between the vehicle- and NAC-treated DOCA-salt rats in the present study, since no differences of LV mass (results not shown) were present between the two groups in the isolated heart study although there were significantly higher NGF levels in the vehicle-treated DOCA-salt rats. Secondly, the underlying mechanisms to cause sympathetic innervation may be different in different types of hypertension. Several animal hypertensive models are associated with increased oxidative stress, including spontaneously hypertensive rats and DOCA-salt rats. ROS (reactive oxygen species), overproduced in spontaneously hypertensive rats, were not associated with increased expression of NGF mRNA [52]. Thus, although the ROS has been proposed as a key mediator of the NGF expression in DOCA-salt rats, the mechanistic heterogeneity in different species should be further investigated. Thirdly, reserpine is an adrenergic blocking agent which can deplete catecholamines from peripheral sympathetic nerve endings [53]. In contrast, hydralazine has been shown to increase plasma noradrenaline levels [54]. To exclude the interaction between reserpine and hydralazine on plasma noradrenaline, we directly measured the myocardial noradrenaline level, a marker of the sympathetic nerve function.

Conclusions

The findings of the present study show that DOCA-salt is associated with increased myocardial oxidative stress, which contributes to the development of myocardial hypertrophy, fibrosis and sympathetic hyperinnervation in a BP-independent manner. Moreover, inhibition of superoxide by administering NAC attenuated the adverse effects induced by DOCA-salt through a replenishment of myocardial GSH. These effects probably are functionally important because they are linked to attenuated severity of fatal arrhythmias. Understanding the mechanism could shed light on the utility of antioxidants in treating human hypertension.

AUTHOR CONTRIBUTION

Tsung-Ming Lee conceived and designed the study, analysed and interpreted the data, and drafted and approved the final version of the paper. Chien-Chang Chen conceived the study, interpreted the data, and revised and approved the final version of the paper. Nen-Chung Chang conceived and designed the study, and revised and approved the final version of the paper.

FUNDING

This work was supported by the Chi-Mei Medical Center [grant number 100CM-TMU04], the National Health Research Institutes [grant number NHRI-EX100-9841SI] and the National Science Council, Taiwan [grant number NSC100-2314-B-384-003].

REFERENCES

- Hennersdorf, M. G. and Strauer, B. E. (2001) Arterial hypertension and cardiac arrhythmias. *J. Hypertens.* **19**, 167–177
- Meredith, I. T., Broughton, A., Jennings, G. L. and Esler, M. D. (1991) Evidence of a selective increase in cardiac sympathetic activity in patients with sustained ventricular arrhythmias. *N. Engl. J. Med.* **325**, 618–624
- Wallin, B. G., Esler, M., Dorward, P., Eisenhofer, G., Ferrier, C., Westerman, R. and Jennings, G. (1992) Simultaneous measurements of cardiac noradrenaline spillover and sympathetic outflow to skeletal muscle in humans. *J. Physiol.* **453**, 45–58
- Guyenet, P. G. (2006) The sympathetic control of blood pressure. *Nat. Rev. Neurosci.* **7**, 335–346
- Grassi, G., Turri, C., Dell’Oro, R., Stella, M. L., Bolla, G. B. and Mancia, G. (1998) Effect of chronic angiotensin converting enzyme inhibition on sympathetic nerve traffic and baroreflex control of the circulation in essential hypertension. *J. Hypertens.* **16**, 1789–1796
- Cao, J. M., Fishbein, M. C., Han, J. B., Lai, W. W., Lai, A. C., Wu, T. J., Czer, L., Wolf, P. L., Denton, T. A., Shintaku, I. P. et al. (2000) Relationship between regional cardiac hyperinnervation and ventricular arrhythmias. *Circulation* **101**, 1960–1969
- Snider, W. D. (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. *Cell* **77**, 627–638
- Shelton, D. L. and Reichardt, L. F. (1984) Expression of the beta-nerve growth factor gene correlates with the density of sympathetic innervation in effector organs. *Proc. Natl. Acad. Sci. U.S.A.* **81**, 7951–7955
- Gloster, A. and Diamond, J. (1992) Sympathetic nerves in adult rats regenerate normally and restore pilomotor function during an anti-NGF treatment that prevents their collateral sprouting. *J. Comp. Neurol.* **326**, 363–374
- Weber, C. S., Thayer, J. F., Rudat, M., Perschel, F. H., Buchholz, K. and Deter, H. C. (2008) Salt-sensitive men show reduced heart rate variability, lower norepinephrine and enhanced cortisol during mental stress. *J. Hum. Hypertens.* **22**, 423–431
- Yatabe, M. S., Yatabe, J., Yoneda, M., Watanabe, T., Otsuki, M., Felder, R. A., Jose, P. A. and Sanada, H. (2010) Salt sensitivity is associated with insulin resistance, sympathetic overactivity, and decreased suppression of circulating renin activity in lean patients with essential hypertension. *Am. J. Clin. Nutr.* **92**, 77–82
- Callera, G. E., Tostes, R. C., Yogi, A., Montezano, A. C. and Touyz, R. M. (2006) Endothelin-1-induced oxidative stress in DOCA-salt hypertension involves NADPH-oxidase-independent mechanisms. *Clin. Sci.* **110**, 243–253
- Bhatia, J., Tabassum, F., Sharma, A. K., Bharti, S., Golechha, M., Joshi, S., Sayeed Akhtar, M., Srivastava, A. K. and Arya, D. S. (2011) *Emblica officinalis* exerts antihypertensive effect in a rat model of DOCA-salt-induced hypertension: role of (p) eNOS, NO and oxidative stress. *Cardiovasc. Toxicol.* **11**, 272–279
- Qin, F., Liang, M. C. and Liang, C. S. (2005) Progressive left ventricular remodeling, myocyte apoptosis, and protein signaling cascades after myocardial infarction in rabbits. *Biochim. Biophys. Acta* **1740**, 499–513
- Dringen, R., Gutterer, J. M. and Hirrlinger, J. (2000) Glutathione metabolism in brain. Metabolic interaction between astrocytes and neurons in the defense against reactive oxygen species. *Eur. J. Biochem.* **267**, 4912–4916
- Healy, C. and Guideri, G. (1985) DOCA-salt induced myocardial sensitization to ventricular fibrillation by isoprenaline in rats. Role of the autonomic nervous system. *J. Auton. Pharmacol.* **5**, 271–278
- Bourraindeloup, M., Adamy, C., Candiani, G., Cailleret, M., Bourin, M. C., Badoual, T., Su, J. B., Adubeiro, S., Roudot-Thoraval, F., Dubois-Rande, J. L. et al. (2004) N-acetylcysteine treatment normalizes serum tumor necrosis factor- α level and hinders the progression of cardiac injury in hypertensive rats. *Circulation* **110**, 2003–2009
- Anderson, S., Rennke, H. G. and Brenner, B. M. (2000) Therapeutic advantage of converting enzyme inhibitors in arresting progressive renal disease associated with systemic hypertension in the rat. *J. Clin. Invest.* **77**, 1993–2000
- Lee, T. M., Lin, M. S. and Chang, N. C. (2008) Effect of ATP-sensitive potassium channel agonists on ventricular remodeling in healed rat infarcts. *J. Am. Coll. Cardiol.* **51**, 1309–1318
- Yan, C. Y., Ferrari, G. and Greene, L. A. (1995) N-acetylcysteine-promoted survival of PC12 cells is glutathione-independent but transcription-dependent. *J. Biol. Chem.* **270**, 26827–26832
- Yan, C. Y. and Greene, L. A. (1998) Prevention of PC12 cell death by N-acetylcysteine requires activation of the Ras pathway. *J. Neurosci.* **18**, 4042–4049
- Kang, C. S., Chang, N. C., Chang, S. T., Lin, C. C. and Lee, T. M. (2009) Effect of pravastatin on nephroprotection in deoxycorticosterone acetate-salt hypertensive rats. *J. Hypertens.* **27**, 2232–2243
- Lang, R. M., Bierig, M., Devereaux, R. B., Flachskampf, F. A., Foster, E., Pellikka, P. A., Picard, M. H., Roman, M. J., Seward, J., Shanewise, J. S. et al. (2005) Recommendations for chamber quantification: a report from the American Society of Echocardiography’s Guidelines and Standards Committee and the Chamber Quantification Writing Group, developed in conjunction with the European Association of Echocardiography, a branch of the European Society of Cardiology. *J. Am. Soc. Echocardiogr.* **18**, 440–463
- Nakata, T., Takeda, K., Itho, H., Hirata, M., Kawasaki, S., Hayashi, J., Oguro, M., Sasaki, S. and Nakagawa, M. (1989) Paraventricular nucleus lesions attenuate the development of hypertension in DOCA/salt-treated rats. *Am. J. Hypertens.* **2**, 625–630
- Osborn, J. W., Fink, G. D., Sved, A. F., Toney, G. M. and Raizada, M. K. (2007) Circulating angiotensin II and dietary salt: converging signals for neurogenic hypertension. *Curr. Hypertens. Rep.* **9**, 228–235
- Bélichard, P., Savard, P., Cardinal, R., Nadeau, R., Gosselin, H., Paradis, P. and Rouleau, J. L. (1994) Markedly different effects on ventricular remodeling result in a decrease in inducibility of ventricular arrhythmias. *J. Am. Coll. Cardiol.* **23**, 505–513
- Yu, X., Patterson, E., Huang, S., Garrett, M. W. and Kem, D. C. (2005) Tumor necrosis factor α , rapid ventricular tachyarrhythmia, and infarct size in canine models of myocardial infarction. *J. Cardiovasc. Pharmacol.* **45**, 153–159
- Lee, T. M., Lai, P. Y. and Chang, N. C. (2010) Effect of N-acetylcysteine on sympathetic hyperinnervation in post-infarcted rat hearts. *Cardiovasc. Res.* **85**, 137–146
- Taniwaki, T. and Schwartz, J. P. (1995) Somatostatin enhances neurofilament expression and neurite outgrowth in cultured rat cerebellar granule cells. *Brain Res. Dev. Brain Res.* **88**, 109–116
- Stegemann, H. and Stalder, K. (1967) Determination of hydroxyproline. *Clin. Chim. Acta* **18**, 267–273
- Matsumura, Y., Hashimoto, N., Taira, S., Kuro, T., Kitano, R., Ohkita, M., Oppenorth, T. J. and Takaoka, M. (1999) Different contributions of endothelin-A and endothelin-B receptors in the pathogenesis of deoxycorticosterone acetate-salt-induced hypertension in rats. *Hypertension* **33**, 759–765
- Bax, J. J., Kraft, O., Buxton, A. E., Fjeld, J. G., Parizek, P., Agostini, D., Knuuti, J., Flotats, A., Arrighi, J., Muxi, A. et al. (2008) ^{123}I -mIBG scintigraphy to predict inducibility of ventricular arrhythmias on cardiac electrophysiology testing: a prospective multicenter pilot study. *Circ. Cardiovasc. Imaging* **1**, 131–140

- 33 Herman, L. M., Dai, S., Hartman, N. G. and McNeill, J. H. (1994) Meta-iodobenzylguanidine uptake in the hypertensive-diabetic rat heart: a marker for myocardial dysfunction? *Can. J. Physiol. Pharmacol.* **72**, 1162–1167
- 34 Fields, N. G., Yuan, B. X. and Leenen, F. H. (1991) Sodium-induced cardiac hypertrophy. Cardiac sympathetic activity versus volume load. *Circ. Res.* **68**, 745–755
- 35 Yemane, H., Busauskas, M., Burris, S. K. and Knuepfer, M. M. (2010) Neurohumoral mechanisms in deoxycorticosterone acetate (DOCA)-salt hypertension in rats. *Exp. Physiol.* **95**, 51–55
- 36 de Champlain, J., Bouvier, M. and Drolet, G. (1987) Abnormal regulation of the sympathoadrenal system in deoxycorticosterone acetate hypertensive rats. *Can. J. Physiol. Pharmacol.* **65**, 1605–1614
- 37 Benigni, A., Zoja, C., Noris, M., Corna, D., Benedetti, G., Bruzzi, I., Todeschini, M. and Remuzzi, G. (1999) Renoprotection by nitric oxide donor and lisinopril in the remnant kidney model. *Am. J. Kidney Dis.* **33**, 746–753
- 38 Mervaala, E., Müller, D. N., Schmidt, F., Park, J. K., Gross, V., Bader, M., Breu, V., Ganten, D., Haller, H. and Luft, F. C. (2000) Blood pressure-independent effects in rats with human renin and angiotensinogen genes. *Hypertension* **35**, 587–594
- 39 Chahine, R., Chen, X., Yamaguchi, N., de Champlain, J. and Nadeau, R. (1991) Myocardial dysfunction and norepinephrine release in the isolated rat heart injured by electrolysis-induced oxygen free radicals. *J. Mol. Cell. Cardiol.* **23**, 279–286
- 40 Li, L., Fink, G. D., Watts, S. W., Northcott, C. A., Galligan, J. J., Pagano, P. J. and Chen, A. F. (2003) Endothelin-1 increases vascular superoxide via endothelin_A-NADPH oxidase pathway in low-renin hypertension. *Circulation* **107**, 1053–1058
- 41 Ceccatelli, S., Lundberg, J. M., Zhang, X., Aman, K. and Hokfelt, T. (1994) Immunohistochemical demonstration of nitric oxide synthase in the peripheral autonomic nervous system. *Brain Res.* **656**, 381–395
- 42 Browning, K. N., Zheng, Z. L., Kreulen, D. L. and Travagli, R. A. (1998) Effects of nitric oxide in cultured prevertebral sympathetic ganglion neurons. *J. Pharmacol. Exp. Ther.* **286**, 1086–1093
- 43 Xiao, H., Chen, Z., Liao, Y., Cheng, X., Liu, K., Wang, Y., Wang, M. and Guo, H. (2008) Positive correlation of tumor necrosis factor- α early expression in myocardium and ventricular arrhythmias in rats with acute myocardial infarction. *Arch. Med. Res.* **39**, 285–291
- 44 Wang, J., Wang, H., Zhang, Y., Gao, H., Nattel, S. and Wang, Z. (2004) Impairment of HERG K⁺ channel function by tumor necrosis factor- α . *J. Biol. Chem.* **279**, 13289–13292
- 45 Dudley, S. C. Jr, Hoch, N. E., McCann, L. A., Honeycutt, C., Diamandopoulos, L., Fukai, T., Harrison, D. G., Dikalov, S. I. and Langberg, J. (2005) Atrial fibrillation increases production of superoxide by the left atrium and left atrial appendage: role of the NADPH and xanthine oxidases. *Circulation* **112**, 1266–1273
- 46 Cook, N. R., Albert, C. M., Gaziano, J. M., Zaharris, E., MacFadyen, J., Danielson, E., Buring, J. E. and Manson, J. E. (2007) A randomized factorial trial of vitamins C and E and beta carotene in the secondary prevention of cardiovascular events in women: results from the Women's Antioxidant Cardiovascular Study. *Arch. Intern. Med.* **167**, 1610–1618
- 47 Colangelo, A. M., Johnson, P. F. and Mocchetti, I. (1998) Beta-adrenergic receptor-induced activation of nerve growth factor gene transcription in rat cerebral cortex involves CCAAT/enhancer-binding protein δ . *Proc. Natl. Acad. Sci. U.S.A.* **95**, 10920–10925
- 48 Abate, C., Patel, L., Rauscher, III, F. J. and Curran, T. (1990) Redox regulation of fos and jun DNA-binding activity *in vitro*. *Science* **249**, 1157–1161
- 49 Kamata, H., Tanaka, C., Yagisawa, H., Matsuda, S., Gotoh, Y., Nishida, E. and Hirata, H. (1996) Suppression of nerve growth factor-induced neuronal differentiation of PC12 cells. N-acetylcysteine uncouples the signal transduction from ras to the mitogen-activated protein kinase cascade. *J. Biol. Chem.* **271**, 33018–33025
- 50 Brugada, P., Green, M., Abdollah, H. and Wellens, H. J. (1984) Significance of ventricular arrhythmias initiated by programmed ventricular stimulation: the importance of the type of ventricular arrhythmia induced and the number of premature stimuli required. *Circulation* **69**, 87–92
- 51 Du, X. J., Cox, H. S., Dart, A. M. and Esler, M. D. (1999) Sympathetic activation triggers ventricular arrhythmias in rat heart with chronic infarction and failure. *Cardiovasc. Res.* **43**, 919–929
- 52 Falckh, P. H., Harkin, L. A. and Head, R. J. (1992) Nerve growth factor mRNA content parallels altered sympathetic innervation in the spontaneously hypertensive rat. *Clin. Exp. Pharmacol. Physiol.* **19**, 541–545
- 53 Mandela, P., Chandley, M., Xu, Y. Y., Zhu, M. Y. and Ordway, G. A. (2010) Reserpine-induced reduction in norepinephrine transporter function requires catecholamine storage vesicles. *Neurochem. Int.* **56**, 760–767
- 54 Fogari, R., Zoppi, A., Mugellini, A., Tettamanti, F., Lusardi, P. and Corradi, L. (1995) Effects of lisinopril vs hydralazine on left ventricular hypertrophy and ambulatory blood pressure monitoring in essential hypertension. *Eur. Heart J.* **16**, 1120–1125

Received 14 February 2012/10 April 2012; accepted 17 April 2012
Published as Immediate Publication 17 April 2012, doi:10.1042/CS20120080