ATTENUATION OF CARDIOVASCULAR REMODELLING IN DOCA-SALT RATS BY THE VASOPEPTIDASE INHIBITOR, OMAPATRILAT

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Short title: Dual ACE/NEP inhibition and cardiovascular remodelling

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ABSTRACT

Omapatrilat, a vasopeptidase inhibitor, inhibits both neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE) with similar potency. The aim of this study was to investigate whether omapatrilat prevents or reverses cardiovascular remodelling and hypertension in deoxycorticosterone acetate (DOCA)-salt rats. Male Wistar rats (313±2 g, n=114) were uninephrectomized (UNX) with or without further treatment with DOCA and 1% NaCl in the drinking water. Compared with UNX control rats, DOCA-salt rats developed hypertension, cardiovascular hypertrophy, perivascular and interstitial cardiac fibrosis and inflammation, endothelial dysfunction and prolongation of ventricular action potential duration within 4 weeks. Administration of omapatrilat (40 mg/kg/day po) for two weeks commencing two weeks after surgery attenuated the development of cardiovascular hypertrophy, inflammation, fibrosis and ventricular action potential prolongation. In contrast, omapatrilat treatment did not lower systolic blood pressure nor improve endothelial dysfunction. We conclude that the renin-angiotensin-aldosterone, natriuretic peptide and bradykinin systems are directly involved in the pathogenesis of cardiovascular remodelling in the DOCA-salt model of hypertension in rats, which may be independent of their effects on blood pressure.

Key Terms: DOCA-salt rat; Omapatrilat; Hypertension; Fibrosis; Hypertrophy.

INTRODUCTION

Dual inhibition of angiotensin converting enzyme (ACE) and neutral endopeptidase (NEP), termed vasopeptidase inhibition, augments the beneficial actions of natriuretic peptides, adrenomedullin and bradykinin on regulating blood pressure and cardiovascular function, whilst preventing the detrimental effects of angiotensin II (1). Vasopeptidase inhibitors, which include omapatrilat, offer a new approach to the treatment of hypertension and heart failure (2-4). In experimental hypertension, omapatrilat lowered blood pressure irrespective of renin and volume status (5) and the response elicited was significantly greater than selective inhibition of either NEP or ACE alone (6,7). Blood pressure control alone, however, is no longer considered adequate therapeutic management of hypertensive patients. Novel antihypertensives should also exert beneficial effects on cardiovascular remodelling and other associated end-organ damage, key risk factors in the morbidity and mortality of heart disease.

Considering the complex interplay between the neurohumoral systems, omapatrilat represents a potentially important therapeutic approach to hypertension and associated end-organ damage (1-5). It has the advantage of blocking a mechanism inducing remodelling via ACE inhibition, whilst, as a NEP inhibitor, simultaneously stimulating a mechanism to reduce remodelling by reducing the degradation of natriuretic peptides, bradykinin and adrenomedullin. One potential drawback is that NEP is also involved in the degradation of endothelin (8,9) and thus NEP inhibition could be deleterious by increasing circulating levels of this potent vasoconstrictor and trophic peptide.

This study has administered the potent, balanced vasopeptidase inhibitor, omapatrilat, to DOCA-salt hypertensive rats for two weeks starting two weeks after induction of hypertension. This is a well-established model of volume-dependent hypertension (10), characterized by low plasma renin activity and elevated atrial natriuretic peptide

(ANP) levels (11). In addition, increased endothelin is believed to be a key mechanism for the induction of cardiac and vascular damage in DOCA-salt hypertensive rats via augmentation of vascular superoxide production by NADPH oxidase (12-14). Our aim was to determine whether omapatrilat prevents the further development of hypertension and cardiovascular remodelling, reverses existing structural changes and normalises cardiovascular function in this rat model of human hypertension.

METHODS

Ethical clearance

All experimentation was approved by the Animal Experimentation Ethics Committee of The University of Queensland under the guidelines of the National Health and Medical Research Council of Australia.

DOCA-salt hypertensive rats

Male Wistar rats weighing 300-330 grams (~8 weeks old) were obtained from the Central Animal Breeding House of The University of Queensland. All rats were uninephrectomied under anaesthesia with intraperitoneal tiletamine (25 mg/kg) and zolazepam (25 mg/kg)(Zoletil®) combined with xylazine (10 mg/kg)(Ilium Xylazil®). Kidneys were visualised by a left lateral abdominal incision. The left kidney was removed after ligation of adjoining renal vasculature and ureter with sutures. Uninephrectomized rats were given either no further treatment (UNX rats) or 1% NaCl in the drinking water with subcutaneous injections of deoxycorticosterone acetate (DOCA; 25mg in 0.4ml dimethylformamide every fourth day) (DOCA-salt rats). After 14 days, subgroups of UNX and DOCA-salt rats received daily oral gavaging of omapatrilat (40 mg/kg) or no treatment, for a further 14 days. Experiments were performed either 14 or 28 days after surgery.

Assessment of physiological parameters

Systolic blood pressure was measured by tail-cuff plethysmography (ADInstruments) in rats lightly anaesthetised with intraperitoneal tiletamine (10 mg/kg) and zolazepam (10 mg/kg). Rats were euthanased with pentobarbitone (200 mg/kg ip). After adequate anaesthesia and prior to death, blood was taken from the abdominal vena cava, just

caudal to the insertion of renal veins, centrifuged and the plasma immediately frozen for subsequent measurement of sodium and potassium concentrations by flame photometry. The heart was removed and weighed immediately after death, and heart weight was expressed as a ratio of the tissue weight (mg) to the total body weight (g).

Isolated Langendorff heart preparation

Rats were anaesthetized with sodium pentobarbitone (100 mg kg^{-1} ip) and heparin (200 IU) was administered via the femoral vein. After allowing two minutes for the heparin to circulate, the heart was excised and placed in cooled (0°C) crystalloid perfusate (modified Krebs-Henseleit solution of the following composition in mM: NaCl 119.1, KCl 4.75, $MgSO_4$ 1.19, KH_2PO_4 1.19, $CaCl_2$ 2.16, $NaHCO_3$ 25.0, glucose 11.0). A cannula was then placed in the heart with its tip immediately above the coronary ostia of the aortic stump. The cannula was used to perfuse the heart in a non-recirculating Langendorff fashion at 100cm of hydrostatic pressure. The perfusate temperature was maintained at 37°C and bubbled with 95% O_2 / 5% CO_2. The apex of the heart was pierced to facilitate thebesian drainage and paced at 250 bpm by electrodes placed on the surface of the right atrium.

Left ventricular developed pressure was measured using a balloon catheter inserted into the left ventricle through the mitral orifice. The catheter was connected via a three-way tap to a micrometer syringe and to a MLT844 Physiological Pressure Transducer (ADInstruments) and PowerLab data acquisition unit (ADInstruments). The outer diameter of the catheter was similar to the mitral annulus to prevent ejection of the balloon during the systolic phase. After a 5 minute stabilization period, steady-state left ventricular pressure was recorded from isovolumetrically beating hearts. Increments in balloon volume were applied to the heart with left ventricular end-diastolic pressure recorded at approximately 0, 5, 10, 15, 20 and 30 mmHg. At the end of the experiment, the atria and right ventricle were dissected away leaving the left ventricle and septum, which were blotted then weighed. Myocardial diastolic stiffness was calculated as the diastolic stiffness constant (k, dimensionless), the slope of the linear relation between tangent elastic modulus (E, dyne/cm^2) and stress (σ, dyne/cm^2) (15, 16).

Isolated thoracic aortic rings

Thoracic aortic rings (approximately 4 mm in length) were suspended in 25mL organ baths with a resting tension of 10 mN. Force of contraction was measured isometrically with Grass FT03C force transducers. Cumulative concentration-response curves were performed for noradrenaline and either acetylcholine or sodium nitroprusside in the presence of a submaximal (~70%) contraction to noradrenaline.

Quantification of left ventricular collagen

Collagen content was determined by image analysis of picrosirius red-stained sections of the hearts (17). Tissue collagen content was also measured by a modified hydroxyproline assay (18). Approximately 5.0 mg samples of left ventricle were dried for 6 hours at 40°C. Tissues and standards were then hydrolyzed in 6M HCl at 107°C for 18 hours. The acid was blown off by filtered compressed air and the hydrolysate reconstituted in distilled water. Chloramine T reagent was added to each sample for the oxidation step to progress, followed by Ehrlich's reagent to enable chromophore development. Absorbance of each sample was read at 550nm in a spectrophotometer and hydroxyproline content established from a standard curve.

Width of media in thoracic aorta

The width of the media in the thoracic aorta of rats was measured by image analysis of picrosirius red-stained sections. Section preparation, staining, image acquisition and analysis were similar to those mentioned above. Three different cross-sectional areas of each aorta were measured and the results averaged.

Grading of inflammation in left ventricle

The degree of left ventricular inflammation was determined by blinded semi-quantitative analysis of haematoxylin and eosin-stained transverse sections. Slides were visualised under bright field illumination at 40x magnification. A zero to four grading scale was used to quantify the degree of inflammatory cell infiltration in the left ventricle. 0 = no inflammatory cells present; 1 = low level of inflammatory cells throughout the left ventricle; 2 = moderate levels of inflammatory cells throughout the left ventricle and concentrated in mild scarring; 3 = high levels of inflammatory cells throughout the left ventricle and concentrated in moderate scarring; 4 = high levels of inflammatory cells throughout the left ventricle and concentrated in heavy scarring.

Microelectrode studies of isolated left ventricular papillary muscles

Electrophysiological recordings of cardiac action potentials were obtained by microelectrode single cell impalements of *ex vivo*, left ventricular papillary muscles, as described previously (17).

Data analysis

All results are given as mean ± SEM. The negative log EC_{50} of the increase in force of contraction in mN was determined from the concentration giving half-maximal responses in individual concentration-response curves. These results were analysed by one-way analysis of variance followed by the Tukey post test to determine differences between treatment groups; $p < 0.05$ was considered significant.

Drugs

Omapatrilat (BMS-186716) was provided by Bristol-Myers Squibb, Princeton, NJ, USA. Deoxycorticosterone acetate, 4-aminopyridine, acetylcholine, sodium nitroprusside and noradrenaline were purchased from Sigma Chemical Company, St Louis, MO, USA. Noradrenaline, sodium nitroprusside and acetylcholine were dissolved in distilled water and deoxycorticosterone acetate was dissolved in dimethylformamide with mild heating.

RESULTS

Over the four-week treatment period, DOCA-salt rats gained significantly less weight than UNX controls and became hypernatraemic, hypokalaemic and hypertensive (Table 1, Figure 1). These rats developed significant cardiac remodelling, including left ventricular and aortic hypertrophy, inflammation, perivascular collagen deposition and cardiac action potential prolongation, in addition to an increased remnant kidney wet weight (Tables 1 and 2, Figure 2). These changes were already evident after two weeks of DOCA-salt administration. An additional two weeks of DOCA-salt administration saw further significant increases in these parameters of cardiac remodelling, as well as elevations in left ventricular interstitial collagen and hydroxyproline contents and kidney weight (Table 1, Figure 2).

Omapatrilat therapy for two weeks commencing two weeks after surgery attenuated the additional increase in left ventricular wet weight and prevented further cardiac collagen deposition and inflammatory cell infiltration without changes in body weight, systolic blood pressure or kidney weight (Tables 1, Figures 1 and 2). In addition, further aortic medial hypertrophy in DOCA-salt rats was prevented by omapatrilat intervention (Table 1). Omapatrilat did not significantly decrease diastolic stiffness in hypertensive rats (Table 1). The vasopeptidase inhibitor was without effect on plasma sodium concentrations, but normalised plasma potassium concentrations in DOCA-salt rats and also augmented the concentrations of this electrolyte in UNX animals (Table 1).

The maximal contractile responses to noradrenaline and relaxant responses to acetylcholine in isolated thoracic aortic rings were unchanged in two week DOCA-salt rats, but were significantly reduced after four weeks of DOCA-salt treatment (Figure 3A and B). There was also an increased potency of noradrenaline after both two and four weeks of DOCA-salt treatment (Figure 3A, Table 2). Responses to sodium nitroprusside were unchanged between groups except in two week DOCA-salt animals, where relaxation was augmented at concentrations of 1µM and 3 µM (Figure 3C). Omapatrilat was without effect in DOCA-salt rats on these indicators of vascular function (Figure 3A, B and C, Table 2).

In microelectrode recordings of isolated left ventricular papillary muscles, resting membrane potential and action potential amplitude were unchanged in the five study groups (Table 3). Two week DOCA-salt rat papillary muscles, however, demonstrated prolonged action potentials at 90% of repolarization (APD_{90}). Four week DOCA-salt controls exhibited further lengthening of APD_{90}, as well as APD_{20} and APD_{50} (Table 3). Omapatrilat therapy from 2 to 4 weeks of the study period prevented further lengthening of APD_{90} in DOCA-salt rats (Table 3).

DISCUSSION

In the treatment of cardiovascular disease, omapatrilat has the advantage of concomitantly blocking mitogenic and hypertensive responses to angiotensin II as an ACE inhibitor while also stimulating anti-mitogenic and hypotensive mechanisms via NEP inhibition. In the current study, we observed that omapatrilat therapy attenuated further increases in left ventricular hypertrophy, fibrosis and inflammation, cardiac action potential prolongation, and aortic medial hypertrophy without antihypertensive action in DOCA-salt rats and without reversing existing remodelling.

Previous studies have shown that omapatrilat reduced blood pressure irrespective of renin status (5) with NEP inhibition most valuable in low renin forms of hypertension, where ACE inhibition is less effective, such as the DOCA-salt hypertensive rat (5, 19-21). This is consistent with the observation that hypertension is accelerated in this model with administration of antibodies to ANP (22). In the current study, however, we showed no change in blood pressure with omapatrilat in DOCA-salt rats using a reversal protocol, despite using a relatively high dose of the drug as in the previous studies (5,19-21). However, our blood pressure findings must be interpreted with caution given the limitations of tail cuff plethysmography versus telemetry. Nonetheless, our results are in agreement with Elmarakby *et al*. (9) who demonstrated with telemetry that omapatrilat was ineffective at lowering arterial pressure in the DOCA-salt hypertensive rat whether given after the establishment of hypertension, as in our study, or for the duration of DOCA-salt treatment. These authors hypothesized that abrogated metabolism of endothelin by NEP inhibition (8) may limit the antihypertensive effects of the dual ACE/NEP inhibitor in this setting. Consistent with this, they found urinary endothelin excretion in DOCA-salt rats increased almost two-fold with omapatrilat treatment suggesting augmented endothelin survival (9). Thus, with the DOCA-salt model characterized by elevated levels of vascular and circulating endothelin-1 (23,24) and the peptide implicated in the development and maintenance of hypertension in this model (9,12-14,24), omapatrilat therapy may potentially increase circulating levels of this potent vasoconstrictor to prevent a reduction in blood pressure.

However, intervention with omapatrilat attenuated further cardiovascular hypertrophy and fibrosis. We have previously shown that cardiac fibrosis, but not hypertrophy, associated with DOCA-salt hypertension was corrected by inhibition of the renin-angiotensin system with the ACE inhibitor captopril, the AT1 receptor antagonist candesartan or the aldosterone antagonist spironolactone, without reduction of blood pressure (15). Our findings suggest that NEP inhibition may play an additional role in the prevention of both cardiac and vascular remodelling, particularly of left ventricular and aortic medial hypertrophy. *In vitro*, natriuretic peptides inhibited the hypertrophy and proliferation of cardiomyocytes (25,26), vascular smooth muscle cells (27) as well as the proliferation and collagen matrix production by fibroblasts (28-30). *In vivo*, knockout inactivation of the natriuretic peptide receptor (NPR)-A in mice (*Npr1-/-*) increased ventricular mass and fibrosis disproportionately with the small rise in blood pressure (31,32), suggesting that natriuretic peptides can regulate cardiovascular remodelling independent of blood pressure. Further, given their anti-mitogenic effects on cardiac fibroblasts and myocytes, there is a possible role for bradykinin (33) and adrenomedullin (34,35) in the cardioprotective effects of omapatrilat in the current study.

NEP inhibition, and therefore presumably natriuretic peptides, may show anti-inflammatory responses since treatment with omapatrilat or the NEP inhibitor, CGS 25462, suppressed the increased expression of NF-κB, monocyte chemotactic protein (MCP)-1, surface adhesion molecules and macrophage infiltration associated with cardiac fibrosis in DOCA-salt rats (20). This is in agreement with our results, where omapatrilat prevented further inflammatory cell infiltration into the left ventricular interstitium.

Action potential prolongation is a common electrophysiological disturbance in hypertrophied myocardium (36), including DOCA-salt hypertensive rats (37). Depression of the calcium-independent transient outward K^+ current (I_{to}) in DOCA-salt rats was responsible for this prolongation, such that an absence of enhanced I_{to} channel expression concurrent with hypertrophy resulted in a reduced channel density per unit surface area (37). Furthermore, non-pharmacological regression of LV hypertrophy in DOCA-salt rats normalized the I_{to} current and APD (37). Thus, the

prevention of further cardiac action potential prolongation with omapatrilat is most likely secondary to its amelioration of left ventricular hypertrophy. Importantly, this improvement in cardiac electrophysiology with omapatrilat would presumably render the rats less susceptible to ventricular arrhythmias, as shown with the ACE inhibitor, captopril, in a renovascular model of left ventricular hypertrophy (38).

In our study, isolated aortic rings from 4 week DOCA-salt treated rats showed a reduced responsiveness to acetylcholine, which acts through an endothelium-dependent mechanism. This indicates a diminished reactivity of endothelial cells to the activation or production of nitric oxide or both. In addition, dual ACE/NEP inhibition with omapatrilat failed to attenuate this endothelial dysfunction. This is in contrast to previous work where omapatrilat therapy improved endothelial function in hypertensive rat models (39-41), including DOCA-salt hypertensive rats (21). Unlike our results, these studies also demonstrated hypotensive responses with omapatrilat, which is the most likely mechanism responsible for this discrepancy. DOCA-salt (2 and 4 week) rats also exhibited normal or enhanced responses to the nitric oxide-donor, SNP, indicating preservation or even augmentation of the downstream pathway of nitric oxide, guanylate cyclase (and cGMP) in the vascular smooth muscle of these animals.

This study shows that omapatrilat attenuated the signs of further cardiovascular remodelling, especially aortic medial thickening, myocardial inflammation and fibrosis, left ventricular hypertrophy and action potential prolongation, in DOCA-salt hypertensive rats. Further, these effects of omapatrilat may be independent of an anti-hypertensive action. This suggests that the renin-angiotensin-aldosterone, natriuretic peptide and bradykinin systems may be directly involved in the pathogenesis of cardiovascular remodelling in the DOCA-salt model of hypertension in rats.

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Table 1: Physiological parameters in UNX, DOCA-salt and omapatrilat-treated rats.

	UNX	UNX+OMA	DOCA-salt (2 week)	DOCA-salt (4 week)	DOCA-salt +OMA
Initial body weight (g)	316±5 (n=8)	309±4 (n=10)	314±2 (n=12)	315±3 (n=13)	312±6 (n=11)
Final body weight (g)	417±5 (n=8)	393±7 (n=10)	332±7* (n=12)	321±6* (n=13)	340±7* (n=11)
LV+septum weight (mg/g)	1.81±0.04 (n=8)	1.74±0.04 (n=10)	2.27±0.05*# (n=12)	3.00±0.07* (n=13)	2.54±0.07*# (n=11)
Remnant kidney weight (mg/g)	5.0±0.2 (n=8)	4.8±0.1 (n=10)	7.5±0.2* (n-12)	10.1±0.5*# (n=13)	9.4±0.3*# (n=11)
Plasma Na^+ concentration (mM)	129.6±0.5 (n=10)	132.5±0.4 (n=10)	136.5±0.3* (n=10)	135.6±1.3* (n=10)	136.3±0.8* (n=10)
Plasma K^+ concentration (mM)	3.5±0.1 (n=10)	5.1±0.4* (n=10)	2.2±0.2* (n=10)	1.9±0.2* (n=10)	3.0±0.2# (n=10)
Diastolic Stiffness Constant (κ)	21.4±0.4 (n=9)	21.7±0.4 (n=9)	22.1±0.3# (n=8)	24.6±0.5* (n=9)	23.2±0.4* (n=10)
Left Ventricular Hydroxyproline Content (mg/g)	1.07±0.05 (n=7)	1.20±0.05 (n=9)	1.20±0.08# (n=10)	1.60±0.08* (n=8)	1.20±0.05# (n=10)
Aortic Media Thickness (μm)	78.5±4.2 (n=7)	82.9±3.7 (n=7)	91.1±2.5# (n=7)	121.6±5.2* (n=7)	103.8±3.7*# (n=8)
Inflammatory Score	0.7±0.2 (n=7)	0.7±0.2 (n=8)	2.4±0.5*# (n=7)	3.8±0.1* (n=7)	2.3±0.2*# (n=8)

* $p < 0.05$ compared to UNX, # $p < 0.05$ compared to DOCA-salt (4 week), OMA=omapatrilat

Table 2: Potency of vascular reactions to noradrenaline, acetylcholine and sodium nitroprusside

	UNX	UNX +OMA	DOCA-salt (2 week)	DOCA-salt (4 week)	DOCA-salt +OMA
Noradrenaline -log EC50 (M)	7.1±0.1 (n=15)	7.0±0.1 (n=15)	7.7±0.1* (n=15)	7.9±0.1* (n=15)	7.8±0.1* (n=14)
Acetylcholine -log EC50 (M)	6.8±0.1 (n=12)	6.7±0.1 (n=14)	6.6±0.1 (n=14)	6.6±0.1 (n=14)	6.7±0.1 (n=14)
Sodium Nitroprusside -log EC50 (M)	7.3±0.1 (n=15)	7.5±0.1 (n=15)	7.3±0.1 (n=14)	7.1±0.1 (n=14)	7.3±0.1 (n=13)

* $p < 0.05$ compared to UNX; EC50 = concentration giving a half maximal response; OMA = omapatrilat

Table 3: Cardiac electrophysiological parameters in UNX, DOCA-salt and omapatrilat-treated rats.

	UNX (n=7)	UNX +OMA (n=6)	DOCA-salt (2 week) (n=9)	DOCA-salt (4 week) (n=9)	DOCA-salt +OMA (n=9)
Resting Membrane Potential (mV)	-73±3	-74±5	-69±2	-74±2	-75±2
Action Potential Amplitude (mV)	93±3	93±2	87±2	93±3	91±2
APD_{20} (ms)	8.3±1.1	8.4±0.7	12.5±1.0	17.6±1.9*	14.4±1.5*
APD_{50} (ms)	18.4±1.6	17.8±1.4	29.6±1.9#	45.3±3.9*	38.1±3.0*
APD_{90} (ms)	50.2±2.1	48.5±3.7	84.1±5.6*#	115.1±4.2*	92.8±5.0*#

* $p < 0.05$ compared to UNX, # $p < 0.05$ compared to DOCA-salt (4 week); APD_{20}, APD_{50}, and APD_{90} = Action Potential Duration at 20%, 50% and 90% of repolarization respectively; OMA = omapatrilat

FIGURE 1.
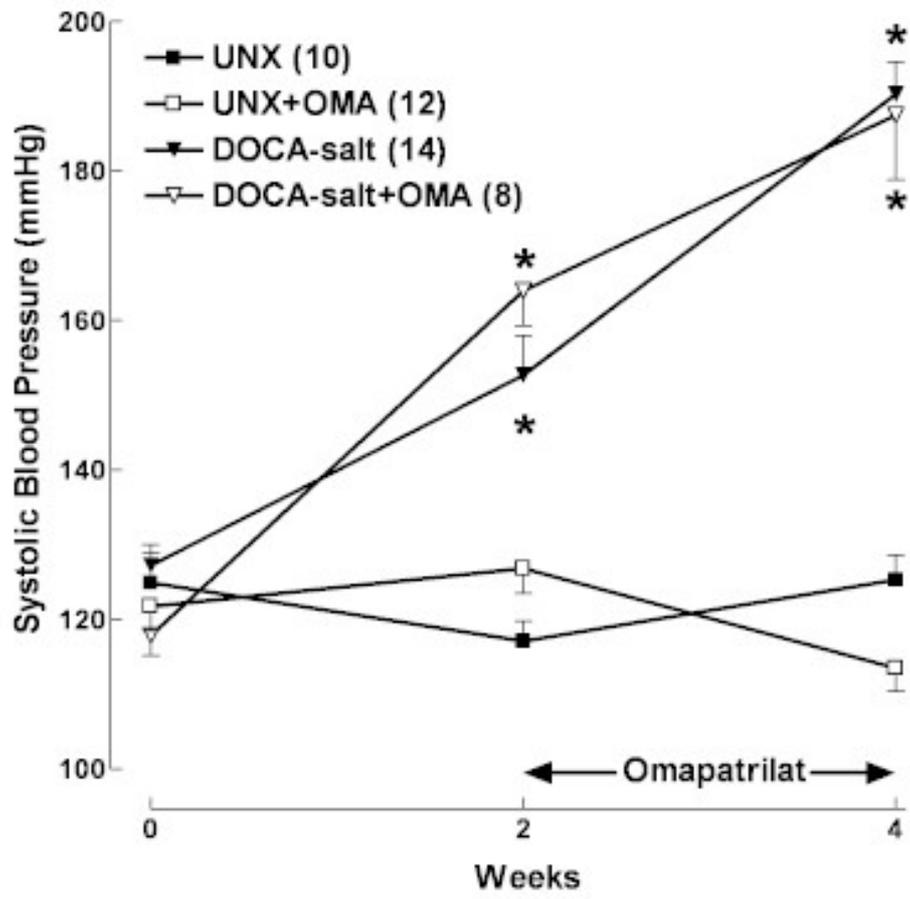
FIGURE 2.
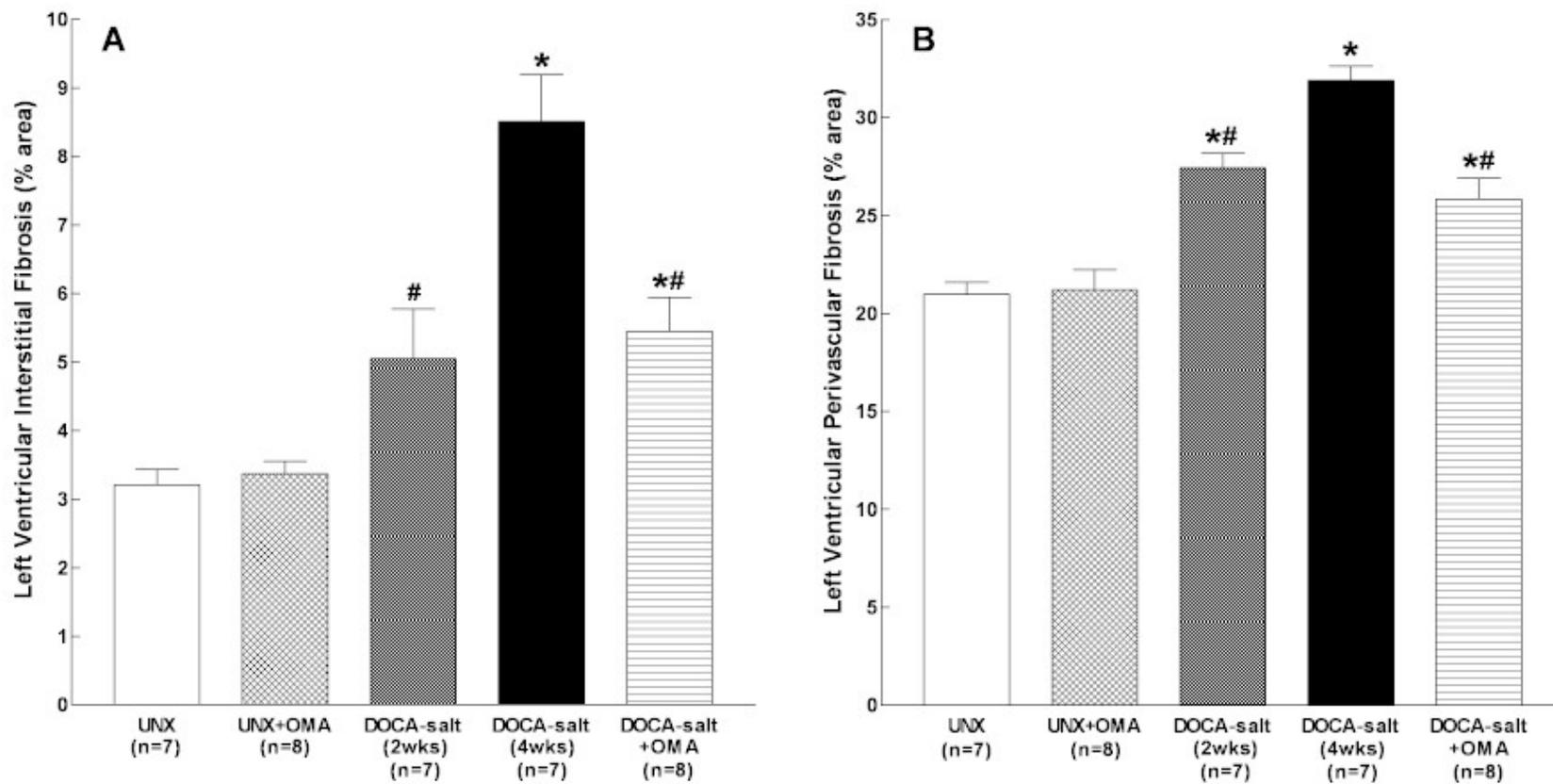
FIGURE 3.
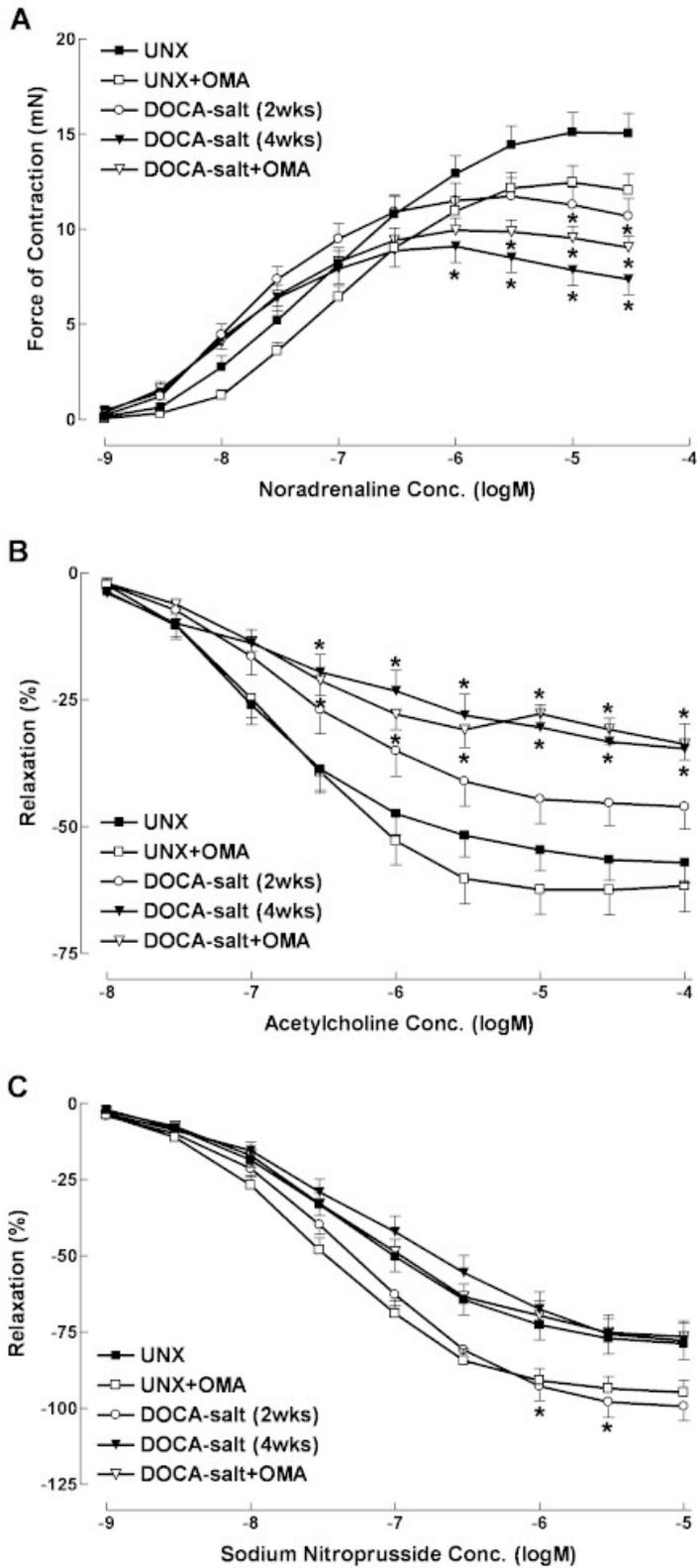
Legends to figures

FIGURE 1: Effect of omapatrilat (OMA) therapy on blood pressure. Data represent the comparison of systolic blood pressure in UNX, OMA-treated UNX, DOCA-salt hypertensive and OMA-treated DOCA-salt hypertensive groups over the 4 week protocol period. Values are mean ± SEM; * $p<0.05$ vs UNX; # $p<0.05$ vs DOCA-salt (4 week). Numbers in parentheses represent animal numbers.

FIGURE 2: Graphical representations of left ventricular interstitial collagen area (A) and perivascular collagen area (B) in UNX, omapatrilat (OMA)-treated UNX, DOCA-salt hypertensive (2 and 4 weeks) and OMA-treated DOCA-salt hypertensive groups over the 4 week protocol period. Values are mean ± SEM; * $p<0.05$ vs UNX; # $p<0.05$ vs DOCA-salt (4 week). Numbers in parentheses represent animal numbers.

FIGURE 3: Concentration-response curves to noradrenaline (A) for UNX (filled square, n=15), omapatrilat (OMA)-treated UNX (open square, n=15), 2 week DOCA-salt (open circle, n=15), 4 week DOCA-salt (filled triangle, n=15) and OMA-treated DOCA-salt (open triangle, n=14). Concentration-response curves to acetylcholine (B) for UNX (filled square, n=12), OMA-treated UNX (open square, n=14), 2 week DOCA-salt (open circle, n=14), 4 week DOCA-salt (filled triangle, n=14) and OMA-treated DOCA-salt (open triangle, n=14). Concentration-response curves to sodium nitroprusside (C) for UNX (filled square, n=15), OMA-treated UNX (open square, n=15), 2 week DOCA-salt (open circle, n=14), 4 week DOCA-salt (filled triangle, n=14) and OMA-treated DOCA-salt (open triangle, n=13); * $p<0.05$ vs UNX.