ORIGINAL ARTICLE

Time course of cardiac inflammation during nitric oxide synthase inhibition in SHR: impact of prior transient ACE inhibition

Lauren A Biwer[1], Karen M D'souza[1], Ali Abidali[1], Danni Tu[1], Ashley L Siniard[2], Matthew DeBoth[2], Matthew Huentelman[2] and Taben M Hale[1]

We have previously demonstrated that angiotensin-converting enzyme (ACE) inhibition with enalapril produces persistent effects that protect against future nitric oxide synthase (NOS) inhibitor (L-arginine methyl ester, L-NAME)-induced cardiac dysfunction and outer wall collagen deposition in spontaneously hypertensive rats (SHR). In the present study, we dissect the cytokine/chemokine release profile during NOS inhibition, its correlation to pathological cardiac remodeling and the impact of transient ACE inhibition on these effects. Adult male SHR were treated with enalapril (E+L) or tap water (C+L) for 2 weeks followed by a 2-week washout period. Rats were then subjected to 0, 3, 7 or 10 days of L-NAME treatment. The temporal response to NOS inhibition was evaluated by measuring arterial pressure, cardiac remodeling and cytokine/chemokine levels. L-NAME equivalently increased blood pressure and myocardial and vascular injury in C+L and E+L rats. However, pulse pressure (PP) was only transiently altered in C+L rats. The levels of several inflammatory mediators were increased during L-NAME treatment. However, interleukin-6 (IL-6) and IL-10 and monocyte chemoattractant protein-1 were uniquely increased in C+L hearts; whereas IL-4 and fractalkine were only elevated in E+L hearts. By days 7 and 10 of L-NAME treatment, there was a significant increase in the cardiac density of macrophages and proliferating cells, respectively only in C+L rats. Although myocardial injury was similar in both treatment groups, PP was not changed and there was a distinct cardiac chemokine/cytokine signature in rats previously treated with enalapril that may be related to the lack of proliferative response and macrophage infiltration in these hearts.
Hypertension Research (2016) 39, 8–18; doi:10.1038/hr.2015.107; published online 22 October 2015

INTRODUCTION

Heart disease continues to be the leading cause of morbidity and mortality in the United States.[1] While overall survival from myocardial infarction has improved, there has been an associated increase in the number of individuals that progress to heart failure—a debilitating and costly disease affecting 5.2 million Americans.[1] Prolonged inflammation following myocardial injury promotes continued tissue destruction and cardiac remodeling that ultimately leads to declining heart function.[2,3] Angiotensin-converting enzyme (ACE) inhibitors have been widely shown both clinically and experimentally to be cardio-protective. Treatment with these agents results in a reduction in blood pressure, an attenuation of pathological cardiac remodeling and macrophage infiltration, as well as preserved or improved cardiac function.[4–7] These beneficial effects have largely been attributed to a reduction in angiotensin II levels, as this peptide has been demonstrated to activate inflammatory pathways, fibroblast proliferation and

extracellular matrix production—key processes involved in pathological myocardial remodeling.[8,9]

We recently demonstrated that prior transient ACE inhibition in adult spontaneously hypertensive rats (SHR) produced persistent changes in the heart that conferred protection against future nitric oxide synthase (NOS) inhibitor-induced cardiac dysfunction[10] and fibrosis of the mid-outer myocardium.[11] Associated with this decreased fibrosis was a significant reduction in cellular proliferation and macrophage infiltration throughout the left ventricles (LVs) of those rats that were previously treated with an ACE inhibitor.[11] Importantly, these 'protected' rats had ceased ACE inhibitor treatment 2 weeks before the initiation of NOS inhibition.[10,11] This suggested that a short-term ACE inhibitor treatment may change the cardiac phenotype and the eventual response to tissue injury—even after the activity of the renin angiotensin system had been restored.

[1]Department of Basic Medical Sciences, University of Arizona, College of Medicine—Phoenix, Phoenix AZ, USA and [2]Neurogenomics Division, The Translational Genomics Research Institute, Phoenix, AZ, USA
Correspondence: Dr TM Hale, Department of Basic Medical Sciences, University of Arizona, College of Medicine—Phoenix, 425N 5th Street, Room 327, Phoenix, AZ 85004, USA.
E-mail: taben.hale@arizona.edu

NOS inhibition is widely used as an experimental tool to induce hypertension and acute myocardial injury.[11–18] In particular, treating SHR with a relatively high dose of a non-selective NOS inhibitor produces a marked elevation in mean arterial pressure (MAP) as well as detrimental effects on the myocardium, including multifocal necrosis, increases in pro-inflammatory mediators, inflammatory cell infiltration and ultimately a fibrotic scar.[11,12,14–16] Previous studies have demonstrated an inflammatory response characterized by increased monocyte chemoattractant protein-1 (MCP-1) levels and infiltration of CD3-positive cells and macrophages within 7 days of NOS inhibition, followed by further myocardial remodeling and fibrosis.[14,19–23] Given the fundamental role of inflammation in the remodeling that underlies heart failure, understanding the early processes regulating inflammatory cell recruitment and infiltration will lay the groundwork for future therapeutic discovery. Moreover, we hypothesize that our previously-described protection afforded by prior ACE inhibition[10,11] is related to differences in the inflammatory response to myocardial injury. Thus, in the present study we compared the temporal response to NOS inhibition with respect to arterial pressure, cardiac hypertrophy, cardiac cytokine/chemokine levels, cellular proliferation and macrophage density in rats that were and were not previously treated with an ACE inhibitor.

METHODS

Animals and treatments
Male SHR obtained from Charles River (Portage, MI, USA) were housed two per cage with standard rat chow and water (ad libitum) and were allowed a minimum of 48 h to acclimate before initiating the study. Rats were divided into two treatment groups: Control+L-arginine methyl ester (L-NAME) (C+L, $n = 32$) and Enalapril+L-NAME (E+L, $n = 32$). At 11 weeks of age, rats were provided vehicle (regular drinking water) or enalapril (30 mg kg^{-1} per day, in the drinking water) for 14 days, followed by a 14-day washout period. The 14-day washout period was based on our previous studies that showed a return to a stable off-treatment blood pressure and restoration of the renin angiotensin system.[24,25] After the washout, C+L and E+L rats were administered the non-selective NOS inhibitor N$^\omega$-Nitro-L-arginine methyl ester (L-NAME, 15 mg kg^{-1} per day, in the drinking water) for up to 10 days. Rats were euthanized after 0 ($n = 6$ per treatment group), 3 ($n = 10$ per treatment group), 7 ($n = 10$ per treatment group) or 10 ($n = 6$ per treatment group) days of L-NAME treatment. This protocol was based on our previous studies that demonstrated persistent reductions in blood pressure and LV to body weight ratios following cessation of ACE inhibitor treatment[4] and protection against L-NAME-induced cardiac fibrosis and dysfunction.[10,11] The duration of L-NAME treatment was based on previous studies, which showed inflammatory responses that peaked within 1 week of NOS inhibition.[14,16,19–23] All drugs were dissolved in the drinking water and concentrations were adjusted as previously described.[10,11] At the end of the treatment period, the rats were anesthetized and the hearts were excised, blotted dry and weighed. An equatorial cross-section was immersion-fixed overnight (16–20 h) in HistoChoice tissue fixative (Amresco, Solon, OH, USA). The right ventricle was trimmed away from the remaining tissue and the LVs were snap frozen in liquid nitrogen. All procedures were in accordance with Arizona State University and University of Arizona institutional guidelines. All animals used in this study were cared for in accordance to the recommendations in The Guide for the Care and Use of Laboratory Animals, National Institute of Health, Publ. No. 85-23, 1986.

Arterial pressure
The impact of this treatment protocol on MAP was previously reported.[10] In the present manuscript, we revisit that data set,[10] which was obtained at the same time as the time course analysis for inflammatory mediators. Arterial pressure data presented herein focuses on systolic blood (SBP), diastolic blood (DBP) and pulse pressures (PP); as SBP and PP have been demonstrated to be clinically important predictors of heart failure,[26,27] with the latter being particularly important in coronary artery disease.[28] Briefly, 9- to 10-week-old

male SHR ($n = 9$ per treatment group: C+L, E+L) were used for in vivo radiotelemetric arterial pressure assessments. Radiotelemetry pressure transducers (TA11PA-C40; Data Sciences International, St. Paul, MN, USA) were implanted in the abdominal aorta as previously described.[10,29] After a 1-week recovery period, baseline measurements were taken, and the treatment protocol described above was initiated when rats were 11–12 weeks of age.

RNA sequencing
RNA was isolated from LV of control (C+L0) as well as SHR treated for 2 weeks with enalapril followed by a 2-week washout (E+L0). RNA sequencing was performed using 1.0 µg of total RNA quantified via Nanodrop (Thermo Scientific, Pittsburgh, PA, USA). A sequencing library was prepared with Illumina's Truseq RNA Sample Preparation Kit v2 (Illumina, San Diego, CA, USA) following the manufacturer's protocol. In brief, poly-A containing mRNA molecules were purified using poly-T oligo attached magnetic beads. The mRNA was then thermally fragmented and converted to double-stranded cDNA. The cDNA fragments were end-repaired, a single 'A' nucleotide was incorporated, sequencing adapters were ligated, and fragments were enriched with 15 cycles of PCR. Final PCR-enriched fragments were validated on a 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) and quantified by qPCR using Kapa's Library Quantification Kit (Kapa Biosystems, Woburn, MA, USA) on the 7900HT (Applied Biosystems, Foster City, CA, USA). The final library was sequenced by 50 bp paired-end sequencing on a HiSeq2500 instrument (Illumina).

Histology and immunohistochemistry
Fixed transverse sections of heart (2–3 mm) were processed according to routine histological procedures and embedded in paraffin (Formula R, Leica Microsystems Surgipath, Buffalo Grove, IL, USA). Histology was examined in a blinded manner in Hematoxylin and Eosin-stained sections (5 µm). To correlate the degree of myocardial injury to macrophage infiltration and cellular proliferation, we calculated the percent area of infarct per LV. Using Image J (http://rsbweb.nih.gov/ij/), each focus of infarction (based on cardiomyocyte loss) was traced and area calculated. The sum of each area was divided by the total cross-sectional area of the LV.

In separate sections, we evaluated macrophage (ED-1)[30] density and proliferating cells (proliferating cell nuclear antigen, PCNA). Following antigen retrieval (ED-1: 0.1M citrate buffer at 96 °C, PCNA: 0.01M Tris-HCl pH 8.6 buffer in microwave pressure cooker) sections were blocked and exposed to monoclonal mouse anti-rat ED-1 antibody (AbD Serotec, Raleigh, NC, USA) or monoclonal mouse anti-PCNA (Dako, Carpinteria, CA, USA) overnight at 4 °C, followed by secondary antibody (Dako), then Streptavidin-HRP (Dako). Positive cells were counted by a blind observer at ×200 magnification, normalized for left ventricular cross-sectional area and data were expressed as percent change from day 0 for each treatment group (that is, C+L, E+L). Given their role in mediating fibrosis through cytokine release,[31] mast cells were labeled with toludene blue and counted in LV cross sections from C+L and E+L rats following 0 or 10 days of L-NAME treatment. All histological and immunohistological assessments were performed on the LV ($n = 6$-10 per group).

Cytokine levels
A rat-specific cytokine array (QAR-Cyt-3, Ray Biotech, Norcross, GA, USA) was performed, according to the manufacturer's instructions. Briefly, the LV ($n = 4$ per group) was pulverized in liquid nitrogen and protein was extracted. Five hundred micrograms of protein was applied to the cytokine array slide to assess levels of a variety of inflammatory mediators. Messenger RNA levels of MCP-1 (Fwd: CAGGTCTCTGTCACGCTTCT, Rev: AGTATTCATGCAAGGG AATAG) relative to β-actin (Fwd: GAAGCTGTGCTATGTTGCCCT, Rev: TTC TGCATCCTGTCAGCAATG) were measured by quantitative real-time PCR.

Statistical analysis
All time course analyses were based on a two-way analysis of variance (ANOVA) with a Bonferroni post-test (Graph Pad Prism Software, La Jolla, CA, USA). The two-way ANOVA investigated the impact of duration of L-NAME treatment (that is, time), prior drug treatment, and the interaction of

these two variables on the result, while the Bonferroni post-test evaluated whether there was a statistically significant difference as compared with L-NAME day 0 within a given treatment group. A subsequent Bonferroni *post hoc* test evaluated whether there were differences across treatment groups at a given time point. MAP analyses assessing changes from baseline were based on repeated measures two-way ANOVA, all other time course analyses were based on regular, unmatched two-way ANOVA. Messenger RNA expression changes were assessed using quantitative real-time PCR and analyzed using the delta delta Ct method. Comparisons between slopes were based on an unpaired Student's *T*-test. Statistical analysis on PCR data was performed using a one sample *t*-test (Graph Pad Prism Software). Data are expressed as mean ± s.e.m. and differences were deemed statistically significant with $P < 0.05$.

With respect to RNA sequencing analysis, RNA-Seq reads were trimmed with AlienTrimmer0.3.2 and mapped to the Rattus norvegicus genome (rn5) with STAR (ver2.3.1z4). Reads mapping to genes were counted with feature-Counts (ver1.4.4). Library-size normalization and differential expression analysis were conducted in DESeq2 (ver1.6.3) from the Bioconductor suite of packages for the R statistical programming environment. Statistically or biologically significant genes were selected for plotting with ggplot2.

RESULTS

Persistent effects induced by prior ACE inhibition

We previously demonstrated in the rats from the present manuscript that enalapril induced a significant reduction (−30%) in MAP during
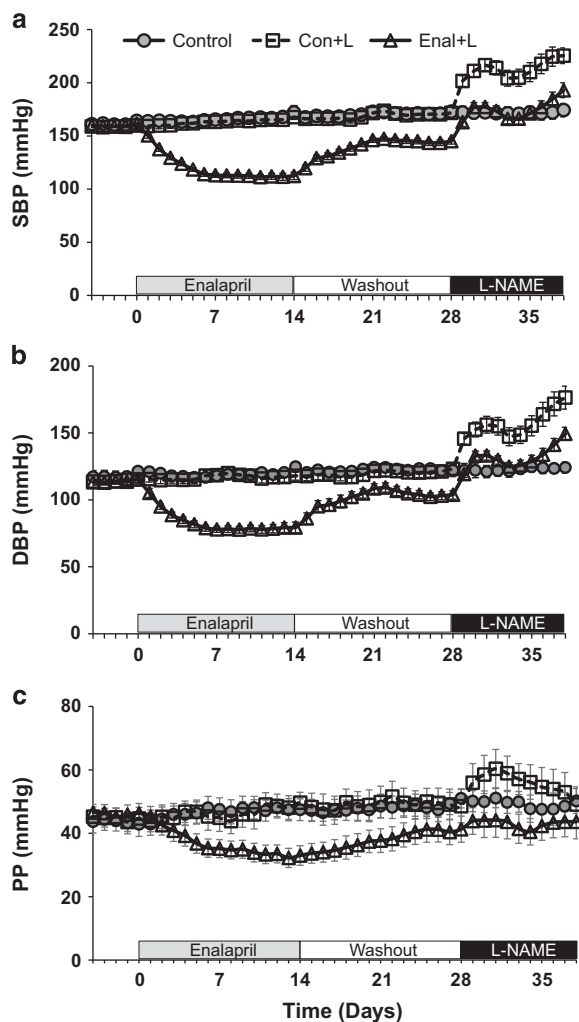

Figure 1 Daily average SBP (**a**), DBP (**b**) and PP (**c**) throughout the treatment period. Data represent average 24 h pressures ± s.e.m.

treatment.[10] Following cessation of treatment, pressure rose and stabilized at a new baseline that was 16% below control levels before initiation of L-NAME treatment.[10] These same rats were re-analyzed to evaluate the time course of L-NAME-induced changes in SBP, DBP and PP (Figure 1, Table 1). As was previously described for MAP,[10] ACE inhibition produced a reduction in SBP and DBP that persisted even after cessation of treatment. In contrast to blood pressure levels that re-established a new baseline within 1 week after stopping treatment, PP rose much more slowly ultimately reaching a level that was not significantly different from untreated rats (Figure 1, Table 1).

Body weight was not different between control SHR (319 ± 2.0 g) and those previously treated with enalapril (319 ± 8.4 g) at baseline (day 0 of L-NAME treatment), yet prior ACE inhibition resulted in a persistent reduction in heart weight relative to body weight (Table 2). We performed next-generation sequencing in order to determine whether prior ACE inhibition had any persistent impact on components of the intra-cardiac RAS. Two weeks after stopping enalapril treatment there was no significant change in gene expression for any of the major enzymes or receptors involved in local angiotensin II production or action (Figure 2).

Impact of NOS inhibition on arterial pressure and cardiac mass

L-NAME significantly increased SBP and DBP over the course of the 10-day treatment (Figure 1). The present analysis revealed that although the two groups started at a different baseline (C+L0 *vs.* E+L0), the percent increase in SBP and DBP due to L-NAME administration was equivalent between treatment groups at days 3, 7 and 10 (Table 1). In contrast, PP was transiently increased on days 3 and 7 in C+L rats, but not changed in E+L (Figure 1, Table 1).

The impact of NOS inhibition on body weight and cardiac hypertrophy was evaluated in C+L and E+L rats. Moreover L-NAME did not significantly alter body weight over the course of 10 days regardless of prior treatment (Table 2). The heart weight-to-body weight ratio remained significantly lower in the E+L rats than age-matched C+L rats throughout the study period (Table 2). L-NAME treatment induced a significant increase in cardiac mass by day 10 in the SHR that did not receive enalapril (C+L), whereas in SHR that were previously treated (E+L), L-NAME induced an increase in cardiac mass as early as day 3 that was sustained throughout the treatment period (Table 2).

Histology

Hematoxylin and eosin staining was performed to evaluate the extent of myocardial and coronary artery injury resulting from NOS inhibition. Before starting L-NAME, the myocardium exhibited normal histological features. The myocardium displayed 0–2 small foci of cardiomyocyte loss or injury at days 0 and 3 of L-NAME treatment. However, these areas increased in size and number over 7 and 10 days of L-NAME, regardless of pre-treatment (Table 2, Figure 3). Similarly, L-NAME induced coronary artery injury. As early as 3 days of NOS inhibition there was evidence of injury in at least one large coronary artery in the majority of the LVs of C+L and E+L rats (Figure 4). The injured vessels showed marked reduction in nuclear staining in the medial wall and there was evidence of increased cellularity surrounding the artery. The vascular damage progressed over 7 and 10 days of L-NAME treatment (Figure 4) and the incidence was not different between treatment groups.

Cytokine profiles

To measure the relative extent of inflammation following 0, 3, 7 and 10 days of L-NAME treatment, we measured several inflammatory

Table 1 Impact of prior enalapril treatment and L-NAME on systolic blood pressure (SBP), diastolic blood pressure (DBP) and pulse pressure (PP)

	SBP	SBP % change from day 0	DBP	DBP % change from day 0	Pulse pressure	PP % change from day 0
C+L0	172 ± 3.1	—	123 ± 3.4	—	49 ± 5.3	—
C+L3	$216 \pm 5.6^*$	26 ± 2.5	$156 \pm 6.2^*$	27 ± 3.2	$60 \pm 6.0^*$	26 ± 4.2
C+L7	$210 \pm 8.9^*$	22 ± 3.9	$155 \pm 7.8^*$	26 ± 4.6	$55 \pm 6.1^*$	12 ± 3.3
C+L10	$226 \pm 7.3^*$	31 ± 3.7	$176 \pm 8.6^*$	43 ± 5.6	49 ± 5.6	7 ± 2.8
E+L0	$145 \pm 3.0^\ddagger$	—	$103 \pm 3.6^\ddagger$	—	41 ± 4.6	—
E+L3	$177 \pm 4.4^{\dagger\ddagger}$	22 ± 1.8	$133 \pm 3.6^{\dagger\ddagger}$	29 ± 2.3	44 ± 5.4	$6 \pm 6.0^\ddagger$
E+L7	$171 \pm 5.8^{\dagger\ddagger}$	18 ± 2.8	$129 \pm 4.7^{\dagger\ddagger}$	25 ± 5.2	42 ± 4.5	3 ± 4.8
E+L10	$193 \pm 7.5^{\dagger\ddagger}$	33 ± 3.8	$149 \pm 5.2^{\dagger\ddagger}$	46 ± 6.6	44 ± 5.4	8 ± 5.8

Abbreviations: ANOVA, analysis of variance; L-NAME, L-arginine methyl ester.
Values are presented as mean ± s.e.m. SBP: two-way ANOVA: pre-treatment $P < 0.05$, time $P < 0.05$, interaction $P = 0.23$; DBP: two-way ANOVA: pre-treatment $P < 0.05$, time $P < 0.05$, interaction $P = 0.66$. PP: two-way ANOVA: time $P < 0.05$, interaction $P < 0.05$. Bonferroni post-test following repeated measures two-way ANOVA: $^*P < 0.05$ vs. C+L0, $^\dagger P < 0.05$ vs. E+L0, $^\ddagger P < 0.05$ vs. age matched C+L.

Table 2 Impact of prior enalapril and L-NAME treatment on body weight (BW), heart weight (HW) and heart weight-to-body weight ratio (HW/BW)

	BW (g)	HW (g)	HW/BW (g kg^{-1})	Infarct/LV (%)
C+L0	319 ± 2.0	1.08 ± 0.012	3.37 ± 0.025	0.33 ± 0.209
C+L3	329 ± 2.9	1.12 ± 0.015	3.39 ± 0.047	0.40 ± 0.146
C+L7	333 ± 6.0	1.13 ± 0.015	3.40 ± 0.038	5.66 ± 2.492
C+L10	314 ± 5.7	$1.17 \pm 0.027^*$	$3.73 \pm 0.106^*$	2.64 ± 1.742
E+L0	319 ± 8.4	$0.93 \pm 0.021\ddagger$	$2.93 \pm 0.031\ddagger$	0.11 ± 0.047
E+L3	326 ± 8.4	$1.03 \pm 0.020\dagger\ddagger$	$3.15 \pm 0.051\dagger\ddagger$	0.16 ± 0.104
E+L7	328 ± 5.6	$1.03 \pm 0.017\dagger\ddagger$	$3.14 \pm 0.033\dagger\ddagger$	5.88 ± 3.124
E+L10	333 ± 8.2	$1.10 \pm 0.023\dagger$	$3.29 \pm 0.050\dagger\ddagger$	0.71 ± 0.387

Abbreviations: ANOVA, analysis of variance; L-NAME, L-arginine methyl ester; LV, left ventricle.
Values are presented as mean ± s.e.m. Bonferroni post-test following two-way ANOVA: $^*P < 0.05$ vs. C+L0, $^\dagger P < 0.05$ vs. E+L0, $^\ddagger P < 0.05$ vs. age-matched C+L. HW/BW: two-way ANOVA: pre-treatment $P < 0.05$, time $P < 0.05$, interaction $P = 0.10$. Infarct/LV: two-way ANOVA: time $P < 0.05$.

mediators in whole LV homogenate. While some mediators did not show marked changes as a result of prior ACE inhibition, or during NOS inhibition (for example, ICAM-1, TNF-α, TIMP-1, CNTF, CINC-2, CINC-3, interleukin-alpha (IL-1α); data not shown), others were found to transiently or progressively increase over the course of the 10-day treatment. Many of these mediators showed different profiles in the hearts from rats previously treated with enalapril. IL-1β levels were found to be significantly influenced by duration of L-NAME treatment; however, post-tests only revealed a statistically significant increase at day 3 in both C+L and E+L LV ($P < 0.05$) (Figure 5a). IL-6 levels tended to increase in C+L LV and there was an overall trend toward a drug effect ($P = 0.086$, Figure 5b). Although granulocyte macrophage-colony stimulating factor (GM-CSF) was elevated by fourfold as early as day 3 in C+L, the increase only reached statistical significance at day 10 in both C+L and E+L hearts (Figure 5c). Monocyte chemoattractant protein-1 (MCP-1) levels were found to be elevated as early as 3 days and remained elevated at day 10 in the C+L LV (Figure 5d). The MCP-1 levels in the E+L LV were not significantly increased over baseline at any time point. Quantitative real-time PCR also revealed a trend toward increased mRNA expression of the gene encoding MCP-1 during L-NAME treatment in both treatment groups, although this did not achieve statistical significance (data not shown). Thymus chemokine-1 (TCK-1) was significantly increased by day 3 in both C+L and E+L, and elevated again at day 10 in the E+L hearts (Figure 5e).

IL-10 levels were significantly increased at day 3 only in C+L LV (Figure 6a), whereas IL-4 levels were significantly increased by day 10 only in the E+L LV (Figure 6b). Finally, fractalkine levels were found to be significantly increased after 3 and 10 days of L-NAME in E+L LV (Figure 6c).

Immunohistochemistry
NOS inhibition significantly increased the number of proliferating cells at day 10 (day 7: ∼ 3-fold, $P > 0.05$; day 10: ∼ 5-fold, $P < 0.05$) in the LV of C+L rats as compared with baseline (C+L0). In contrast, in the LV of E+L SHR there was no significant change in the number of proliferating cells with respect to baseline (E+L0), at any of the time points studied (Figure 7). The number of proliferating cells was significantly correlated with the overall area of infarct only in C+L LVs (R^2: C+L 0.35, $P < 0.05$; E+L 0.046, $P > 0.05$; Figure 7). In addition, the number of proliferating cells was found to be significantly correlated with the concentration of MCP-1 in C+L (R^2: 0.37, $P < 0.05$), but not E+L (R^2: 0.019, $P > 0.05$) LVs (data not shown).

When compared with baseline, macrophage density showed a significant increase by day 7 (5-fold, $P < 0.05$) of NOS inhibition that was maintained at day 10 (3.6-fold, $P < 0.05$) in C+L LV (Figure 8). In contrast, there was no significant change in macrophage density in the LV of E+L SHR (Figure 8). The area of infarct was found to significantly correlate with the number of macrophages in the LV of both C+L (R^2: 0.92, $P < 0.05$) and E+L (R^2: 0.43, $P < 0.05$) rats. However, the slopes of the regression lines were significantly different (C+L: $1.1 \times 10^{-3} \pm 5.3 \times 10^{-5}$ vs. E+L $2.0 \times 10^{-3} \pm 3.6 \times 10^{-4}$, $P < 0.05$) indicating that for a given area of infarct, there were significantly fewer macrophages infiltrating the E+L hearts (Figure 8). Mast cell numbers were not different as a result of prior
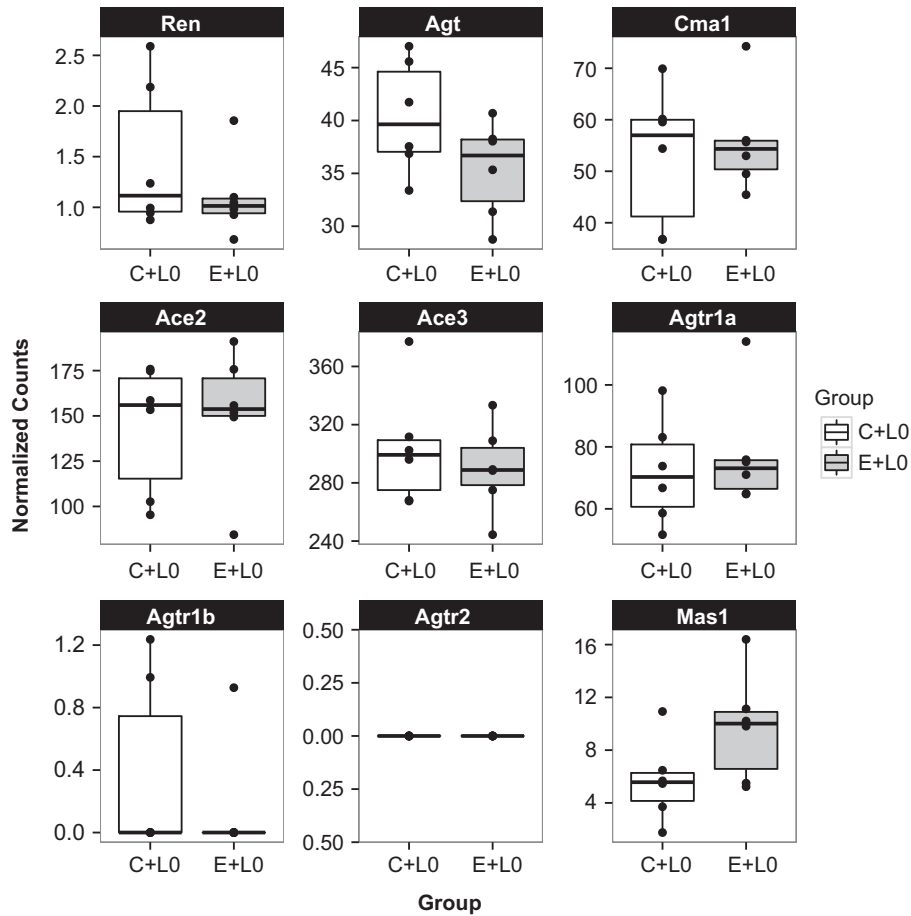
Figure 2 Gene expression of components of the intra-cardiac renin angiotensin system in LV from control (C+L0) and rats previously treated with an ACE inhibitor followed by washout (E+L0). Prior ACE inhibition did not significantly alter expression of renin (Ren), angiotensinogen (Agt), chymase (Cma1), ACE2, ACE3, angiotensin II type 1a receptor (Agtr1a), angiotensin II type 1b receptor (Agtr1b), angiotensin II type 2 receptor (Agtr2) or the Mas1 receptor.
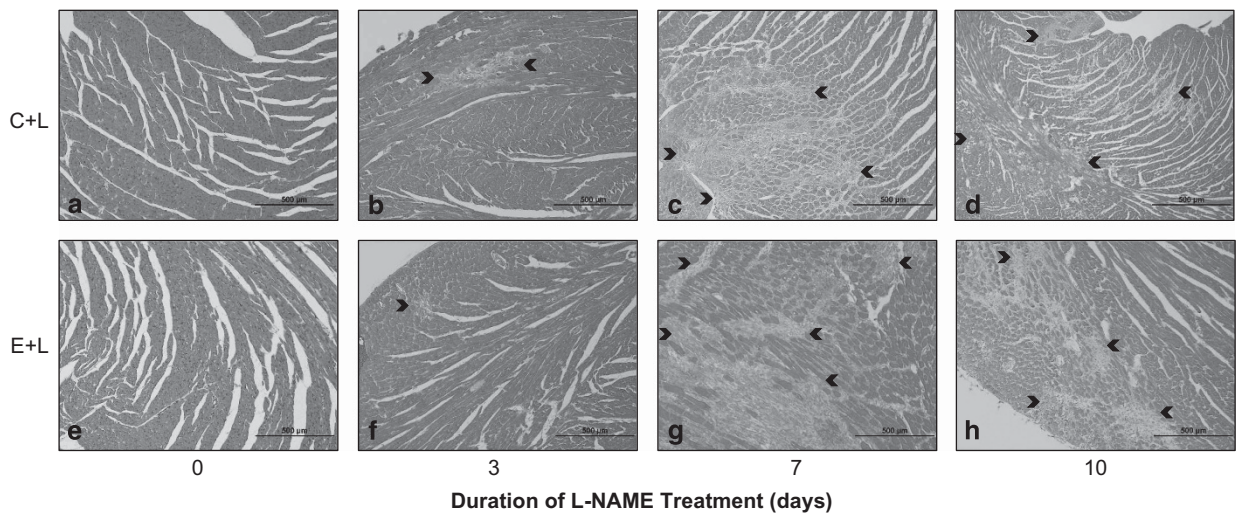

Figure 3 Histological examination of myocardium from rats treated with L-NAME (C+L) (**a–d**) or enalapril followed by L-NAME (E+L) (**e–h**) for 0, 3, 7 and 10 days. Myocardium displayed normal histological features in both C+L and E+L at day 0. In both treatment groups, there were 1–2 small foci of infarct as early as day 3 that was characterized by cardiomyocyte loss and increased cellular infiltrate (**b, f**). The progression of this damage was characterized by increases in size and number of foci of infarction in myocardium from both treatment groups over the course of 10 days. Representative sections stained with Hematoxylin and Eosin, ×100 magnification. Arrowheads point to foci of infarction. A full color version of this figure is available at *Hypertension Research* journal online.
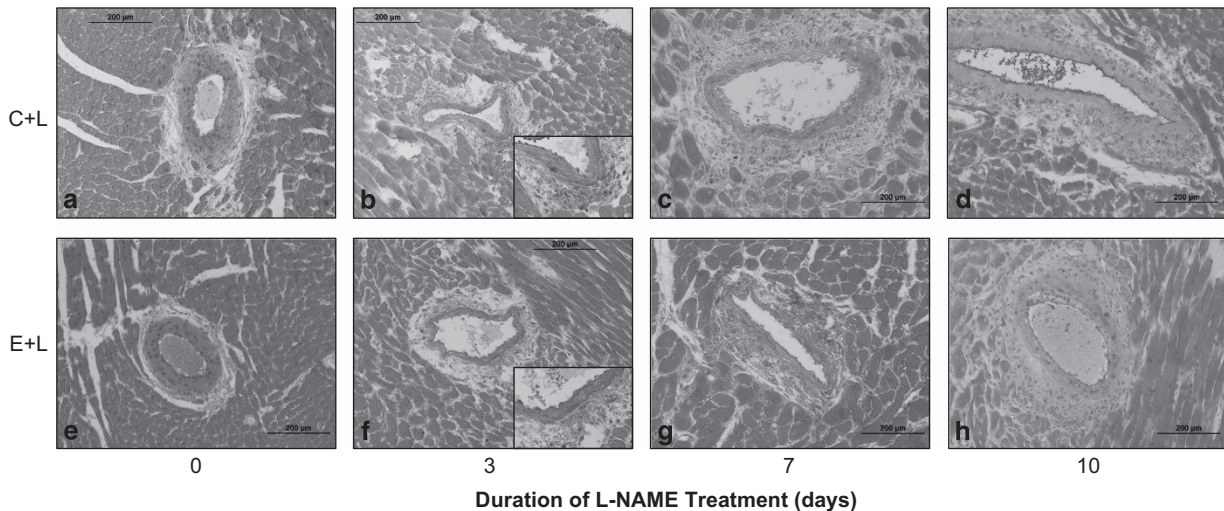
Figure 4 Histological examination of coronary arteries from rats treated with L-NAME (C+L) (**a–d**) or enalapril followed by L-NAME (E+L) (**e–h**) for 0, 3, 7 and 10 days. Vessels displayed normal histological features in both C+L and E+L at day 0. In both treatment groups, there was evidence of injury as early as day 3 that was characterized by loss of nuclear staining in the medial wall and increased cellular infiltrate surrounding the vessel (**b**, **f**, insets). This damage progressed in arteries from both treatment groups over the course of 10 days. Representative sections stained with Hematoxylin and Eosin, ×200 magnification, insets: ×400 magnification. A full color version of this figure is available at *Hypertension Research* journal online.

ACE inhibition or NOS inhibition (C+L0: 102 ± 11.0, C+L10: 105 ± 25.9, E+L0: 102 ± 10.4, E+L10: 109 ± 8.3).

DISCUSSION

This is the first study to perform a temporal analysis of multiple cytokine and chemokine profiles in the LVs of NOS inhibitor-treated rats. The present study demonstrates that prior ACE inhibition induces persistent changes that impact the PP and cardiac inflammatory and remodeling responses to the first 10 days of NOS inhibition. Despite equivalent impact on arterial pressure, cardiac hypertrophy, coronary artery and myocardial wall injury in response to L-NAME, cardiac cell proliferation and macrophage density were unchanged in rats previously treated with enalapril. That is, for an equivalent degree of injury, there was a markedly reduced inflammatory and proliferative response to NOS inhibition in the hearts of rats that underwent prior, transient ACE inhibition.

In the present study, and consistent with previous studies[14] we demonstrate that within 3 days of L-NAME treatment there is a significant increase in blood pressure, evidence of coronary artery injury and increases in pro-inflammatory cytokines and chemokines. MCP-1 and GM-CSF are known to be involved in macrophage recruitment and activation.[32–38] Regulating cardiac macrophage infiltration and function has been identified as an important target for heart disease treatment.[7,39–41] When inflammatory macrophages persist at sites of injury, they promote ongoing tissue destruction resulting from prolonged cytokine secretion and production of proteases, growth factors and superoxide.[2,3] In the myocardium, this vicious cycle leads to fibroblast proliferation, infarct expansion and excessive fibrosis. In the present study, we found that in the C+L rats, the macrophage-recruiting chemokine MCP-1 was increased ninefold at day 3, before significant cardiomyocyte loss and macrophage infiltration at day 7. GM-CSF also tended to be elevated at these early time points. Levels of MCP-1, GM-CSF, macrophage density and cellular proliferation remained significantly elevated (↑12-fold, ↑6-fold, ↑4-fold and ↑5-fold *vs.* baseline, respectively) after 10 days of NOS inhibition in this treatment group. In contrast, there was no significant change in MCP-1 levels, macrophage accumulation or

proliferating cells in hearts from rats previously treated with an ACE inhibitor. Moreover, in the LVs of E+L rats, there were significantly fewer macrophages per given area of infarct, as compared with C+L. In addition, MCP-1 levels significantly correlated with the number of proliferating cells only in the C+L hearts. MCP-1 has been shown to induce proliferation of several cell types including VSMC and fibroblasts, although whether this is due to direct or indirect actions of this chemokine remains equivocal.[16,42–44] Furthermore, Anti-MCP-1 therapy has been shown to decrease cardiac cell proliferation during NOS inhibition[16] and aortic constriction.[44] In the present study, although the extent of injury did not differ between groups, the macrophage and proliferative response to injury did. It may be that the attenuation of macrophage infiltration and cellular proliferation is related to the lack of increase in the macrophage-recruiting chemokine MCP-1 in these hearts. However, the correlation between MCP-1 and cell proliferation in the C+L, but not E+L hearts potentially suggests a phenotypic difference in the cardiac cells from the two treatment groups. Specifically, the cellular response to injury with respect to chemokine production, subsequent macrophage infiltration, and cellular proliferation was altered in rats previously treated with enalapril. It should be noted however that the E+L hearts did exhibit a significant increase in GM-CSF at day 10, however, this was not associated with any change in cardiac MCP-1 or macrophage levels. In addition, we have previously found in older rats undergoing a similar treatment protocol that there was no significant increase in macrophage density over baseline in E+L rats even after 14 days of L-NAME (unpublished observations).

Other pro-inflammatory cytokines were also found to be increased within 3 days of L-NAME treatment. Specifically, IL-1β and IL-6 have an early and critical role in the inflammatory response to tissue injury by inducing cytokines and chemokines to facilitate inflammatory cell infiltration.[45–47] While Il-1β was increased in both treatment groups, Il-6 tended to be uniquely increased in the C+L hearts. TCK-1, a variant of CXCL7 that has been shown to be involved in neutrophil activation and the vascular repair response to injury, was transiently increased in both treatment groups.[48]
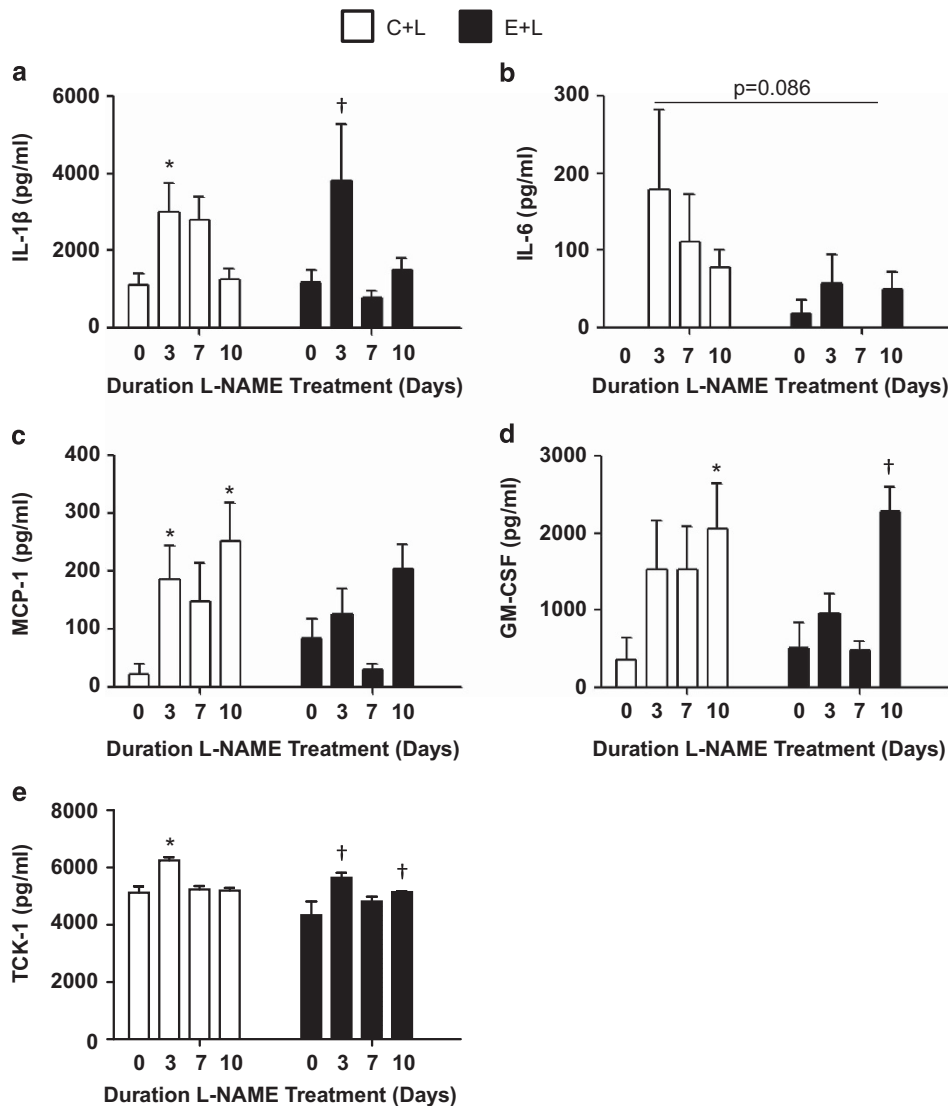
Figure 5 Left ventricular levels of (**a**) IL-1β (two-way: time $P<0.05$), (**b**) IL-6 (two-way: pre-treatment $P=0.086$), (**c**) MCP-1 (two-way: time $P<0.05$), (**d**) GM-CSF (two-way: time $P<0.05$) and (**e**) TCK-1 (two-way: time $P<0.05$, drug $P<0.05$) protein after 0, 3, 7 and 10 days of L-NAME treatment in previously untreated (C+L: white bars) and previously enalapril-treated (E+L: black bars) rats. Mean ± s.e.m., Bonferroni post-test following two-way ANOVA: *$P<0.05$ vs. C+L0, †$P<0.05$ vs. E+L0.

Several cytokines and chemokines have also been associated with resolving inflammation and facilitating repair processes.[49,50] For example, fractalkine, IL-4 and IL-10 have been shown to be involved in the recruitment and activation of the so-called 'reparative' subset of macrophages.[37,51] This monocyte subset functions in part to activate fibroblasts to promote collagen deposition and scar formation.[51] Fractalkine levels appeared to be increased overall in both treatment groups, however, they were only statistically significantly elevated in the E+L hearts. Further study will be required to evaluate the impact of these elevated fractalkine levels. IL-4 and IL-10 are typically considered as anti-inflammatory mediators based on their ability to inhibit secretion of pro-inflammatory cytokines and promote tissue repair involving collagen deposition and fibrosis.[49,50] IL-4 was increased only in the E+L hearts in the present study at a time point that correlates with the increased level of GM-CSF in this group. IL-10 has also been shown to inhibit macrophage infiltration following

myocardial infarction in rats.[52] While the levels of IL-10 were found to be statistically significantly elevated at day 3 of NOS inhibition in C+L hearts, this was clearly not sufficient to inhibit the subsequent macrophage infiltration in these rats. Given the multiple effects described for each mediator, it may be more important to evaluate the net impact of a collection of cytokines, rather than each in isolation. That is, identifying inflammatory and anti-inflammatory signatures may be a more appropriate strategy for investigating the potential for disease progression.

The mechanisms by which prior ACE inhibition provides sustained cardiac protection against a future insult remain to be fully determined. Given the extensive impact of this antihypertensive treatment, there are likely multiple factors that contribute to these effects. For example, before L-NAME treatment, E+L rats were operating at a more advantageous baseline level of left ventricular mass and arterial pressure. We have previously determined that these effects persist for
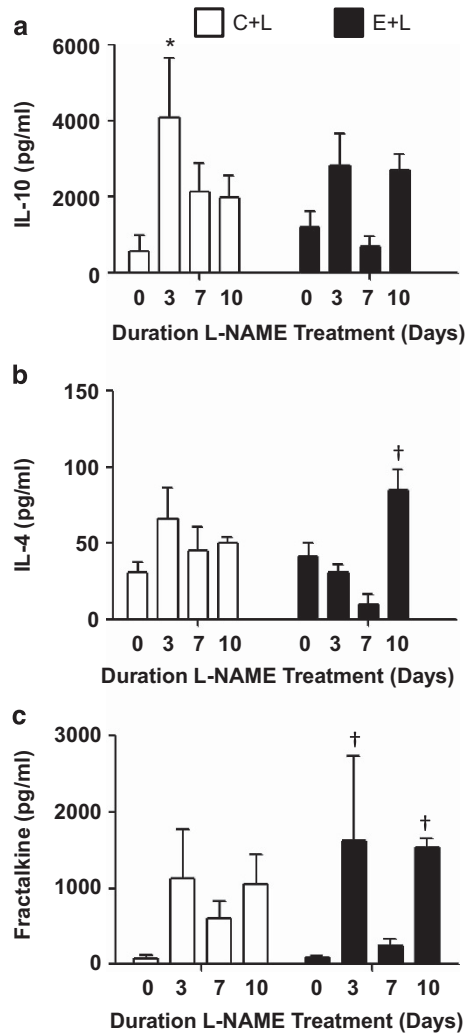
Figure 6 Left ventricular levels of (**a**) IL-10 (two-way: time $P<0.05$), (**b**) IL-4 (two-way: time $P<0.05$, interaction $P<0.05$) and (**c**) Fractalkine (two-way: time $P<0.05$) protein after 0, 3, 7 and 10 days of L-NAME treatment in previously untreated (C+L: white bars) and previously enalapril-treated (E+L: black bars) rats. Mean ± s.e.m., Bonferroni post-test following two-way ANOVA: *$P<0.05$ vs. C+L0, †$P<0.05$ vs. E+L0.

Figure 7 Representative images (×200 magnification) of LV from C+L10 (**a**) and E+L10 (**b**) rats immunolabeled for PCNA and counterstained with hematoxylin and eosin. Brown indicates positive staining and arrows point to representative positive cells. (**c**) Quantification of immunohistochemistry, data presented as the average percent increase in labeled cells mm^{-2} from day 0 of L-NAME treatment ± s.e.m. Two-way ANOVA: time $P<0.05$, interaction $P<0.05$, Bonferroni post-test following two-way ANOVA: *$P<0.05$ vs. C+L0, ‡$P<0.05$ vs. C+L day 10. (**d**) Number of PCNA-positive cells plotted against area of infarct in each LV. Slope of C+L line is significantly non-zero ($P<0.05$). A full color version of this figure is available at *Hypertension Research* journal online.

up to 20 weeks off-treatment despite restoration of the renin angiotensin system.[25] Moreover, we showed using a crossover kidney transplant study design that ACE inhibitor-induced changes in structurally-based renal vascular resistance were the likely mechanism underlying this persistent reduction in arterial pressure.[25] In the present study, despite different baselines with respect to arterial pressure and LV mass in C+L0 and E+L0 rats, L-NAME treatment resulted in a similar incidence of vascular and myocardial injury, as well as equivalent increases in pressure and cardiac hypertrophy relative to the respective baseline. In fact, the impact on cardiac mass was realized 7 days earlier in the E+L hearts. This is perhaps not surprising given that we previously showed that the sustained reduction in left ventricular hypertrophy was blood pressure dependent.[25] Although the relative increase in blood pressure was equivalent, it is possible that the lower absolute level of arterial pressure in E+L rats is in part responsible for the attenuated inflammatory response to injury in the present study. However, others have shown that preventing L-NAME-induced hypertension does not
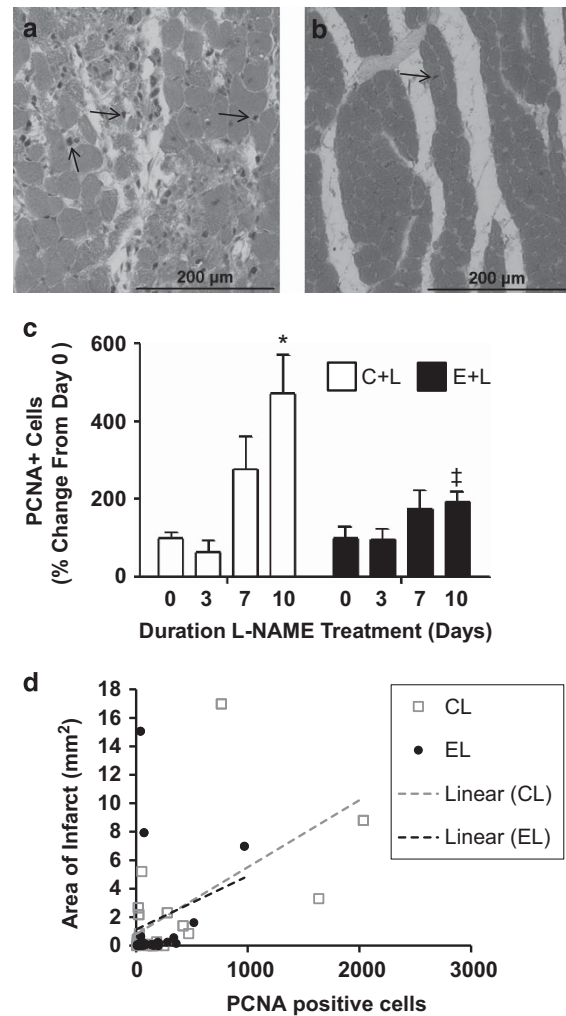
impact MCP-1 production or macrophage infiltration in response to NOS inhibition.[53] Nonetheless, it is noteworthy that in the present study many of the cytokine levels tended to decrease at day 7—particularly in the E+L rats. Arterial pressure dips between days 3 and 8 of L-NAME treatment, reaching a nadir on days 5 and 6.[10] This occurs in both treatment groups and the mechanism for this tri-phasic blood pressure response is not known. PP has also been positively associated with acute coronary heart disease[28] and may represent another potential reason for the differential impact of cardiac inflammation in C+L and E+L rats. This parameter was not different at baseline between treatment groups; however, the rise in systolic pressure during NOS inhibition was faster than the rise in diastolic pressure in C+L rats resulting in a transient increase in PP at time points that correlated with cytokine and chemokine increases (day 3) and macrophage infiltration (day 7) in the C+L treatment
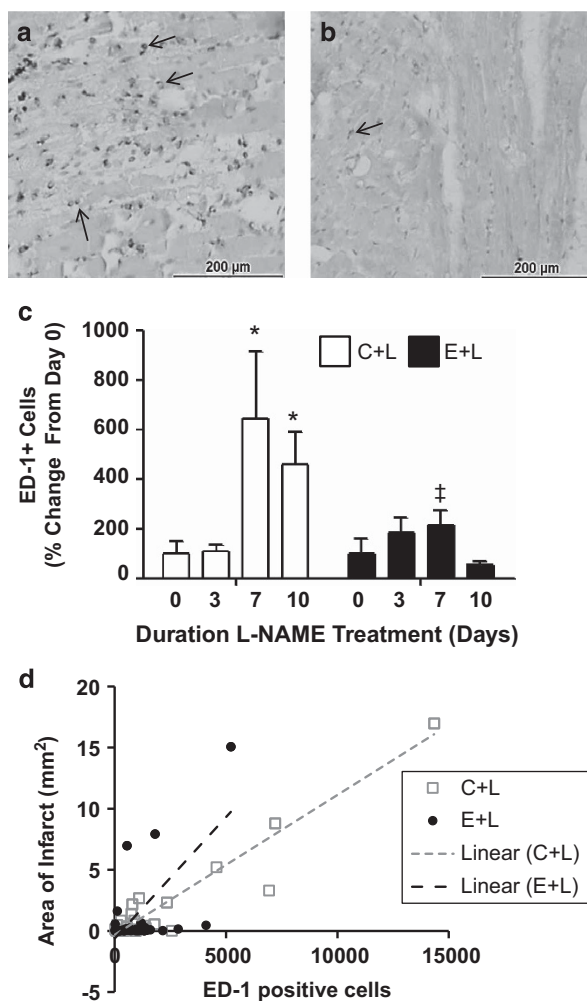
Figure 8 Representative images (×200 magnification) of ED-1 positive macrophages in the LVs of C+L10 (**a**) and E+L10 (**b**). Brown indicates positive staining and arrows point to representative positive cells. (**c**) Quantification of immunohistochemistry, data presented as the average percent increase in labeled cells mm^{-2} from day 0 of L-NAME treatment ± s.e.m. Two-way ANOVA: interaction $P < 0.05$, Bonferroni post-test following two-way ANOVA: *$P < 0.05$ *vs.* C+L0, ‡$P < 0.05$ *vs.* C+L day 7. (**d**) Number of PCNA-positive cells plotted against area of infarct in each LV. Slope of both C+L and E+L lines is significantly non-zero ($P < 0.05$). Slope of E+L line is significantly greater than C+L slope ($P < 0.05$). A full color version of this figure is available at *Hypertension Research* journal online.

group. A third potential mechanism for the beneficial effects following transient ACE inhibition may be a persistent alteration in the RAS. Indeed, RAS inhibition has been shown to prevent L-NAME-induced hypertension,[23,54,55] increases in cardiac[23] and renal[56] macrophage infiltration and MCP-1 levels. While our previous study[25] showed that there was not a sustained RAS inhibition following cessation of enalapril, the present study investigated the long-term impact on the cardiac-specific RAS. The present findings demonstrate that the sustained cardio-protection is not related to an alteration in expression of cardiac enzymes involved in angiotensin II production or of angiotensin receptors. Taken together with the abovementioned published studies,[23,54–56] the present findings suggest that ongoing RAS inhibition is necessary to prevent the hypertensive, but not the inflammatory response to L-NAME. However, although not

statistically significant, there was a trend toward an increase in the expression of the Mas receptor. Activation of this receptor by Ang(1–7) has been previously demonstrated to be cardio-protective[57,58] and this tendency toward increased expression may potentially mediate, at least in part the beneficial effects observed in the present study. Finally, enalapril-induced changes in cardiac cells may underlie the long-term protection against pathological cardiac remodeling. For example, ACE inhibitor treatment has been shown to induce apoptosis in 30% of left ventricular fibroblasts.[59,60] It may be that there is a sub-population of fibroblasts that is hyperproliferative and promotes excessive fibrosis and inflammation. Prior ACE inhibitor treatment may have either deleted this particular subset of fibroblasts via apoptosis, or induced epigenetic changes that ultimately alter the fibroblast response to L-NAME treatment. This is a particularly intriguing hypothesis given that cardiac fibroblasts have emerged as important mediators of the inflammatory response following ischemic injury.[61–63]

Despite improved treatments and diagnostic tools, heart disease remains the leading cause of morbidity and mortality in the United States.[1] Our present and previous findings[10,11] suggest that the heart of a hypertensive rat with a predisposition to damage can be modified such that the response to a pathogenic stimulus is reduced. Although E+L rats were operating at a lower blood pressure and reduced left ventricular mass, L-NAME induced myocardial infarction and coronary artery injury to a similar extent in both treatment groups. Importantly, in response to this injury there was no change in the number of proliferating cells, and the number of macrophages infiltrating per unit area of infarct was lower in E+L when compared with C+L hearts. The protection observed in the hearts of SHR previously treated with the ACE inhibitor likely involves a complex network of cytokines; however, differential regulation of MCP-1 has emerged as a predominate candidate. The relatively short duration of the washout period is a limitation of the present study. Future investigations involving longer off-treatment periods are an important future direction that will ultimately determine whether there is potential for long-term clinical benefit to transient ACE inhibition. While it remains to be determined how long the cardio-protection lasts, Ishiguro *et al*[64,65] have shown that transient RAS inhibition protects against L-NAME-induced renal injury and diabetic nephropathy for at least 6–7 weeks off-treatment. In addition, we have previously demonstrated that the persistent reduction in arterial pressure and left ventricular hypertrophy persisted for more than 20 weeks after cessation of a 2-week ACE inhibitor treatment. Future work emanating from these studies will reveal a greater understanding of the inflammatory processes involved in macrophage recruitment and in pathological cardiac remodeling. Moreover, determining the mechanisms by which prior ACE inhibition produce cardiac protection may lead to novel strategies for heart failure treatment and prevention.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

1 Schocken DD, Benjamin EJ, Fonarow GC, Krumholz HM, Levy D, Mensah GA, Narula J, Shor ES, Young JB, Hong Y. Prevention of heart failure: a scientific statement from the American Heart Association Councils on Epidemiology and Prevention, Clinical Cardiology, Cardiovascular Nursing, and High Blood Pressure Research; Quality of Care and Outcomes Research Interdisciplinary Working Group; and Functional Genomics and Translational Biology Interdisciplinary Working Group. Circulation 2008; 117: 2544–2565.

2 DiPietro LA. Wound healing: the role of the macrophage and other immune cells. Shock 1995; 4: 233–240.

3 Chen GY, Nunez G. Sterile inflammation: sensing and reacting to damage. Nat Rev Immunol 2010; 10: 826–837.

4 Hale TM, Shoichet MJ, Bushfield TL, Adams MA. Time course of vascular structural changes during and after short-term antihypertensive treatment. Hypertension 2003; 42: 171–176.

5 Perret-Guillaume C, Joly L, Jankowski P, Benetos A. Benefits of the RAS blockade: clinical evidence before the ONTARGET study. J Hypertens Suppl 2009; 27: S3–S7.

6 Susic D, Varagic J, Frohlich ED. Pharmacologic agents on cardiovascular mass, coronary dynamics and collagen in aged spontaneously hypertensive rats. J Hypertens 1999; 17: 1209–1215.

7 Leuschner F, Panizzi P, Chico-Calero I, Lee WW, Ueno T, Cortez-Retamozo V, Waterman P, Gorbatov R, Marinelli B, Iwamoto Y, Chudnovskiy A, Figueiredo JL, Sosnovik DE, Pittet MJ, Swirski FK, Weissleder R, Nahrendorf M. Angiotensin-converting enzyme inhibition prevents the release of monocytes from their splenic reservoir in mice with myocardial infarction. Circ Res 2010; 107: 1364–1373.

8 Marko L, Kvakan H, Park JK, Qadri F, Spallek B, Binger KJ, Bowman EP, Kleinewietfeld M, Fokuhl V, Dechend R, Muller DN. Interferon-gamma signaling inhibition ameliorates angiotensin II-induced cardiac damage. Hypertension 2012; 60: 1430–1436.

9 Rosin NL, Falkenham A, Sopel MJ, Lee TD, Legare JF. Regulation and role of connective tissue growth factor in AngII-induced myocardial fibrosis. Am J Pathol 2013; 182: 714–726.

10 Biwer LA, Broderick TL, Xu H, Carroll C, Hale TM. Protection against L-NAME-induced reduction in cardiac output persists even after cessation of angiotensin-converting enzyme inhibitor treatment. Acta Physiol (Oxf) 2013; 207: 156–165.

11 Hale TM, Robertson SJ, Burns KD, deBlois D. Short-term ACE inhibition confers long-term protection against target organ damage. Hypertens Res 2012; 35: 604–610.

12 Ono Y, Ono H, Matsuoka H, Fujimori T, Frohlich ED. Apoptosis, coronary arterial remodeling, and myocardial infarction after nitric oxide inhibition in SHR. Hypertension 1999; 34 (4 Pt 1): 609–616.

13 Tomita H, Egashira K, Ohara Y, Takemoto M, Koyanagi M, Katoh M, Yamamoto H, Tamaki K, Shimokawa H, Takeshita A. Early induction of transforming growth factor-beta via angiotensin II type 1 receptors contributes to cardiac fibrosis induced by long-term blockade of nitric oxide synthesis in rats. Hypertension 1998; 32: 273–279.

14 Tomita H, Egashira K, Kubo-Inoue M, Usui M, Koyanagi M, Shimokawa H, Takeya M, Yoshimura T, Takeshita A. Inhibition of NO synthesis induces inflammatory changes and monocyte chemoattractant protein-1 expression in rat hearts and vessels. Arterioscler Thromb Vasc Biol 1998; 18: 1456–1464.

15 Miguel-Carrasco JL, Mate A, Monserrat MT, Arias JL, Aramburu O, Vazquez CM. The role of inflammatory markers in the cardioprotective effect of L-carnitine in L-NAME-induced hypertension. Am J Hypertens 2008; 21: 1231–1237.

16 Koyanagi M, Egashira K, Kitamoto S, Ni W, Shimokawa H, Takeya M, Yoshimura T, Takeshita A. Role of monocyte chemoattractant protein-1 in cardiovascular remodeling induced by chronic blockade of nitric oxide synthesis. Circulation 2000; 102: 2243–2248.

17 Li Z, Ma JY, Kerr I, Chakravarty S, Dugar S, Schreiner G, Protter AA. Selective inhibition of p38alpha MAPK improves cardiac function and reduces myocardial apoptosis in rat model of myocardial injury. Am J Physiol Heart Circ Physiol 2006; 291: H1972–H1977.

18 Bertera FM, Del Mauro JS, Lovera V, Chiappetta D, Polizio AH, Taira CA, Hocht C. Enantioselective pharmacokinetics and cardiovascular effects of nebivolol in L-NAME hypertensive rats. Hypertens Res 2014; 37: 194–201.

19 Neves SR, Machado CR, Pinto AM, Borges AH, Cunha FQ, Camargos ER. Macrophage populations and cardiac sympathetic denervation during L-NAME-induced hypertension in rats. Histol Histopathol 2006; 21: 803–812.

20 Egashira K, Ni W, Inoue S, Kataoka C, Kitamoto S, Koyanagi M, Takeshita A. Pravastatin attenuates cardiovascular inflammatory and proliferative changes in a rat model of chronic inhibition of nitric oxide synthesis by its cholesterol-lowering independent actions. Hypertens Res 2000; 23: 353–358.

21 Kitamoto S, Egashira K, Kataoka C, Koyanagi M, Katoh M, Shimokawa H, Morishita R, Kaneda Y, Sueishi K, Takeshita A. Increased activity of nuclear factor-κB participates in cardiovascular remodeling induced by chronic inhibition of nitric oxide synthesis in rats. Circulation 2000; 102: 806–812.

22 Koyanagi M, Egashira K, Kubo-Inoue M, Usui M, Kitamoto S, Tomita H, Shimokawa H, Takeshita A. Role of transforming growth factor-beta1 in cardiovascular inflammatory changes induced by chronic inhibition of nitric oxide synthesis. Hypertension 2000; 35 (1 Pt 1): 86–90.

23 Usui M, Egashira K, Tomita H, Koyanagi M, Katoh M, Shimokawa H, Takeya M, Yoshimura T, Matsushima K, Takeshita A. Important role of local angiotensin II activity mediated via type 1 receptor in the pathogenesis of cardiovascular inflammatory changes induced by chronic blockade of nitric oxide synthesis in rats. Circulation 2000; 101: 305–310.

24 Hale TM, Bushfield TL, Woolard J, Pang JJ, Rees-Milton KJ, Adams MA. Changes critical to persistent lowering of arterial pressure in spontaneously hypertensive rat occur early in antihypertensive treatment. J Hypertens 2011; 29: 113–122.

25 Smallegange C, Hale TM, Bushfield TL, Adams MA. Persistent lowering of pressure by transplanting kidneys from adult spontaneously hypertensive rats treated with brief antihypertensive therapy. Hypertension 2004; 44: 89–94.

26 Haider AW, Larson MG, Franklin SS, Levy D. Systolic blood pressure, diastolic blood pressure, and pulse pressure as predictors of risk for congestive heart failure in the Framingham Heart Study. Ann Intern Med 2003; 138: 10–16.

27 Vaccarino V, Holford TR, Krumholz HM. Pulse pressure and risk for myocardial infarction and heart failure in the elderly. J Am Coll Cardiol 2000; 36: 130–138.

28 Glasser SP, Halberg DL, Sands C, Gamboa CM, Muntner P, Safford M. Is Pulse Pressure an Independent Risk Factor for Incident Acute Coronary Heart Disease Events? The REGARDS Study. Am J Hypertens 2013; 27: 555–563.

29 Hale TM, Okabe H, Bushfield TL, Heaton JP, Adams MA. Recovery of erectile function after brief aggressive antihypertensive therapy. J Urol 2002; 168: 348–354.

30 Chen X, Mori T, Guo Q, Hu C, Ohsaki Y, Yoneki Y, Zhu W, Jiang Y, Endo S, Nakayama K, Ogawa S, Nakayama M, Miyata T, Ito S. Carbonyl stress induces hypertension and cardio-renal vascular injury in Dahl salt-sensitive rats. Hypertens Res 2013; 36: 361–367.

31 Kanellakis P, Ditiatkovski M, Kostolias G, Bobik A. A pro-fibrotic role for interleukin-4 in cardiac pressure overload. Cardiovasc Res 2012; 95: 77–85.

32 Biwa T, Sakai M, Shichiri M, Horiuchi S. Granulocyte/macrophage colony-stimulating factor plays an essential role in oxidized low density lipoprotein-induced macrophage proliferation. J Atheroscler Thromb 2000; 7: 14–20.

33 Chen BD, Clark CR, Chou TH. Granulocyte/macrophage colony-stimulating factor stimulates monocyte and tissue macrophage proliferation and enhances their responsiveness to macrophage colony-stimulating factor. Blood 1988; 71: 997–1002.

34 Burgess AW, Metcalf D. The nature and action of granulocyte-macrophage colony stimulating factors. Blood 1980; 56: 947–958.

35 Tanimoto A, Murata Y, Wang KY, Tsutsui M, Kohno K, Sasaguri Y. Monocyte chemoattractant protein-1 expression is enhanced by granulocyte-macrophage colony-stimulating factor via Jak2-Stat5 signaling and inhibited by atorvastatin in human monocytic U937 cells. J Biol Chem 2008; 283: 4643–4651.

36 Jost MM, Ninci E, Meder B, Kempf C, Van RN, Hua J, Berger B, Hoefer I, Modolell M, Buschmann I. Divergent effects of GM-CSF and TGFbeta1 on bone marrow-derived macrophage arginase-1 activity, MCP-1 expression, and matrix metalloproteinase-12: a potential role during arteriogenesis. FASEB J 2003; 17: 2281–2283.

37 Martinez FO, Sica A, Mantovani A, Locati M. Macrophage activation and polarization. Front Biosci 2008; 13: 453–461.

38 Kusunoki H, Taniyama Y, Otsu R, Rakugi H, Morishita R. Anti-inflammatory effects of hepatocyte growth factor on the vicious cycle of macrophages and adipocytes. Hypertens Res 2014; 37: 500–506.

39 Leuschner F, Rauch PJ, Ueno T, Gorbatov R, Marinelli B, Lee WW, Dutta P, Wei Y, Robbins C, Iwamoto Y, Sena B, Chudnovskiy A, Panizzi P, Keliher E, Higgins JM, Libby P, Moskowitz MA, Pittet MJ, Swirski FK, Weissleder R, Nahrendorf M. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. J Exp Med 2012; 209: 123–137.

40 Maekawa Y, Anzai T, Yoshikawa T, Asakura Y, Takahashi T, Ishikawa S, Mitamura H, Ogawa S. Prognostic significance of peripheral monocytosis after reperfused acute myocardial infarction:a possible role for left ventricular remodeling. J Am Coll Cardiol 2002; 39: 241–246.

41 Hong YJ, Jeong MH, Ahn Y, Yoon NS, Lee SR, Hong SN, Moon JY, Kim KH, Park HW, Kim JH, Cho JG, Park JC, Kang JC. Relationship between peripheral monocytosis and nonrecovery of left ventricular function in patients with left ventricular dysfunction complicated with acute myocardial infarction. Circ J 2007; 71: 1219–1224.

42 Frangogiannis NG, Dewald O, Xia Y, Ren G, Haudek S, Leucker T, Kraemer D, Taffet G, Rollins BJ, Entman ML. Critical role of monocyte chemoattractant protein-1/CC chemokine ligand 2 in the pathogenesis of ischemic cardiomyopathy. Circulation 2007; 115: 584–592.

43 Kruglov EA, Nathanson RA, Nguyen T, Dranoff JA. Secretion of MCP-1/CCL2 by bile duct epithelia induces myofibroblastic transdifferentiation of portal fibroblasts. Am J Physiol Gastrointest Liver Physiol 2006; 290: G765–G771.

44 Kuwahara F, Kai H, Tokuda K, Takeya M, Takeshita A, Egashira K, Imaizumi T. Hypertensive myocardial fibrosis and diastolic dysfunction: another model of inflammation? Hypertension 2004; 43: 739–745.

45 Bujak M, Frangogiannis NG. The role of IL-1 in the pathogenesis of heart disease. Arch Immunol Ther Exp (Warsz) 2009; 57: 165–176.

46 Brasier AR. The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. Cardiovasc Res 2010; 86: 211–218.

47 Kobara M, Noda K, Kitamura M, Okamoto A, Shiraishi T, Toba H, Matsubara H, Nakata T. Antibody against interleukin-6 receptor attenuates left ventricular remodelling after myocardial infarction in mice. Cardiovasc Res 2010; 87: 424–430.

48 Gleissner CA, von Hundelshausen P, Ley K. Platelet chemokines in vascular disease. Arterioscler Thromb Vasc Biol 2008; 28: 1920–1927.

49 te Velde AA, Huijbens RJ, Heije K, de Vries JE, Figdor CG. Interleukin-4 (IL-4) inhibits secretion of IL-1 beta, tumor necrosis factor alpha, and IL-6 by human monocytes. Blood 1990; 76: 1392–1397.

50 Wang P, Wu P, Siegel MI, Egan RW, Billah MM. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. J Biol Chem 1995; 270: 9558–9563.

51 Nahrendorf M, Pittet MJ, Swirski FK. Monocytes: protagonists of infarct inflammation and repair after myocardial infarction. *Circulation* 2010; **121**: 2437–2445.

52 Stumpf C, Seybold K, Petzi S, Wasmeier G, Raaz D, Yilmaz A, Anger T, Daniel WG, Garlichs CD. Interleukin-10 improves left ventricular function in rats with heart failure subsequent to myocardial infarction. *Eur J Heart Fail* 2008; **10**: 733–739.

53 Toba H, Nakagawa Y, Miki S, Shimizu T, Yoshimura A, Inoue R, Asayama J, Kobara M, Nakata T. Calcium channel blockades exhibit anti-inflammatory and antioxidative effects by augmentation of endothelial nitric oxide synthase and the inhibition of angiotensin converting enzyme in the N(G)-nitro-L-arginine methyl ester-induced hypertensive rat aorta: vasoprotective effects beyond the blood pressure-lowering effects of amlodipine and manidipine. *Hypertens Res* 2005; **28**: 689–700.

54 Katoh M, Egashira K, Mitsui T, Chishima S, Takeshita A, Narita H. Angiotensin-converting enzyme inhibitor prevents plasminogen activator inhibitor-1 expression in a rat model with cardiovascular remodeling induced by chronic inhibition of nitric oxide synthesis. *J Mol Cell Cardiol* 2000; **32**: 73–83.

55 Pereira LM, Bezerra DG, Machado DL, Mandarim-de-Lacerda CA. Enalapril attenuates cardiorenal damage in nitric-oxide-deficient spontaneously hypertensive rats. *Clin Sci (Lond)* 2004; **106**: 337–343.

56 Kashiwagi M, Masutani K, Shinozaki M, Hirakata H. MCP-1 and RANTES are expressed in renal cortex of rats chronically treated with nitric oxide synthase inhibitor. Involvement in macrophage and monocyte recruitment. *Nephron* 2002; **92**: 165–173.

57 Benter IF, Yousif MH, Al-Saleh FM, Raghupathy R, Chappell MC, Diz DI. Angiotensin-(1-7) blockade attenuates captopril- or hydralazine-induced cardiovascular protection in spontaneously hypertensive rats treated with NG-nitro-L-arginine methyl ester. *J Cardiovasc Pharmacol* 2011; **57**: 559–567.

58 Benter IF, Yousif MH, Anim JT, Cojocel C, Diz DI. Angiotensin-(1-7) prevents development of severe hypertension and end-organ damage in spontaneously hypertensive rats treated with L-NAME. *Am J Physiol Heart Circ Physiol* 2006; **290**: H684–H691.

59 Tea BS, Dam TV, Moreau P, Hamet P, deBlois D. Apoptosis during regression of cardiac hypertrophy in spontaneously hypertensive rats. Temporal regulation and spatial heterogeneity. *Hypertension* 1999; **34**: 229–235.

60 Der Sarkissian S, Marchand EL, Duguay D, Hamet P, deBlois D. Reversal of interstitial fibroblast hyperplasia via apoptosis in hypertensive rat heart with valsartan or enalapril. *Cardiovasc Res* 2003; **57**: 775–783.

61 Smith RS, Smith TJ, Blieden TM, Phipps RP. Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. *Am J Pathol* 1997; **151**: 317–322.

62 Kawaguchi M, Takahashi M, Hata T, Kashima Y, Usui F, Morimoto H, Izawa A, Takahashi Y, Masumoto J, Koyama J, Hongo M, Noda T, Nakayama J, Sagara J, Taniguchi S, Ikeda U. Inflammasome activation of cardiac fibroblasts is essential for myocardial ischemia/reperfusion injury. *Circulation* 2011; **123**: 594–604.

63 van Nieuwenhoven FA, Turner NA. The role of cardiac fibroblasts in the transition from inflammation to fibrosis following myocardial infarction. *Vascul Pharmacol* 2013; **58**: 182–188.

64 Ishiguro K, Sasamura H, Sakamaki Y, Hayashi K, Saruta T, Itoh H. Differential effects of transient treatment of spontaneously hypertensive rats with various antihypertensive agents on the subsequent development of diabetic nephropathy. *Nephron Exp Nephrol* 2008; **109**: e20–e28.

65 Ishiguro K, Sasamura H, Sakamaki Y, Itoh H, Saruta T. Developmental activity of the renin-angiotensin system during the 'critical period' modulates later L-NAME-induced hypertension and renal injury. *Hypertens Res* 2007; **30**: 63–75.