Reduction of Ventricular Hypertrophy and Fibrosis in Spontaneously Hypertensive Rats by L-Arginine

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Abstract

The purpose of this experiment was to explore long-term L-arginine administration on ventricular hypertrophy and cardiac fibrosis in spontaneously hypertensive rats (SHR) and normotensive Wistar Kyoto (WKY) rats. Twenty-four rats of each strain at eight wks of age were divided into two groups - one receiving L-arginine and the other vehicle for twelve wks. Arterial pressure (AP) and heart rate were monitored. At 20 wks of age, the rats' rings of thoracic aorta were isolated to record isometric tension. The study measured left ventricular weight (LVW), body weight (BW), left ventricular (LV) contents of cGMP, and collagen volume fraction (LVCVF). Histological examination of the LV tissue determined changes in cardiomyocytes. Administration of L-arginine did not alter the AP change in SHR, but reduced the AP in WKY after six wks. Our results showed a significantly higher LVW/BW ratio and LVCVF in vehicle-treated SHR compared to levels in corresponding WKY, whereas, the LV cGMP and nitrite/nitrate measurements were higher in vehicle-treated WKY than in SHR. L-Arginine treatment decreased LVW/BW ratio and LVCVF, while increasing the levels of LV cGMP and nitrite/ nitrate only in SHR, consistent with histopathological examinations that showed L-arginine prevented cardiomyocytes from thickness and hypertrophy. Our results suggested that the mechanism of reduction in ventricular hypertrophy and fibrosis following long-term L-arginine administration in SHR may stem from increased myocardial nitric oxide-cGMP signaling, independent of AP and EDV of thoracic aorta.

Key Words: L-arginin, cyclic GMP, nitric oxide, cardiomyocytes, ventricular hypertrophy, fibrosis

Introduction

L-arginine is the substrate of nitric oxide (NO), catalyzed by NO synthase (NOS) to produce NO and L-citrulline (29, 31). Three NOS isoforms - endothelial NOS, neuronal NOS, and inducible NOS (20, 22), have been identified in cardiomyocytes (32, 37). NO stimulates soluble guanylate cyclase to increase intracellular levels of cyclic guanosine 5'-monophosphate (cGMP) and causes a reduction in intracellular calcium (7, 21, 24), and the continuous release of NO from the endothelium plays a significant role in the regulation of arterial pressure and peripheral resistance (2, 4, 16, 25). Previous and recent studies have revealed that chronic inhibition of NO synthesis leads to sustained hypertension (1, 3, 28), cerebral vascular changes (13, 14), cardiac hypertrophy (28, 33), and interstitial ventricular fibrosis (30). One study by Kuo *et al*. (19) found that spontaneously hypertensive rats (SHR) contained lower cardiac levels of cGMP and cGMP-dependent protein kinase than normotensive rats, suggesting that cardiac hypertrophy of SHR may result, at least in part, from an abnormal NO-cGMP producing system. Two previous studies declared that L-arginine administration did not influence blood pressure and the degree of cardiac hypertrophy in hypertensive rats (18, 34), while, Mastuoka *et al*. (23) reported that chronic L-arginine

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Received: July 14, 2004; Revised: September 22, 2004; Accepted: September 25, 2004.

treatment could attenuate cardiac hypertrophy in SHR.

The effects of long-term L-arginine administration on cardiac hypertrophy remains controversial, compounded by the fact that cardiac fibrosis usually developed in the progression of ventricular hypertrophy (9). Whether chronic L-arginine treatment can also reduce cardiac fibrosis, based upon NO inhibition of collagen synthesis *in vitro* (17), awaits further investigation. In SHR, however, the extent of cardiac hypertrophy corresponds with arterial impedance (pulsatile hemodynamics) and does not significantly correlate to arterial pressure (AP) or total peripheral resistance (steady hemodynamics) (15). The arterial impedance reflects mainly the Windkessel function of the aorta and large arteries (4, 15, 16), implying that endothelium-dependent vasodilation (EDV) of thoracic aorta might also contribute to the pathogenesis of cardiac hypertrophy.

This study examines the effects of long-term L-arginine administration on ventricular hypertrophy and cardiac fibrosis. We also attempted to elucidate possible mechanisms of these changes.

Materials and Methods

Experimental Animals

SHR and age-matched normotensive Wistar Kyoto strain (WKY) were purchased from the National Animal Center and housed in the University Animal Center with constant room temperature at 22 ± 1°C under a 12/12 hour light/dark regimen. The rats were fed a standard rodent diet in pellet form (NaCl content < 1%), purchased from PMI Feeds, Inc. of Richmond, IN. Food and water were provided *ad libitum*. The care and experiments with animals were carried out in accordance with the principles of the National Animal Center and Laboratory Animal Center of Tzu Chi University.

Drugs

L-Arginine, norepinephrine, acetylcholine and other chemicals were purchased from Sigma Chemical Co (St. Louis, MO, USA). L-Arginine was dissolved in distilled water to make a daily dose of 43 mM. The other drugs were dissolved in normal saline. We prepared these agents immediately before use.

Long-term L-Arginine Administration

Twenty-four male SHR (8 wks old) and the same number of male age-matched WKY rats were randomly divided into four groups. Each group consisted of 12 weight and age matched rats. Vehicle control groups were provided distilled water without L-arginine, while treated groups of SHR and WKY received distilled water containing L-arginine (7.5 g/L = 43 mM). At this concentration, the daily intake of L-arginine was approximately 0.7 mmol/kg. Administration of L-arginine continued for a period of 12 wks. Therefore, the age of treated and vehicle control rats was 20 wks old at the time of entering the acute experiment.

Measurement of Arterial Pressure, Heart Rate, and Body Weight

In each group, the systolic arterial pressure (SAP) and heart rate (HR) were measured once a week by the tail-cuff method with a photoelectric volume oscillometer (UR-50100; Ueda, Tokyo, Japan) as previously described (3). Before measurement, each rat was pre-warmed to 37°C for 10-15 mins. We repeated the procedure every 2-3 min, taking four measurements as an individual mean for data analysis. The body weight (BW) was recorded every day, before and during the experiment.

Isometric Tension Recording of Isolated Thoracic Aortic Rings

After oral administration of L-arginine or vehicle for 12 wks, rats were sacrificed with an overdose of intraperitoneal sodium pentobarbital (120 mg/kg). The thorax was opened to isolate a segment of thoracic aorta, and then the aortic ring was immediately placed in oxygenated (95% O_2, 5% CO_2) Kreb's solution (mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 11, pH 7.4). The ring segments were connected to force-displacement transducers (FTO3; Grass) for isometric tension recording in organ baths filled with oxygenated Kreb's solution (37°C), maintaining basal tension at 2 g. After stabilizing for one hour, the rings were contracted with norepinephrine (10^{-6} M) and then relaxed by acetylcholine (10^{-6} M), to test the integrity of endothelial cells. After washout and a 30-min stabilization, aortic rings were precontracted with norepinephrine (10^{-5} M), and cumulative concentration-response curves were constructed in four rings from each rat with acetylcholine (10^{-8} ~10^{-6} M) to obtain the endothelium-dependent vasorelaxation. We expressed the endothelium-dependent vasorelaxation as a percentage of the precontractile tension.

Evaluation of Ventricular Hypertrophy and Measurement of Cardiac cGMP and NO Contents

The study used the left ventricular weight-to-body weight ratio (LVW/BW) to determine the degree of ventricular hypertrophy, according to the method

previously described (15), which involved weighing the left ventricle following removal of the aorta and atrium, and then immediately storing the tissue in liquid nitrogen. A part of the frozen tissue was taken to quantify the ratio of frozen to dry weight. The remaining tissue was homogenized in cold 6% trichloracetic acid to obtain a final 10% homogenate, which we centrifuged for 20 min at 10,000 rpm. The supernatant was recovered and washed four times with five volumes of water-saturated ethyl ether. We dried the final aqueous extract at 60°C under a stream of nitrogen and then determined the left ventricular (LV) cGMP level by enzymatic assay (Amersham, Life Science, UK), expressing the data as fmol/mg dry weight. The level of LV nitrite/nitrate, as an indicator of NO production, was measured using a spectrophotometric assay (10).

Histopathological Examination and Evaluation of Ventricular Fibrosis

To evaluate ventricular fibrosis, five pieces of excised left ventricular tissue from each group were fixed in 6% formaldehyde and dehydrated with graded concentrations of alcohol for embedding in paraffin. We sectioned paraffin slices from each ventricle at 5 μm thicknesses and stained them with hematoxylin and eosin (HE). Histological changes of the myocardium were examined with a light microscope.

LV interstitial collagen volume fraction (LVCVF) was measured using picrosirius red staining (38). For the quantitative measurement of LVCVF, sections were analyzed under the microscope using a x20 lens. A total of 20 fields was analyzed per section. The collagen content was determined by measuring the area of stained tissue within a set field and expressed as a percentage of the total field area. A CCD video camera (BioScan Inc. USA) recorded images, excluding fields containing artifacts, vessels or damaged tissue.

Statistical Analysis

Data was expressed as mean ± SEM with n = number of rats. Comparisons within and among groups were made by analysis of variance and *Scheffe's* test. P values < 0.05 were considered to be statistically significant.

Results

Changes in SAP with Age

Figure 1 displays the changes of SAP in SHR and WKY before and during the 12 wks of oral administration with L-arginine or vehicle, illustrating a gradual increase in SAP of vehicle-treated animals since the experiment began. In WKY, SAP maintained a constant level of approximately 150 mmHg after the sixth wk of treatment (14 wks old), while the SAP in SHR did not reach a plateau at any time during the 12-wk treatment. At the end of the experiment, the difference of SAP between vehicle-treated SHR and WKY was 67 mmHg (218 ± 3 *vs.* 151 ± 3 mmHg, P < 0.01).

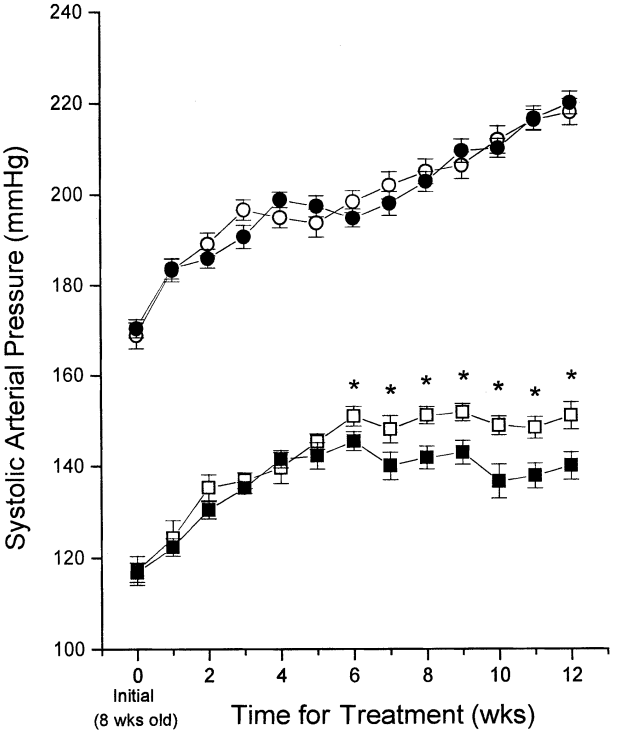

Fig. 1. Systolic arterial pressure (SAP) changes in SHR and WKY before (initial) and during 12 wks of oral L-arginine administration. Open circles represent vehicle-SHR; closed circles, L-arginine-SHR; open squares, vehicle-WKY; and closed squares, L-arginine-WKY. Values are means ± SEM (n = 12 in each group). Note the decrease in SAP after six wks of L-arginine treatment in WKY (*P < 0.05).

Effects of L-Arginine on SAP and HR

The 12-wk L-arginine administration did not significantly alter the changes in SAP and HR in SHR (Table 1 and Fig. 1), suggesting that chronic L-arginine treatment did not exert a depressor effect in SHR. In addition, chronic L-arginine treatment did not affect the HR in WKY (Table 1). The difference was that L-arginine did not change the SAP from the first to fifth wks of treatment in WKY, but L-arginine-treated WKY displayed a lower SAP than vehicle-treated WKY after the sixth wk - at 14 wks old (Fig. 1). The values of SAP were 140 ± 3 *vs.* 151 ± 3 mmHg, respectively at the end of the experiment (L-

Table 1. Changes in SAP, HR, BW and LVW following 12-wk administration of L-arginine in SHR and WKY.

	SAP mmHg	HR beats/min	BW g	LVW mg	LVW/BW mg/g
SHR-vehicle (n = 12)	218 ± 3	330 ± 12	354 ± 4	1127 ± 34	3.21 ± 0.09
SHR-L-arg (n = 12)	220 ± 4	328 ± 10	357 ± 9	1009 ± 28	2.83 ± 0.05
P value	> 0.05	> 0.05	> 0.05	< 0.05	< 0.05
WKY-vehicle (n = 12)	151 ± 3	283 ± 8	379 ± 10	915 ± 18	2.43 ± 0.078
WKY-L-arg (n = 12)	140 ± 3	285 ± 9	388 ± 9	953 ± 73	2.45 ± 0.098
P value	< 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Values are mean ± SE. L-arg, L-arginine; SAP, systolic arterial pressure HR, heart rate; BW, body weight; LVW, left ventricular weight.

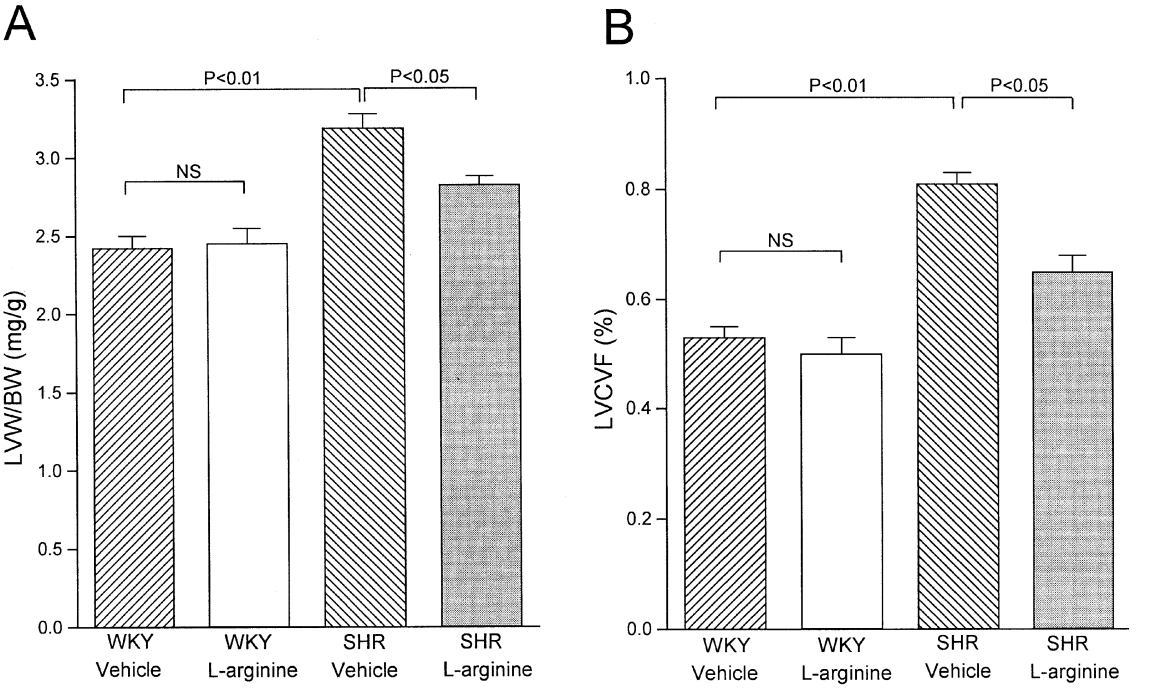

Fig. 2. Histographs showing the effect of L-arginine administration for 12 wks on the left ventricular (LV) weight-to-body weight (LVW/BW) ratio (A); and LV interstitial collagen volume fraction (LVCVF) (B). Note the higher LVW/BW ratio and LVCVF in SHR than WKY, and the reduction in LVW/BW, as well as LVCVF following L-arginine treatment in SHR, but not in WKY. Data are means ± SEM (n = 12 in each group).

arginine-treated WKY *vs*. vehicle-treated WKY, $P < 0.05$; Table 1). L-Arginine treatment significantly reduced the SAP in WKY, but not in SHR.

Effects of L-Arginine on BW, Cardiac Mass, and Fibrosis

At the same age, SHR had lower BW than that in WKY ($P < 0.05$). L-Arginine treatment for 12 wks did not affect the BW in either strain (Table 1). In vehicle-treated groups, LVW, LVW/BW ratio and LVCVF were all significantly higher in SHR than those in WKY ($P < 0.01$; Table 1 and Fig. 2). The findings indicated the presence of ventricular hypertrophy and fibrosis in SHR. LVW, LVW/BW ratio and LVCVF were not affected by L-arginine treatment in WKY, but were significantly decreased in SHR ($P < 0.05$; Table 1 and Fig. 2).

Histopathological examination of the left ventricle revealed enlarged cardiomyocytes in vehicle-treated SHR and showed hyperchromatic nuclei with irregular outlines (Fig. 3A). The latter characterizes ventricular hypertrophy and fibrosis. On the other hand, thickness and fibrosis of myocytes did not occur in L-arginine-treated SHR. Nuclei of myocardial cells were oval and not hyperchromatic (Fig. 3B). In addition, there were no significant histopathological changes of cardiomyocytes in either vehicle-treated and L-arginine-treated WKY (not shown).

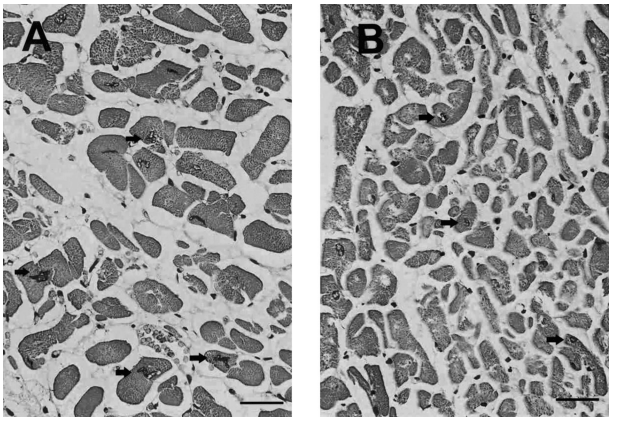

Fig. 3. Histological examination of cardiomyocytes in vehicle-treated (A) and L-arginine-treated (B) SHR. Note the enlargement of cardiomyocytes and hyperchromatic nuclei with an irregular outline (arrows) in vehicle-treated SHR (A), and also the prevention of cardiomyocytes from thickness and fibrosis by L-arginine (B). (HE stain, scale = 100 μm).

Effects of L-Arginine on Cardiac cGMP and NO Contents

In vehicle-treated groups, LV cGMP and nitrite/nitrate contents were less in SHR than in WKY ($P < 0.05$; Fig. 4), indicating impairment of myocardial NO-cGMP production in SHR. However, long-term L-arginine administration increased the levels of LV cGMP and nitrite/nitrate in SHR ($P < 0.05$), but not in WKY.

Effects of L-Arginine on EDV of Isolated Thoracic Aortic Rings

The same vehicle-treated groups reflected a significantly higher degree of concentration-dependent acetylcholine-induced vasodilation of isolated aortic rings in WKY than in SHR ($P < 0.05$; Fig. 5). EDV induced by acetylcholine was 53 ± 2% of the contraction induced by 10^{-5} M norepinephrine in rings from vehicle-treated SHR, while maximum relaxation induced by 10^{-5} M acetylcholine was only slightly increased in L-arginine-treated SHR by an insignificant difference of 3 ± 3%. L-Arginine also did not influence the EDV of isolated aortic rings in WKY at any concentration of acetylcholine.

Discussion

Our study revealed two major discoveries: 1) Following long-term L-arginine administration, BW, HR, AP, and EDV of thoracic aortic rings remained unchanged in SHR. In WKY, BW, HR and EDV were essentially not altered, but SAP was significantly decreased. 2) L-arginine attenuated ventricular hypertrophy and fibrosis and increased cardiac cGMP and nitrite/nitrate contents specifically in SHR, but not in WKY.

Previous studies of long-term oral or intraperitoneal administration with L-arginine in SHR (5, 18, 23) or stroke-prone SHR (SHRSP) (34) show that L-arginine increased NO production, but it did not influence the SAP; however, in these studies they did not compare SHR with WKY. Our results in SHR consistently matched these findings, but on the contrary, we found that L-arginine did not change the SAP during the first to fifth wk of treatment in WKY, while SAP became lower in L-arginine-treated WKY than in vehicle-treated WKY after the sixth wk. In WKY, L-arginine significantly decreased the SAP by 11 mmHg (Fig. 1).

Dickhout and Lee (6) observes the AP and HR development in young SHR and WKY and suggestes that the AP change is a physiological development of AP. The AP of WKY gradually increased throughout time after birth and then remained at a constant level of about 150 mmHg after the sixth wk of treatment (14 wks old, Fig. 1). The physiological change of AP in WKY might minimize the depressor effect of L-arginine before the fifth wk in WKY. When the AP in WKY became constant after the sixth wk, the depressor effect of L-arginine became effective.

In contrast, the hypertensive tendency of AP development in SHR was always larger than the depressor effect of L-arginine (Fig. 1). The discrepancy may explain why L-arginine decreased the SAP in normotensive rats after six wks of treatment, but not in hypertensive rats. Simile to our findings, it is intriguing to note that acute administration of L-arginine reduces the arterial pressure in normotensive and hypertensive humans (12, 27), but, chronic administration did not affect the development of hypertension in SHR (5, 18, 23) and SHRSP (34).

Long-term treatment of SHR with L-arginine did not significantly influence the SAP and EDV of isolated thoracic aortic rings. We found that L-arginine could decrease LVW and LVW/BW ratio and LVCVF in SHR, but did not affect the cardiac weight and collage volume in WKY. Histopathological examination also revealed that L-arginine prevented ventricular fibrosis and hypertrophy in SHR.

This study also showed that in vehicle-treated animals, LV cGMP and nitrite/nitrate contents were lower in SHR than those in WKY, suggesting impaired myocardial NO-cGMP production in SHR. This observation was identical to that of a previous study (19). Interestingly, long-term L-arginine treatment significantly increased LV cGMP and nitrite/nitrate contents in SHR, but not in WKY. The results inferred that the mechanism of reduction in ventricular hypertrophy and fibrosis following long-term L-arginine administration in SHR might be mediated by

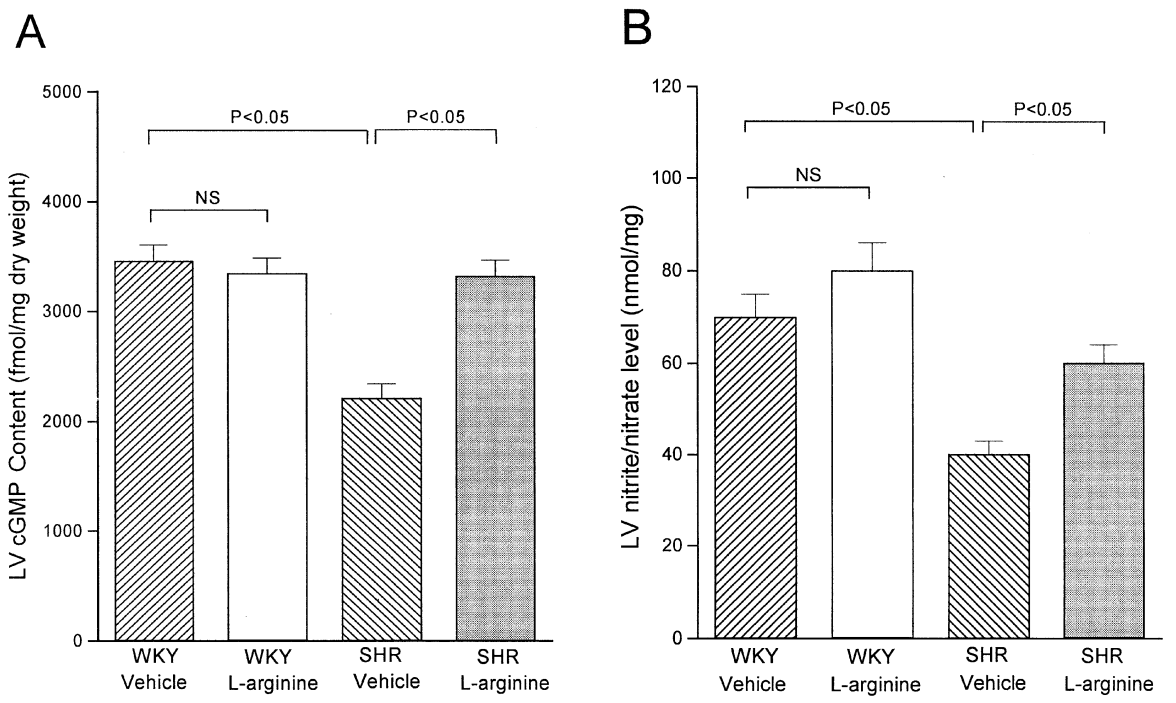

Fig. 4. The left ventricular (LV) contents of cGMP (A) and nitrite/nitrate (B). Note the lower LV cGMP and nitrite/nitrate in SHR, compared to WKY, and the elevation of these two compounds following L-arginine administration in SHR, but not in WKY. Data are means ± SEM (n = 12 in each group).

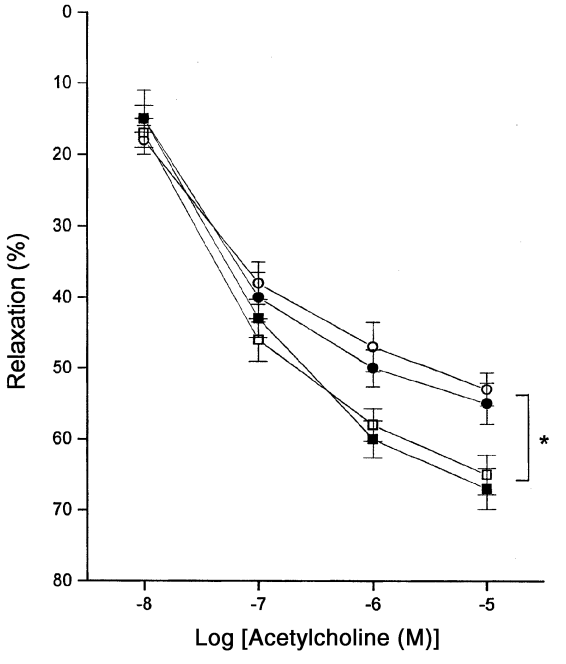

Fig. 5. Relaxations of isolated aortic rings *ex vivo* induced by acetylcholine from SHR and WKY after 12 wks of administration with L-arginine or vehicle (control). Open circles represent vehicle-SHR; closed circles, L-arginine-SHR; open squares, vehicle-WKY; closed squares, L-arginine-WKY. Values are means ± SEM (n = 12 in each group). Note that L-arginine did not alter the acetylcholine-induced relaxation in both WKY and SHR. The relaxation was greater in WKY than that in SHR (*$P < 0.05$).

increased myocardial production of NO-cGMP, signaling its being independent of SAP and EDV of thoracic aorta. Recent studies may lend support to our result. For example, Fagan *et al.* (8) reported that L-arginine administration reduced right heart hypertrophy in hypoxia-induced pulmonary hypertensive animals by increased plasma NO level. Another study conducted by Grieve *et al.* (11) noted a decrease in both coronary endothelial NOS expression and NO bioactivity in an experimental guinea pig model of decompensated pressure-overload left ventricular hypertrophy.

Our results indicated that L-arginine might attenuate not only ventricular hypertrophy but also cardiac fibrosis, albeit specifically to SHR. Matsuoka *et al.* (23) also reports that L-arginine can significantly reduce ventricular hypertrophy in SHR, but they do not examine whether L-arginine could reduce cardiac fibrosis. On the other hand, Susic *et al.* (35) finds that prolonged L-arginine reduces cardiac fibrosis in aged SHR, but they do not further address the possible mechanism. To the best of our knowledge, this was the first report to find that long-term L-arginine administration could alleviate cardiac fibrosis in SHR, and to reasonably explain the possible mechanism.

Although investigations by other researchers report that L-arginine administration fails to reduce blood pressure or attenuate cardiac hypertrophy in SHRSP (34) and SHR (18), the time period of

administration might well account for the disparate results. In this study, L-arginine was given to WKY and SHR for as long as 12 wks, whereas the subjects in other studies received L-arginine for only six wks or less. Another possible explanation lies in the different method and route of L-arginine administration. We dissolved L-arginine in distilled water, while Stier *et al*. (34) uses 1% NaCl solution, which may exacerbate hypertensive ventricular hypertrophy (36). Our daily dose of L-arginine was also higher than that of other studies. Kristek (18) finds that intraperitoneal injection of L-arginine does not influence SAP, HR, cardiac weight, and arterial wall thickness in SHRSP, and that the higher blood pressure in SHRSP than in SHR may minimize the protective effect of L-arginine on ventricular hypertrophy.

In hypertensive animals and humans, ventricular hypertrophy and fibrosis may occur as a consequence of an external load on the heart or other pathogenesis, including autonomic nervous and endocrine systems (26). A previous hemodynamic study from our laboratory has suggested that SAP and total peripheral resistance do not significantly correlate with the degree of ventricular hypertrophy (15). The present study concurred with this view, demonstrating a degree of ventricular hypertrophy not highly dependent upon SAP and EDV of thoracic aorta in SHR. Numaguchi *et al*. (28) also postulates that long-term blockade of NOS causes cardiac hypertrophy and coronary microvascular remodeling in rats, *in vivo*, by a mechanism other than arterial hypertension. However, they do not further address the possible mechanism.

The present study emphasized the importance of NO and its second messenger cGMP in the development of ventricular hypertrophy and fibrosis in SHR. Our study demonstrated that basal cardiac levels of cGMP and NO were lower in SHR than in WKY. Long-term administration of L-arginine in SHR alleviates not only ventricular hypertrophy but also cardiac fibrosis, and increases LV cGMP and NO contents as well. The extent of ventricular hypertrophy and fibrosis could not be significantly correlated with AP and EDV of thoracic aorta in SHR. In contrast, the abnormality of the cardiac L-arginine-NO-cGMP pathway might be involved in the pathogenesis of ventricular hypertrophy and fibrosis in SHR.

Acknowledgments

This work was supported by grants NSC-90-2320-B-320-004 and NSC-91-2320-B-320-015 from the National Science Council. Huai-Ren Chang is a recipient of the MD-PhD Predoctoral Fellowship DD9204C91 from the National Health Research Institutes, Taiwan. With a medical degree from Tzu Chi University, Chang is currently studying for a Ph. D. at the Institute of Physiology, College of Medicine, National Taiwan University, Taipei, Taiwan.

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