ANTIOXIDANTS & REDOX SIGNALING
Volume 10, Number 12, 2008

DOI: 10.1089/ars.2008.2069

Original Research Communication

N-Acetylcysteine Abolishes the Protective Effect of Losartan Against Left Ventricular Remodeling in Cardiomyopathy Hamster

Seiji Matsuhisa, Hajime Otani, Toru Okazaki, Koji Yamashita, Yuzo Akita, Daisuke Sato, Akira Moriguchi, and Toshiji Iwasaka

Abstract

Oxidative stress mediated by activation of angiotensin II type-1 receptor (AT_1R) plays a crucial role in the progression of heart failure. We investigated the effect of *N*-acetylcysteine (NAC) and an AT_1R blocker on oxidative stress and left ventricular (LV) remodeling in BIO14.6 cardiomyopathy hamsters. The cardiomyopathy hamsters were treated with NAC or the AT_1R blocker losartan for 20 weeks. Although NAC and losartan inhibited oxidative stress and upregulation of iNOS in the cardiomyopathy hamster heart, only losartan inhibited LV chamber dilation, myocardial fibrosis, and LV dysfunction in the cardiomyopathy hamster. Co-treatment with NAC abolished the protective effect of losartan against LV remodeling associated with inhibition of phosphatidylinositol 3-kinase (PI3K)/Akt and eNOS activation. An iNOS inhibitor 1400W or a nonselective NOS inhibitor Nω-nitro-L-arginine methyl ester (L-NAME) exacerbated LV remodeling in the cardiomyopathy hamster. However, L-NAME but not 1400W abrogated losartan-mediated inhibition of LV remodeling. These results suggest that redox-sensitive upregulation of iNOS plays a crucial role in preventing LV remodeling in the BIO14.6 cardiomyopathy hamster. Losartan inhibits LV remodeling by switching the cardioprotective mechanism from iNOS- to eNOS-dependence, but NAC abolishes the protective effect of losartan by inhibiting redox-sensitive activation of PI3K/Akt and eNOS in the cardiomyopathy hamster. *Antioxid. Redox Signal*. 11, 1999–2008.

Introduction

Oxidative stress mediated by activation of angiotensin II type-1 receptor (AT_1R) plays a crucial role in the progression of heart failure (3). However, the efficacy of antioxidant therapy in heart failure remains controversial (7, 33). The limited efficacy of antioxidant therapy for heart failure may at least in part be due to the fact that oxidative stress is a double-edged sword that mediates myocardial injury but simultaneously confers cardioprotection through the activation of redox-sensitive survival pathways (17, 18).

Inducible nitric oxide synthase (iNOS) is upregulated in the heart predisposed to oxidative stress under the various pathological conditions by activation of the redox-sensitive transcriptional factors such as NF-κB (31). We have previously demonstrated that cardiomyopathy heart is protected against ischemia/reperfusion injury by exposure to oxidative/nitrosative stress via upregulation of iNOS and the use of a genuine antioxidant, 2-mercaptopropionylglycine, inhibited upregulation of iNOS, and abrogated tolerance to ischemia/reperfusion injury in these hearts (13). It is, therefore, anticipated that indiscriminate elimination of reactive oxygen species (ROS) compromises the cardioprotective signal transduction, offsets a beneficial antioxidative effect, and exaggerates left ventricular (LV) remodeling and heart failure.

In contrast to genuine antioxidants that eliminate both injurious and protective ROS, AT_1R blockers can inhibit only the source of ROS, particularly NADPH oxidase, in the heart (24). Thus, these agents may represent causal antioxidants that eliminate only injurious oxidative stress without interrupting cardioprotective redox-signal transduction mediated by other ROS sources such as mitochondrial K_{ATP} channels (22). In addition to its antioxidant effect, it has been demonstrated that AT_1R blockers can promote activation of endothelial nitric oxide synthase (eNOS) which is also redox-sensitive (1) and plays a cardioprotective role in hypertension and heart failure (14, 28). Therefore, we tested the hy-

The Second Department of Internal Medicine, Division of Cardiology, Kansai Medical University, Moriguchi City, Japan.

pothesis that a genuine antioxidant and an AT_1R blocker provoke a different effect on the progression of LV remodeling and dysfunction in the cardiomyopathic heart despite similar inhibition of oxidative stress and that a genuine antioxidant abrogates the cardioprotective effect of an AT_1R blocker by inhibiting redox-sensitive activation of eNOS.

Materials and Methods

Animals

Male BIO14.6 hamsters which are devoid of ä-sarcoglycan gene (16) and the control BIOFIB hamsters at 5 weeks of age were obtained from BIO Breeders (Fitchburg, MA). All experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996) and approved by the institutional Committee of Animal Care and Use in Kansai Medical University (Moriguchi, Japan).

Experimental protocol

To evaluate the effect of chronic treatment with an antioxidant and an AT_1R blocker, *N*-acetylcysteine (NAC, 1 g/kg/day, p.o.) or losartan (30 mg/kg/day, p.o.) or both was administered for 20 weeks starting from 6 weeks of age. The dose of NAC is relatively higher than reported in the work in which NAC given at 0.5 g/kg/day did not completely prevent hyperglycemia-induced oxidative stress (9), but was lower than that (1.5 g/kg/day) used to prevent L-NAME-induced hypertension in rats (25). The dose of losartan was chosen on the basis of our previous study demonstrating that losartan had no significant cardioprotective effect against ischemia/reperfusion injury in the control rat but inhibited oxidative stress and conferred cardioprotection in the hypertensive rat (15). The role of nitric oxide synthase (NOS) in the progression of LV remodeling and its modulation by losartan were studied using 1400W, which is highly selective for iNOS possessing an IC50 value of 2.0 μM and is 50-fold more potent to iNOS compared to eNOS (10), at a dose 10 mg/kg/day, p.o. or a nonselective NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME; 100 mg/kg/day, p.o). The doses of 1400W and L-NAME were chosen to achieve expected maximum serum concentrations above the IC50 value. These agents were administered alone or co-administered with losartan for 20 weeks, starting from 6 weeks of age. Each treatment group had 10 animals; five animals were assigned for biochemical experiments, and five animals underwent echocardiography followed by histological analysis.

Measurements of myocardial GSH and GSSG

We determined myocardial GSH/GSSG, because the change in GSH/GSSG in the tissue is known to reflect magnitude of oxidative stress and a global change in all of the intracellular antioxidant redox systems (12). The hamsters were euthanized and the hearts were removed as described above. The hearts were snap-frozen in liquid nitrogen and stored at -80°C until use. Myocardial glutathione (GSH) and glutathione disulfide (GSSG) were measured using a BIOXYTECH GSH/GSSG-412™ colorimetric assay kit from Oxis Research (Portland, OR). At an indicated time, mice were anesthetized by overdose sodium pentobarbital, and the heart was rapidly excised and snap-frozen in liquid nitrogen. Frozen myocardial tissue samples were homogenized (g/10 ml) in 5% metaphosphoric acid. The procedure was followed as per manufacturer's instructions and the levels were quantified as micromolar GSH or GSSG based on standard supplied along with the kit.

Determination of myocardial MDA+HNE

We also measured myocardial MDA+HNE as an index of lipid peroxidation (21). Frozen heart samples were ground in a small volume of ice-cold 20 m*M* Tris-HCl buffer pH 7.4, in a 1:10 wt/vol ratio, and homogenized using a Teflon pestle. After centrifugation at 3,000 *g* for 10 min at 4°C, the supernatant was used for the determination of malondialdehyde (MDA) plus 4-hydroxy-noneal (HNE), using an assay kit (BIOXYTECH LPO-586, Oxis Research, Foster, CA). The protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).

Immunoblot analysis

Frozen heart samples were ground with lysis buffer containing 30 m*M* Tris, pH 7.4, 150 m*M* NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 m*M* EDTA, 0.1 m*M* phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail Complete (Roche Diagnostics, Mannheim, Germany). The protein concentration was determined as described before. Equal amount of protein (50 μg) from tissue homogenates was separated on a 7.5% SDS-PAGE and the separated proteins were transferred to a polyvinylidene-difluoride membrane with a transfer buffer containing 25 m*M* Tris, 192 m*M* glycine, and 10% methanol. The membranes were blocked with 5% skimmed milk and incubated with rabbit polyclonal primary antibodies specific for iNOS (Santa Cruz Biotechnology, Santa Cruz, CA), eNOS (Cell Signaling Technology, Beverly, MA), phospho-eNOS (Ser-1177) (Cell Signaling Technology), Akt or phosphorylated Akt (Ser-473) (Cell Signaling Technology) at a 1:1,000 dilution. These membranes were subsequently incubated with peroxidase-conjugated anti-rabbit secondary antibodies at a 1:1,000 dilution and developed using an enhanced chemiluminescence detection system (Amersham Biosciences, Tokyo, Japan) according to the manufacturer's instructions. The immunolabeling was quantified with a densitometric analysis using an image analyzing software Win Roof (Mitani Co., Fukui, Japan). Consistency in the data analysis was ensured by normalization of each immunoblot signal to the corresponding Coomassie Blue stain signal as described previously (23). Phospho-Akt/total Akt and phospho-eNOS/total eNOS were calculated as an indicator for activation of phosphatidylinositol 3-kinase (PI3K) (29) and eNOS (6), respectively.

Echocardiography

The hamster was anesthetized with a mixture of ketamine, xylazine, and acepromazine as described previously (26). Echocardiography was performed using a SONOS-7500 (Philips Medical Systems, Andover, MA) equipped with a 6-15 MHz transducer (Model 21390A, Philips). M-mode measurements of LV internal diameter were made from more than three beats and averaged. Measurements of the LV end-diastolic diameter (LVEDD) were taken at the time of the ap-

parent maximal LV diastolic dimension, while measurements of the LV end-systolic diameter (LVESD) were taken at the time of the most anterior systolic excursion of the posterior wall. LV ejection fraction (LVEF) was calculated according to the cubic method as described previously (26). The echocardiographer was blinded to the groups.

Morphological and histological analysis

At the end of echocardiography, animals were anesthetized with overdose sodium pentobarbital (100 mg/kg), and the hearts were removed. The hearts were dissected into the free wall of the right ventricle and the left ventricle including the septum and both atria and weighed. Then, the hearts were fixed with a 10% solution of formalin in phosphate-buffered saline at 4°C for 24 h, cut perpendicularly to the apex, embedded in paraffin, and sectioned at 6 μm thickness. The section was stained with hematoxylin-eosin, and gross morphology of the heart was viewed under a low power field (×0.5). LV wall thickness and LV diameter were measured at the mid ventricular level. The area of fibrosis was identified by Masson trichrome staining, viewed under a low power field (X2), and quantified using Win Roof.

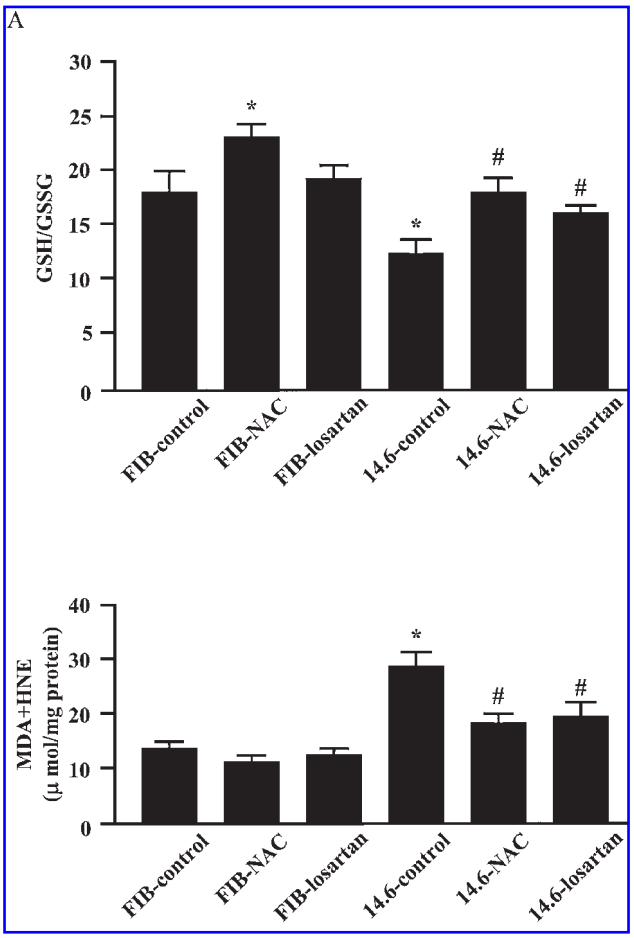

FIG. 1. Actions of *N*-acetylcysteine and losartan. (**A**) The effect of N-acetylcysteine (NAC) and losartan on myocardial glutathione (GSH) and glutathione disulfide (GSSG). (**B**) The effect of NAC and losartan on myocardial malondialdehyde (MDA) plus 4-hydroxy-noneal (HNE) content. Each bar represents mean ± SE of five experiments. *$p < 0.05$ compared to the nontreated BIOFIB hamster (FIB-control); #$p < 0.05$ compared to the nontreated BIO14.6 hamster (14.6-control).

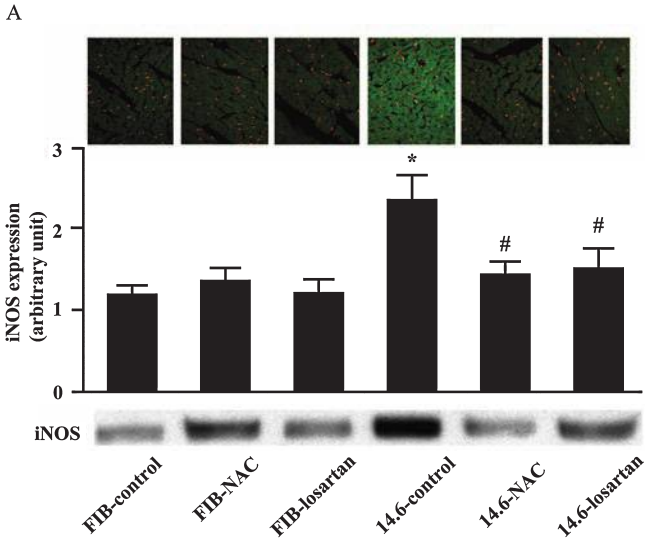

FIG. 2. The effect of *N*-acetylcysteine (NAC) and losartan on iNOS expression in the heart. *Upper panels* are the representative immunohistochemistry images for iNOS. *Lower panels* are the representative immunoblot images for iNOS. *Bars* represent the data of quantitative analysis for iNOS. Each bar represents mean ± SE of five experiments. *$p < 0.05$ compared to the nontreated BIOFIB hamster (FIB-control), #$p < 0.05$ compared to the nontreated BIO14.6 hamster (14.6-control). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

Statistical analysis

All numerical data are expressed as mean ± SE. Statistical analysis was performed by Student *t*-test to analyze the difference between two groups and one-way ANOVA followed by the Bonferroni post hoc test to compare the difference within the groups. The differences were considered significant at a p value of <0.05.

Results

Effect of NAC and losartan on oxidative stress

GSH/GSSG was significantly increased by treatment with NAC but not with losartan in the BIOFIB hamster heart (Fig. 1A). GSH/GSSG was significantly lower in the BIO14.6 hamster heart compared to the BIOFIB hamster heart. Treatment with NAC or losartan significantly inhibited the decrease of GSH/GSSG in the cardiomyopathy heart. In addition, the increase in MDA+HNE was also inhibited by treatment with NAC or losartan in the cardiomyopathy heart (Fig. 1B).

Effect of NAC and losartan on expression of iNOS and activation of eNOS

iNOS expression was increased in the BIO14.6 hamster heart compared to the BIOFIB hamster heart at 26 weeks of age (Fig. 2). Treatment with NAC or losartan had no effect on iNOS expression in the BIOFIB hamster heart, but inhibited it in the cardiomyopathy hamster heart.

Effect of NAC and losartan on activation of phosphatidylinositol 3-kinase and eNOS

Treatment with NAC had no significant effect on the expression of total Akt and phospho-Akt and phospho-Akt/total Akt in the BIOFIB and the BIO14.6 cardiomyopathy hamster heart (Fig. 3A). However, losartan increased the expression of phospho-Akt and phospho-Akt/total Akt in the BIOFIB and the cardiomyopathy hearts, although the magnitude of an increase in phospho-Akt/total Akt was greater in the cardiomyopathy heart. NAC inhibited the increase in phospho-Akt/total Akt induced by losartan in the BIOFIB and the BIO14.6 hamster hearts.

Treatment with NAC or losartan had no significant effect on the expression of total eNOS and phospho-eNOS and phospho-eNOS/total eNOS in the BIOFIB heart (Fig. 3B). Although there was no difference in total eNOS expression between the NAC-treated and the losartan-treated cardiomyopathy heart, losartan but not NAC increased the expression of phospho-eNOS and phospho-eNOS/total eNOS in the cardiomyopathy heart. However, NAC inhibited the increase in phospho-eNOS/total eNOS induced by losartan in the BIO14.6 hamster heart.

Effects of NAC and losartan on gross morphology of the heart, heart weight/body weight, and myocardial fibrosis

Gross morphology of the BIO14.6 hamster heart at 26 weeks of age showed dilation of the LV chamber and thinning of the LV wall compared with BIOF1B hamster heart at the same age associated with an increase in heart weight/body weight (Fig. 4A–C). Treatment of the BIOFIB hamster with NAC or losartan had no significant effect on gross morphology of the heart and heart weight/body weight. Treatment with NAC had no significant effect on dilation of the LV chamber, thinning of the LV wall, and heart weight/body weight in the cardiomyopathy hamster heart. In contrast, treatment with losartan ameliorated dilation of the LV chamber and thinning of the LV wall and decreased heart weight/body weight in the cardiomyopathy hamster heart. Co-treatment with NAC and losartan abrogated losartan-induced amelioration of LV chamber dilation and LV

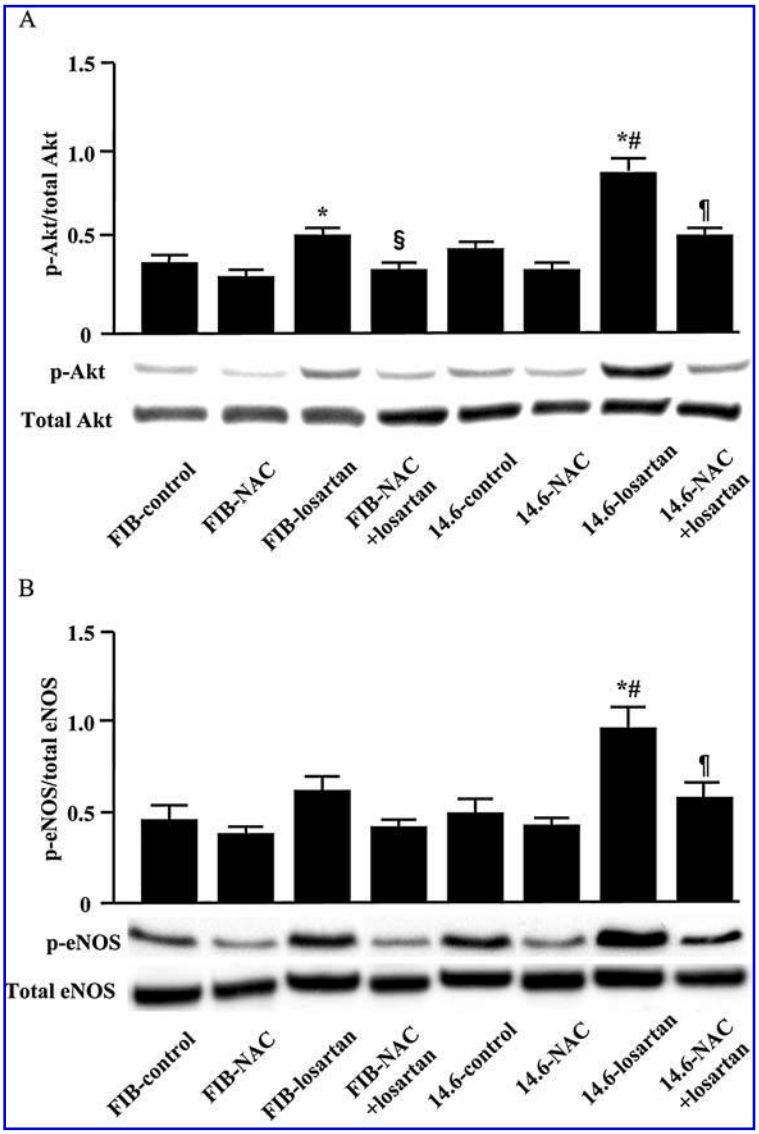

FIG. 3. (A) The effect of *N*-acetylcysteine (NAC) and losartan on phosphorylation of Akt. The *lower panel* shows representative immunoblot images for phospho-Akt (p-Akt) and total Akt. *Upper bars* represent the data of quantitative analysis for p-Akt/total Akt. (**B**) The effect of NAC and losartan on phosphorylation of eNOS. The *lower panel* shows representative immunoblot images for phospho-eNOS (p-eNOS) and total eNOS. *Upper bars* represent the data of quantitative analysis for p-eNOS/total eNOS. Each bar represents mean ± SE of five experiments. $^{*}p < 0.05$ compared to the nontreated BIOFIB hamster (FIB-control); $^{\#}p < 0.05$ compared to the nontreated BIO14.6 hamster (14.6-control); $^{\S}p < 0.05$ compared to the losartan-treated BIOFIB hamster; $^{\P}p < 0.05$ compared to the losartan-treated BIO14.6 hamster.

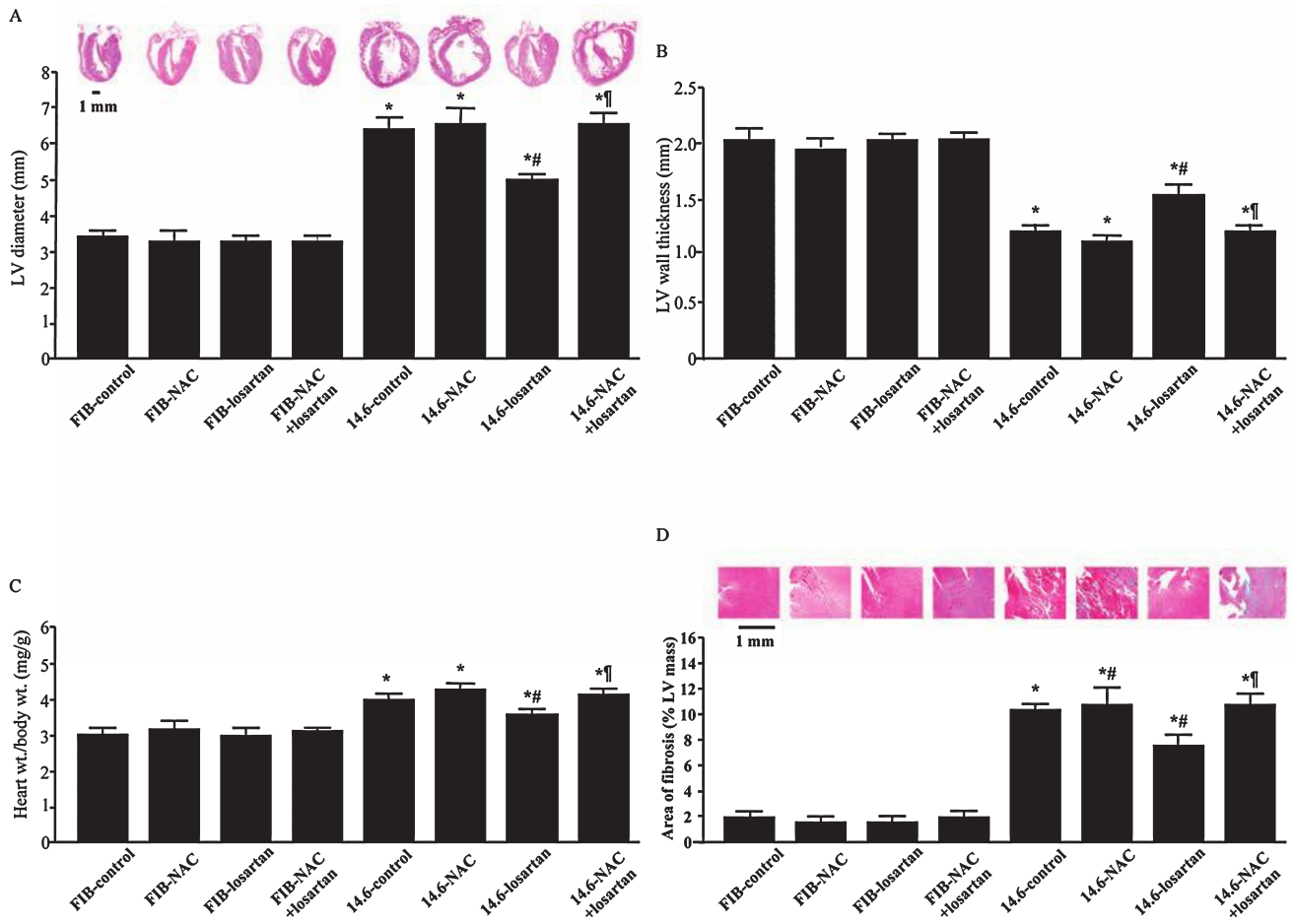

FIG. 4. The effect of *N*-acetylcysteine (NAC) and losartan on gross morphology of the heart (A–C). *Panels* are the representative hematoxylin-eosin staining images of the heart. *Bars* represent the data of quantitative analysis for (**A**) LV diameter, (**B**) LV thickness, and (**C**) heart weight/body weight. The effect of N-acetylcysteine (NAC) and losartan on myocardial fibrosis (**D**). *Panels* are the representative Masson trichrome staining images of the heart. *Bars* represent the data of quantitative analysis for myocardial fibrosis. Each bar graph represents mean ± SE of five experiments.*$p < 0.05$ compared to the nontreated BIOFIB hamster (FIB-control), #$p < 0.05$ compared to the nontreated BIO14.6 hamster (14.6-control), ¶$p < 0.05$ compared to the losartan-treated BIO14.6 hamster. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

wall thinning and decrease of heart weight/body weight in the BIO14.6 hamster heart.

The area of fibrosis in the LV myocardium was increased in the BIO14.6 hamster heart compared to the BIOFIB hamster at 26 weeks of age (Fig. 4D). Treatment with NAC or losartan had no effect on myocardial fibrosis in the BIOFIB hamster. Although treatment with NAC had no significant effect on the area of fibrosis in the LV myocardium in the BIO14.6 hamster heart, treatment with losartan significantly decreased the area of fibrosis in this heart. However, co-treatment with NAC and losartan significantly increased myocardial fibrosis compared to the losartan treatment alone in the cardiomyopathy hamster heart.

Effect of NAC and losartan on LV function

Echocardiography showed an increase in LVEDD and LVESD and a decrease of LVEF in the BIO14.6 hamster heart compared to the BIOFIB hamster heart at 26 weeks of age (Table 1). Treatment of the BIOFIB hamster with NAC or losartan had no significant effect on LV dimension and

TABLE 1. EFFECT OF *N*-ACETYLCYSTEINE AND LOSARTAN ON LEFT VENTRICULAR DIMENSION AND EJECTION FRACTION

	LVEDD	*LVESD*	*LVEF*
BIOFIB			
Control ($n = 5$)	5.1 ± 0.2	2.7 ± 0.2	79 ± 2
NAC ($n = 5$)	5.4 ± 0.2	2.8 ± 0.1	79 ± 1
Losartan ($n = 5$)	5.1 ± 0.1	2.5 ± 0.1	82 ± 1
NAC + losartan ($n = 5$)	5.2 ± 0.2	2.8 ± 0.1	76 ± 2
BIO14.6			
Control ($n = 5$)	7.8 ± 0.4*	6.2 ± 0.4*	41 ± 4*
NAC ($n = 5$)	8.0 ± 0.3*	6.5 ± 0.4*	38 ± 3*
Losartan ($n = 5$)	6.7 ± 0.3*†	4.8 ± 0.3*†	54 ± 3*†
NAC + losartan ($n = 5$)	8.1 ± 0.3*‡	6.5 ± 0.3*‡	38 ± 3*‡

BIOFIB, BIOFIB control hamster; BIO14.6, BIO14.6 cardiomyopathy hamster; LVEDD, left ventricular end-diastolic dimension (mm); LVESD, left ventricular end-systolic dimension (mm); LVEF, left ventricular ejection fraction (%); NAC, *N*-acetylcysteine.

Data are expressed as mean ± SEM; *$p < 0.05$ compared to BIOFIB control; †$p < 0.05$ compared to BIO14.6 control; ‡$p < 0.05$ compared to BIO14.6 losartan.

LVEF. Although treatment with NAC had no significant effect on LV dimension and LVEF, treatment with losartan significantly decreased LVEDD and LVESD and increased LVEF in the cardiomyopathy hamster heart. However, co-treatment with NAC and losartan significantly decreased LVEF compared to the losartan treatment alone in the cardiomyopathy hamster heart.

Effects of 1400W and L-NAME on losartan-induced reduction of LV chamber dilation, heart weight/body weight, and myocardial fibrosis

Treatment with 1400W had no significant effect on LV diameter, LV wall thickness, and heart weight/body weight in the BIOFIB hamster, while the same treatment aggravated LV chamber dilation and LV wall thinning and increased heart weight/body weight in the BIO14.6 cardiomyopathy hamster (Figs. 5A–5C). Treatment with L-NAME tended to increase LV wall thickness and heart weight/body weight in the BIOFIB hamster heart, but significantly decreased LV wall thickness and increased LV diameter and heart weight/body weight in the cardiomyopathy hamster. Treatment with 1400W or L-NAME aggravated LV chamber dilation and LV wall thinning and increased heart weight/body weight in the BIO14.6 hamster. However, co-treatment with 1400W did not prevent losartan-induced amelioration of LV chamber dilation and LV wall thinning and decrease of heart weight/body weight in the cardiomyopathy hamster. In contrast, co-treatment with L-NAME reversed losartan-induced amelioration of LV chamber dilation and LV wall thinning and decrease of heart weight/body weight in the cardiomyopathy hamster.

Treatment with 1400W had no significant effect on myocardial fibrosis in the BIOFIB hamster heart, while the same treatment significantly increased myocardial fibrosis in the BIO14.6 cardiomyopathy hamster heart (Fig. 5D). Treatment

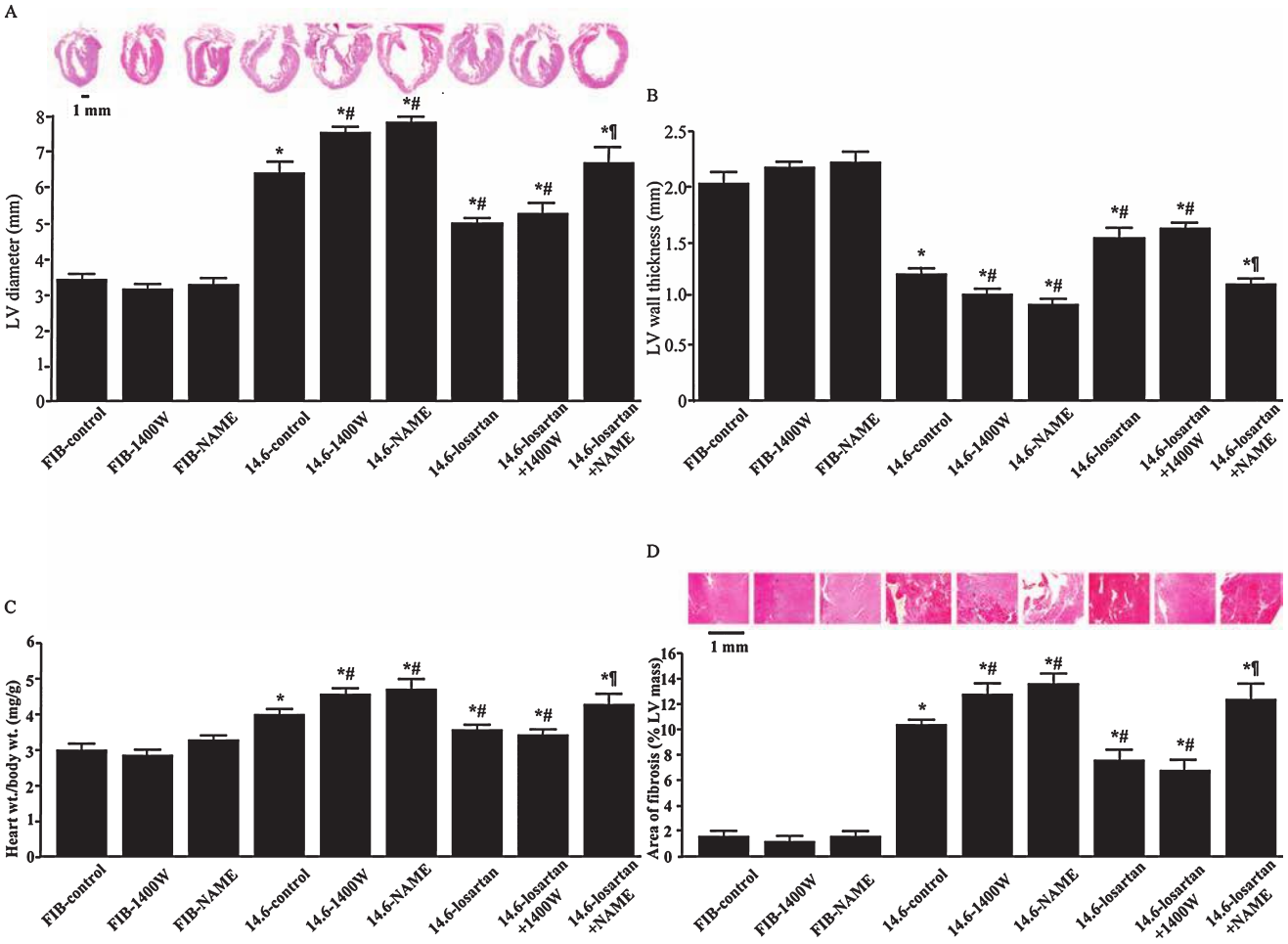

FIG. 5. The effect of 1400W and Nω-nitro-L-arginine methyl ester (NAME) on losartan-mediated modulation of gross morphology of the heart (A–C). *Panels* are the representative hematoxylin-eosin staining images of the heart. *Bars* represent the data of quantitative analysis for (**A**) LV diameter, (**B**) LV thickness, and (**C**) heart weight/body weight. The effect of 1400W and Nω-nitro-L-arginine methyl ester (NAME) on losartan-mediated modulation of myocardial fibrosis (**D**). *Panels* are the representative Masson trichrome staining images of the heart. *Bars* represent the data of quantitative analysis for myocardial fibrosis. Each bar represents mean ± SE of 5 experiments.*$p < 0.05$ compared to the nontreated BIOFIB hamster (FIB-control), #$p < 0.05$ compared to the nontreated BIO14.6 hamster (14.6-control), ¶$p < 0.05$ compared to the losartan-treated BIO14.6 hamster. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

with L-NAME tended to increase myocardial fibrosis in the BIOFIB hamster heart and significantly increased myocardial fibrosis in the cardiomyopathy hamster heart. Co-treatment with 1400W did not prevent losartan-induced inhibition of myocardial fibrosis in the cardiomyopathy hamster heart. In contrast, co-treatment with L-NAME and losartan significantly increased myocardial fibrosis compared to the losartan treatment alone in the cardiomyopathy hamster heart.

Effect of 1400W and L-NAME on losartan-induced improvement of LV function

Treatment with 1400W had no significant effect on LV dimension and LVEF in the BIOFIB hamster, while 1400W significantly increased LVESD and decreased LVEF in the BIO14.6 cardiomyopathy hamster heart (Table 2). Treatment with L-NAME tended to increase LVEDD and LVESD and decrease LVEF in the BIOFIB hamster and significantly increased LVEDD and LVESD and decreased LVEF in the BIO14.6 hamster. Co-treatment with 1400W did not prevent losartan-induced reduction of LVEDD and LVESD and improvement of LVEF in the cardiomyopathy hamster. In contrast, co-treatment with L-NAME and losartan significantly increased LVEDD and LVESD and decreased LVEF compared to the losartan treatment alone in the cardiomyopathy hamster.

Discussion

The salient findings of the present study were: (a) oxidative stress was increased in the BIO14.6 cardiomyopathy hamster heart associated with enhanced expression of iNOS; (b) chronic treatment with NAC and losartan equally prevented oxidative stress and expression of iNOS but only losartan conferred protection against LV remodeling in the cardiomyopathy hamster heart; (c) activation of eNOS was induced by treatment with losartan but not with NAC, and NAC inhibited losartan-induced activation of eNOS and protection against LV remodeling in the cardiomyopathy hamster heart; (d) 1400W and L-NAME aggravated LV remodeling in the cardiomyopathy hamster heart; (e) L-NAME but not 1400W abrogated losartan-induced inhibition of LV remodeling in the cardiomyopathy hamster heart. These results are consistent with the hypothesis that oxidative stress promotes upregulation of iNOS that plays a crucial role in inhibiting LV remodeling in the BIO14.6 cardiomyopathy hamster heart. Therefore, it is conceivable that no protective effect of NAC against LV remodeling is a result of inhibition of iNOS upregulation despite a possible beneficial effect on LV remodeling via inhibition of oxidative stress. On the contrary, it is suggested that similar inhibition of oxidative stress and upregulation of iNOS by losartan confers protection against LV remodeling by switching the cardioprotective mechanism from iNOS- to eNOS-dependent. Moreover, it is noteworthy that the antioxidant may abrogate the AT_1R blocker-mediated cardioprotection presumably through inhibition of eNOS activation.

AT_1R-mediated oxidative stress is known to play a detrimental role in the progression of heart failure. Whaley–Connell and associates (32) demonstrated that the AT_1R blocker valsartan was capable of inhibiting oxidative stress in the heart associated with amelioration of cardiac remodeling and LV dysfunction in a rodent model of chronically elevated tissue levels of angiotensin II. It has also been demonstrated that angiotensin II-mediated oxidative stress and inflammation mediate the age-dependent cardiomyopathy in angiotensin converting enzyme 2 null mice (19). Accordingly, prevention of AT_1R-mediated oxidative stress protects a variety of cellular constituents from degeneration and inactivation and promotes the cell survival under a variety of pathological environments. On the other hand, Das and associates (4) demonstrated that pre-ischemic treatment with angiotensin II exerts cardioprotection against ischemia/reperfusion injury in the isolated and perfused rat heart and such a cardioprotective effect was reversed by co-treatment with a NADPH oxidase inhibitor apocynin or NAC, indi-

TABLE 2. EFFECT OF *1400W AND L-NAME ON* LOSARTAN-MEDIATED MODULATION OF LEFT VENTRICULAR DIMENSION AND EJECTION FRACTION

	LVEDD	*LVESD*	*LVEF*
BIOFIB			
Control ($n = 5$)	5.1 ± 0.2	2.7 ± 0.2	79 ± 2
1400W ($n = 5$)	5.2 ± 0.1	2.5 ± 0.1	81 ± 3
NAME ($n = 5$)	5.5 ± 0.1	3.2 ± 0.1	73 ± 2
NAC + losartan ($n = 5$)			
BIO14.6			
Control ($n = 5$)	7.8 ± 0.4*	6.2 ± 0.4*	41 ± 4*
1400W ($n = 5$)	8.8 ± 0.4*	7.6 ± 0.4*†	27 ± 2*†
NAME ($n = 5$)	9.2 ± 0.4*†	8.2 ± 0.3*†	23 ± 3*†
Losartan ($n = 5$)	6.7 ± 0.4*†	4.8 ± 0.3*†	54 ± 3*†
Losartan + 1400W ($n = 5$)	6.6 ± 0.4*†	4.7 ± 0.4*†	54 ± 4*†
Losartan + NAME ($n = 5$)	8.8 ± 0.4*‡	7.8 ± 0.5*†‡	24 ± 3*†‡

BIOFIB, BIOFIB control hamster; BIO14.6, BIO14.6 cardiomyopathy hamster; LVEDD, left ventricular end-diastolic dimension (mm); LVESD, left ventricular end-systolic dimension (mm); LVEF, left ventricular ejection fraction (%); NAC, *N*-acetylcysteine.

Data are expressed as mean ± SEM; *$p < 0.05$ compared to BIOFIB control; †$p < 0.05$ compared to BIO14.6 control; ‡$p < 0.05$ compared to BIO14.6 losartan.

cating that NADPH oxidase-derived ROS mediates angiotensin II-induced cardioprotection against ischemia/reperfusion injury. The finding that AT_1R-mediated oxidative stress confers cardioprotection is consistent with the hypothesis that ROS and oxidants can function as intracellular signaling molecules that convert a death signal into a survival signal (5). Such redox signaling acutely promotes activation of survival kinases known as reperfusion injury salvage kinase [*i.e.*, PI3K/Akt and p42/p44 extracellular signal-regulated kinase cascades (11)]. The same oxidative stress can chronically confer cardioprotection through activation of redox-sensitive transcriptional factors such as nuclear factor-kappa B, thereby promoting upregulation of iNOS which has consistently been implicated in the mechanism of late cardioprotection by ischemic preconditioning (2). Indeed, our previous study demonstrated that the BIO14.6 cardiomyopathy hamster hearts were markedly tolerant to ischemia/reperfusion injury through the redox-sensitive activation of iNOS (13). The present study demonstrating that treatment with 1400W or L-NAME aggravated LV remodeling in the cardiomyopathy hamster also points to the conclusion that iNOS is a mediator of protection against LV remodeling in the cardiomyopathy hamster. In addition, the fact that losartan inhibited oxidative stress and upregulation of iNOS but improved LV remodeling in the cardiomyopathy hamster heart suggests that generally observed amelioration of LV remodeling and heart failure by AT_1R blockers can not simply be explained by inhibition of oxidative stress but is also attributed to additional mechanisms.

The results of the present study suggest that the salutary effect of losartan on LV remodeling in the BIO14.6 cardiomyopathy hamster heart is mediated by activation of eNOS. AT_1R blockers can exert cardioprotection through multiple mechanisms. It decreases blood pressure, thereby mitigating ventricular wall stress. Contribution of lowering blood pressure by treatment with losartan to the prevention of heart failure is unlikely, because it has been reported that treatment with losartan or NAC equally decreased blood pressure in the cardiomyopathy hamster (8). On the other hand, bradykinin-dependent and –independent mechanisms have been implicated in AT_1R blocker-mediated cardioprotection against ischemia/reperfusion injury (27). Activation of eNOS has been suggested as a downstream event of these mechanisms. Our study demonstrating that activation of eNOS and prevention of LV remodeling by treatment with losartan was blocked by co-treatment with L-NAME but not with 1400W suggests that eNOS is a mediator of cardioprotection by losartan in the cardiomyopathy hamster heart. However, because we did not study the role of nNOS which is known to exist in the heart and is inhibited by L-NAME, the contribution of nNOS to losartan-mediated amelioration of heart failure can not be ignored.

The finding that NAC reverses the redox-sensitive activation of eNOS and the cardioprotective effect of losartan underscores the need for site-specific antioxidant therapy. The redox-sensitive nature of eNOS activation has been demonstrated by Sun and associates (30) who showed that exposure of bovine aortic endothelial cells to 2,4,6-trinitrotoluene was capable of phosphorylating and activating eNOS through the generation of ROS. A major source of ROS in endothelial cells is the NADPH oxidase enzyme complex that is activated in specific membrane rafts specifically in caveolae in response to a variety of humoral factors (34). NADPH-derived ROS are thought to be generated in the proximity of the PI3K/Akt signaling complex, which is responsible for activation of eNOS (20, 30). The PI3K/Akt axis is activated not only by various growth factors and cytokines but also by G-protein-coupled receptor agonists such as bradykinin via transactivation of receptor tyrosine kinase or activation of non-receptor tyrosine kinase such as Src in a ROS-dependent manner (17). Indeed, the present study demonstrated that losartan activated PI3K in a NAC-sensitive manner, suggesting that losartan promotes redox-sensitive activation of the PI3K/Akt axis. Although the source of ROS generated by treatment with losartan remains to be investigated, maintaining ROS generation in a specific cellular compartment would be essential to preserve the ability of losartan to activate PI3K and eNOS. Therefore, indiscriminate elimination of ROS by treatment with NAC may abolish this redox-sensitive signaling for losartan-induced activation of PI3K and eNOS. In line with this notion, antioxidant medicine must be site-specific, being targeted to a specific ROS or cellular compartment, without a deleterious effect on other redox-sensitive signaling pathways necessary for cardioprotection.

In conclusions, redox-sensitive upregulation of iNOS plays a crucial role in preventing LV remodeling and heart failure in the BIO14.6 cardiomyopathy hamster heart. Losartan inhibits LV remodeling by switching the cardioprotective mechanism from iNOS- to eNOS-dependent but NAC abolishes the protective effect of losartan by inhibiting redox-sensitive activation of PI3K and eNOS in the cardiomyopathy hamster.

Acknowledgments

This work was supported in part by Research Grant 16591420 from the Ministry of Education, Science, and Culture of Japan and Promotion and Mutual Aid Corporation for Private Schools of Japan.

Abbreviations

AT_1R, angiotensin II type-1 receptor; eNOS, endothelial nitric oxide synthase; GSH, glutathione; GSSG, glutathione disulfide; HNE, 4-hydroxy-noneal; iNOS, inducible nitric oxide synthase; L-NAME, Nω-nitro-L-arginine methyl ester; LV, left ventricular; LVEDD, LV end-diastolic diameter; LVEF, LV ejection fraction; LVESD, LV end-systolic diameter; MDA, malondialdehyde; NAC, *N*-acetylcysteine; NOS, nitric oxide synthase; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species.

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Address reprint requests to:
Hajime Otani, M.D.
The Second Department of Internal Medicine
Division of Cardiology, Kansai Medical University
10-15 Fumizono-cho
Moriguchi City
570-8507, Japan

E-mail: otanih@takii.kmu.ac.jp

Date of first submission to ARS Central, March 6, 2008; date of final revised submission, April 15, 2008; date of acceptance, May 13, 2008.

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