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# Superoxide-Dependent Cathepsin Activation Is Associated with Hypertensive Myocardial Remodeling and Represents a Target for Angiotensin II Type 1 Receptor Blocker Treatment

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**The elastolytic activity of cathepsins in the myocardium is implicated in hypertensive heart failure (HF). Given that reactive oxygen species are also implicated in protease activation associated with cardiac remodeling, we examined the role of the reactive oxygen species-induced cathepsin activation system in cardiac remodeling during the development of hypertensive HF. Dahl salt-sensitive hypertensive rats maintained on a high-salt diet were treated with vehicle, the cathepsin inhibitor E64d, or the angiotensin receptor blocker olmesartan from 12 to 19 weeks of age. Cathepsin expression and activity were increased in the left ventricle of HF rats; olmesartan inhibited these effects, restored the balance between elastin and collagen in the left ventricle, and suppressed degradation of the elastic lamina of coronary arteries of HF rats. Furthermore, olmesartan inhibited upregulation of NADPH oxidase subunits and activity as well as superoxide generation. These effects of olmesartan were mimicked by E64d and were accompanied by amelioration of cardiac fibrosis. Finally, olmesartan and apocynin reduced angiotensin II-induced increases in cathepsin mRNA and protein levels in cultured rat neonatal cardiac myocytes. These data suggest that cathepsins likely trigger and promote cardiac remodeling and that blocking the angiotensin II type 1 receptor attenuates cathepsin expression and activity by inhibiting the production of superoxide by NADPH oxidase, thereby attenuating cardiac remodeling and dysfunction.** ***(Am J Pathol 2008, 173:358–369; DOI: 10.2353/ajpath.2008.071126)***

The cardiac NADPH oxidase system has been implicated in the development of atherosclerotic lesions and heart failure (HF).[1,2] NADPH oxidase is thought to be a major source of reactive oxygen species (ROS) generated in the cardiovascular system in response to angiotensin II (Ang II).[3–5] Superoxide generation by NADPH oxidase has been shown to result in the activation of matrix metalloproteinases (MMPs) *in vitro* and *in vivo* as well as to contribute to the pathogenesis of atherosclerosis and cardiac fibrosis.[6–9] However, it is unknown whether or not superoxide produced by NADPH oxidase also participates in the activation of other proteases, such as cathepsins.

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Cathepsins are lysosomal cysteine proteases that belong to the family of papain-like peptidases and play important roles in the degradation of the extracellular matrix (ECM).[10,11] An increase in cathepsin gene expression or in the elastolytic activity of these proteases has been found in animals and humans with hypertensive HF or idiopathic dilated cardiomyopathy.[12–14] Cathepsins exhibit pronounced elastolytic and collagenolytic activities *in vitro* and *in vivo*.[15,16] On the other hand, studies of cathepsin L-deficient mice have revealed that this protease is important for the maintenance of heart structure and that its deficiency results in lysosomal impairment as well as in the consequent development of dilated cardiomyopathy.[17] These various observations have thus demonstrated the importance of cathepsins in cardiac remodeling.

The major cardiovascular actions of Ang II have been thought to be mediated by the Ang II type 1 receptor ($AT_1R$), and $AT_1R$ blockers (ARBs) have been widely used as antihypertensive drugs with the expectation of a cardiovascular protective effect.[18] In this study, we explored the possibility that cathepsin activation by NADPH oxidase-derived superoxide may play an important role in hypertensive cardiac remodeling and dysfunction and might represent a target for ARB therapy in the Dahl salt-sensitive (DS) rat model. Moreover, we also evaluated the possible effects of ARB therapy on the NADPH oxidase and cathepsin activation systems in cardiac biopsy specimens from humans with hypertensive HF. Finally, we examined whether or not the Ang II signal cascade enhances cathepsin expression in cultured cardiac myocytes (CMCs) via NADPH oxidase-derived ROS.

## Materials and Methods

### Animals and Treatment

Male inbred DS rats were obtained from Eisai (Tokyo, Japan) and handled in accordance with the revised guidelines of Nagoya University Graduate School of Medicine as well as with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Weaned rats were fed laboratory chow containing 0.3% NaCl until 7 weeks of age. DS rats fed an 8% NaCl diet after 7 weeks manifest compensated concentric left ventricular (LV) hypertrophy secondary to hypertension at 12 weeks and a distinct stage of fatal LV failure with lung congestion at 19 weeks.[12,19] DS rats were therefore fed an 8% NaCl diet from 7 weeks of age and were randomized to an HF group, an E64d group (10 mg per kg of body mass per day, administered intraperitoneally every other day; Sigma-Aldrich, St. Louis, MO), or an olmesartan group (3 mg/kg per day in chow; Sankyo Pharmaceutical, Tokyo, Japan) from 12 to 19 weeks of age ($n = 10$ for each group). The doses of olmesartan (an ARB) and E64d (a synthetic cathepsin inhibitor) were determined in preliminary experiments and previous studies.[20,21] DS rats maintained on the 0.3% NaCl diet served as age-matched controls (control group, $n = 10$). At 19 weeks of age, all of the rats were euthanized by an intraperitoneal overdose of sodium pentobarbital (50 mg/kg), and the hearts were removed for biological and histological analyses.[12] Arterial blood was collected from the abdominal aorta for the measurement of renin activity. Systolic blood pressure and heart rate were measured in conscious rats from 7 weeks of age, every week, using a noninvasive tail-cuff method.[12] In separate experiments, 12-week-old DS rats, fed a low-salt diet from 7 weeks of age, were given vehicle, olmesartan, or E64d in the same manner as in the above experiments ($n = 5$ for each group), and the LV tissues for measuring targeting mRNAs and protein levels were immediately placed in liquid nitrogen and stored at −80°C.

### Echocardiographic and Hemodynamic Analyses

At 19 weeks of age, rats were subjected to transthoracic echocardiography. In brief, M-mode echocardiography was performed with a SONOS 7500 ultrasound system and 5- to 12-MHz ultraband transducer (Philips, Andover, MA). The peak negative myocardial velocity gradient was derived from tissue Doppler imaging as a measurement of diastolic function. After echocardiography, a 2-F high-fidelity manometer-tipped catheter (SPR-407; Millar Instruments, Houston, TX) that had been calibrated relative to atmospheric pressure was introduced through the right carotid artery into the left ventricle. Tracings of LV pressure and the electrocardiogram were digitized to determine LV end-diastolic pressure.[12]

### Quantitation of Gene Expression

Total RNA was extracted from the LV tissue and cell extracts, and the abundance of specific mRNAs was determined by reverse-transcription (RT) and real-time quantitative polymerase chain reaction (PCR) analysis as described previously.[22] The sequences of primers and TaqMan probes for rats [including rat cathepsins S, K, B, D, and L; cystatin C; angiotensin-converting enzyme (ACE); $AT_{1A}R$; MMP-2; and MMP-9] were described previously,[10,12,16,19,23] and those for humans (including ACE, $AT_1R$, $p22^{phox}$, $gp91^{phox}$, and $p47^{phox}$) are described in Table 1. The amount of each mRNA was normalized by the corresponding amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

### Histology and Immunohistochemistry

Transverse tissue sections (3 $\mu$m) were stained with hematoxylin and eosin (H&E) for routine histological examination; van Gieson's solution (Muto, Tokyo, Japan) was used to evaluate elastin deposition, and Picrosirius red (Sigma-Aldrich) was used to examine collagen deposition as described.[12] Quantitative analyses of the cross-sectional area of cardiomyocytes and the areas of elastin and collagen deposition in the interstitial or/and perivascular regions were performed.[12] For immunohistochemistry, the sections from rats and humans were stained with rabbit polyclonal antibodies to cathepsin S or K (1:200 dilutions) as described.[12]

**Table 1.** Sequences of Oligonucleotides Used as Forward Primers, Reverse Primers, and Detection Probes (Human)

| mRNA | Oligonucleotide sequences | GenBank locus |
|---|---|---|
| gp91 phox | | AF_229177 |
| Forward primer (816 to 837) | 5′-TGCAGTCTCAGCCATATCTTC-3′ | |
| Reverse primer (878 to 899) | 5′-TGTGCACGCTGAAAAAGTCTTC-3′ | |
| Probe (848 to 872) | 5′-CACCCCTTCACCCTTACCTCTGCCC-3′ | |
| P22 phox | | M21186 |
| Forward primer (121 to 140) | 5′-GCGCTTCACCCAGTGGTACT-3′ | |
| Reverse primer (172 to 191) | 5′-GGTACTCCAGCAGGCACACA-3′ | |
| Probe (142 to 164) | 5′-TGGTGCCTACTCCATTGTGGCGG-3′ | |
| P47 phox | | M32011 |
| Forward primer (498 to 516) | 5′-TAGCATTGGCCACGAGCAT-3′ | |
| Reverse primer (566 to 588) | 5′-GGCTCATATAGCTTCTGCTTCCA-3′ | |
| Probe (523 to 548) | 5′-TGAGCCCAGACATTCCAAAATCGACA-3′ | |
| AT1 | | M93394 |
| Forward primer (543 to 566) | 5′-TTGATCGATACCTGGCTATTGTTC-3′ | |
| Reverse primer (601 to 622) | 5′-GATGCAGGTGACTTTGGCTACA-3′ | |
| Probe (575 to 597) | 5′-AAGTCCCGCCTTCGACGCACAAT-3′ | |
| ACE | | X16295 |
| Forward primer (691 to 713) | 5′-GAGAGCCATCCTCCAGTTTTACC-3′ | |
| Reverse primer (757 to 779) | 5′-ACGAGTCCCCTGCATCTACATAG-3′ | |
| Probe (737 to 756) | 5′-CAGGCTGCCCGGCTCAATGG-3′ | |

### *Immunoblot Assay*

Membrane and cytosolic fractions were isolated from LV tissues as previously described[10] and subjected to immunoblot analysis with antibodies to $p22^{phox}$, $p47^{phox}$ (1:1000 dilutions; both from Santa Cruz Biotechnology, Santa Cruz, CA), or $gp91^{phox}$ (1:1000 dilution; BD Transduction Laboratories, Lexington, KY). Antibodies to GAPDH (Santa Cruz Biotechnology) were used to confirm equal loading of samples.

### In Situ *Hybridization*

Three 30-mer oligo-DNAs and 45-mer oligo-DNAs complementary to cathepsins S and K mRNA sequences were designed from the mRNA of rat cathepsins S and K. The oligo-DNAs were labeled with digoxigenin (DIG) using a DIG oligonucleotide tailing kit (Roche Diagnostics, Mannheim, Germany).

*In situ* hybridization was performed as previously described.[16] In brief, the sections were immersed in 50% formamide (FA)/5× standard saline citrate (150 mmol/L NaCl and 15 mmol/L sodium citrate, pH 7.4) for 2 hours at 39°C for prehybridization. DIG-labeled oligo-DNA probes were heated for 10 minutes at 95°C to linearize the probes. For immunohistochemical detection of haptenized (DIG-labeled) antisense probes, the sections were first treated with 1% blocking reagent (DIG nucleic acid detection kit; Roche Diagnostics) in buffer containing 100 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4, for 1 hour, and then were incubated with alkaline phosphatase-conjugated sheep anti-DIG IgG (1:500) dissolved in 1% blocking buffer for 1 hour. After being washed three times, the sections were visualized with the color substrate NBT/BCIP (DIG nucleic acid detection kit; Roche Diagnostics) according to the manufacturer's instructions. Control experiments were used to confirm the specificity of the cathepsin S or K mRNA signals. Sense probes were hybridized with some sections as a negative control. The cathepsins S and K antisenses were described previously.[16]

### *Assay of NAPDH Oxidase, Total Superoxide Dismutase (SOD), and Catalase Activities, as well as Superoxide Production*

Specific myocardial NADPH oxidase activity was measured in total homogenates of the fresh LV tissue with the use of a lucigenin-based enhanced chemiluminescence assay as described previously.[24] A low lucigenin concentration (5 $\mu$mol/L) was used to minimize artifactual $O_2^-$ production attributable to redox cycling. In brief, 1 mg of homogenate protein diluted in 1 ml of lysis buffer (in mmol/L, Tris-HCl, 20; NaCl, 150; EDTA, 1; EGTA, 1; and 1% Triton X-100, pH 7.5) was transferred to an assay tube, and NADPH and dark-adapted lucigenin were added to final concentrations of 100 and 5 $\mu$mol/L, respectively, immediately before the measurement of chemiluminescence. The chemiluminescence signal was sampled every minute for 12 minutes with a tube luminometer (20/20; Turner Designs, Sunnyvale, CA), and the respective background counts were subtracted from the experimental values. Dihydroethidium staining for superoxide generation was performed as described.[25] After treatment with acetone for 10 minutes, cryostat myocardial sections (5 $\mu$m) were incubated for 30 minutes at 37°C with 5 $\mu$mol/L dihydroethidium in the presence or absence of an NADPH oxidase inhibitor diphenyliodonium (10 $\mu$mol/L; Sigma-Aldrich), a polyethylene glycol-SOD (500 U/ml; Sigma-Aldrich), and a native SOD (500 U/ml; Sigma-Aldrich), and then examined with a laser-scanning confocal microscope (WinROOF version 5.0; WinROOF, Tokyo, Japan). The levels of SOD and catalase were determined by assay kits obtained from Cayman (Ann Arbor, MI). Total SOD activity was assessed by the disappearance of superoxide detected by a water-soluble tetrazolium salt.[26] Catalase activity was deter-

mined as the conversion of methanol to formaldehyde in the presence of $H_2O_2$ using the method of Johansson and Borg.[27] The Ang II concentration in the LV myocardium was measured by radioimmunoassay from the LV homogenates collected in chilled tubes containing a mixture of 25 mmol/L EDTA (Sigma-Aldrich), 0.44 mmol/L 1,20-orthophenanthrolene monohydrate, 1 mmol/L $Na^+$ parachloromercuribenzoate, and 3 $\mu$mol/L WFML (rat renin inhibitor: acetyl-His-Pro-Phe-Val-Statine-Leu-Phe), and the total amount of Ang II was corrected by LV weight.

### *Human Myocardial Biopsy*

Details of patients' information for human myocardial biopsy are provided in Supplementary Table 1 (see *http://ajp.amjpathol.org*). Human LV endomyocardial biopsy specimens were obtained as described previously.[12] The patients who had cardiomyopathy such as ischemic, valvular, diabetic, alcoholic, inflammatory, systemic, hypertrophic, idiopathic dilated cardiomyopathy were excluded by established clinical, hemodynamic, echocardiographic, and cardiac catheterization criteria. Hypertensive HF patients were treated with ($n = 6$) or without olmesartan ($n = 4$). Endomyocardial biopsy samples were performed for real-time PCR analysis and immunohistochemistry. Baseline characteristics of the humans for this study are summarized in Supplementary Table 1 at *http://ajp.amjpathol.org*). Normal LV myocardial samples ($n = 7$) were obtained from donor hearts not matched for transplantation. For all normal hearts there was no history of cardiac diseases. Informed consent was obtained from all of the patients for use of the myocardial specimens in the present study. All of the patients provided informed consent for use of the myocardial specimens in the present study.

### *Cell Culture and Stimulation*

CMCs were isolated from the LV myocardium of 1-day-old Wistar rats and were cultured in a mixture (50:50, v/v) of Dulbecco's modified Eagle's medium and Ham's F-12 (DMEM/F-12; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and antibiotics as previously described.[12] CMCs ($5 \times 10^4$/well) were cultured in 12-well plates in serum-free DMEM/F-1 for 24 hours. After pretreatment with or without olmesartan (1 $\mu$mol/L), apocynin (APO, 100 $\mu$mol/L), xanthine oxidase (XO) inhibitor allopurinol (ALL, 10 $\mu$mol/L), or PEG-SOD (500 U/ml) for 30 minutes, CMCs were cultured in the presence or absence of Ang II (1 $\mu$mol/L) for 24 hours, and total RNA was extracted for quantitative real-time PCR assay. CMCs were also stimulated with $H_2O_2$ and XO/xanthine (4 $\mu$g/ml and 0.5 mmol/L) for examination of examined targeting gene expression.

### *Elastase Assay*

CMCs were cultured in 24-well plates until confluent. After pretreatment with or without APO (100 $\mu$mol/L) and PEG-SOD (500 U/ml), CMCs were cultured in the presence or absence of Ang II (1 $\mu$mol/L) in serum-free medium containing BODIPY fluorescein-conjugated DQ elastin from bovine neck ligament (300 $\mu$g/ml; Molecular Probes, Eugene, OR) as described previously.[28] After 24 hours of incubation, the cultured medium was analyzed for degraded elastin by Fluoroskan Ascent CF (excitation/emission: 485/530; Labsystems, Helsinki, Finland). The elastolytic activity in the extracts of rat LV tissues was also studied. Data were presented as relative units after adjustment for background levels. Data were representative of at least six independent experiments.

### *Immunocytofluorescence*

Immunocytofluorescence was performed as previously described.[29] Briefly, after pretreatment with APO, PEG-SOD, and *N*-acetyl-L-cysteine (NAC, 5 mmol/L), CMCs ($2 \times 10^4$/ml) were cultured in the presence or absence of $H_2O_2$ or XO/xanthine, in serum-free DMEM/F-12 for 24 hours. After blocking with 3% bovine serum albumin, the cells were treated with antibody to cathepsin S (1:100), washed, and then incubated with fluorescein isothiocyanate-conjugated goat antibodies to rabbit IgG (1:400; Medical & Biological Laboratories, Nagoya, Japan). Coverslips were finally treated with Prolong mounting medium (Molecular Probes) and examined by confocal microscopy.

### *Statistical Analysis*

Data were considered to be normally distributed and are presented as means ± SEM unless indicated otherwise. Differences were analyzed by Student's *t*-test or by one-way analysis of variance followed by Scheffé's multiple comparison test. A $P$ value of $<0.05$ was considered statistically significant.

## *Results*

### *Cardiac Remodeling and Function*

Systolic blood pressure was significantly higher in HF rats than in control rats at 8 weeks of age and thereafter (Supplementary Figure S1, see *http://ajp.amjpathol.org*). Neither olmesartan nor E64d affected blood pressure at any age. Neither heart rate nor body weight differed significantly among the four groups (Supplementary Table 2, see *http://ajp.amjpathol.org*). Echocardiographic and hemodynamic data for DS rats at 19 weeks of age are shown in Supplemental Table 2 (see *http://ajp.amjpathol.org*). The ratio of LV weight to tibial length, an index of LV hypertrophy, was 59% greater in HF rats than in control rats, and this change was attenuated by olmesartan. The ratio of lung weight to tibial length, an index of pulmonary congestion, was 68% greater in HF rats than in controls, indicative of the development of congestive HF; the increase in this parameter was attenuated by olmesartan. The LV end diastolic dimension was greater, whereas LV fractional shortening was smaller, in HF rats than in control rats at 19 weeks of age; all of these

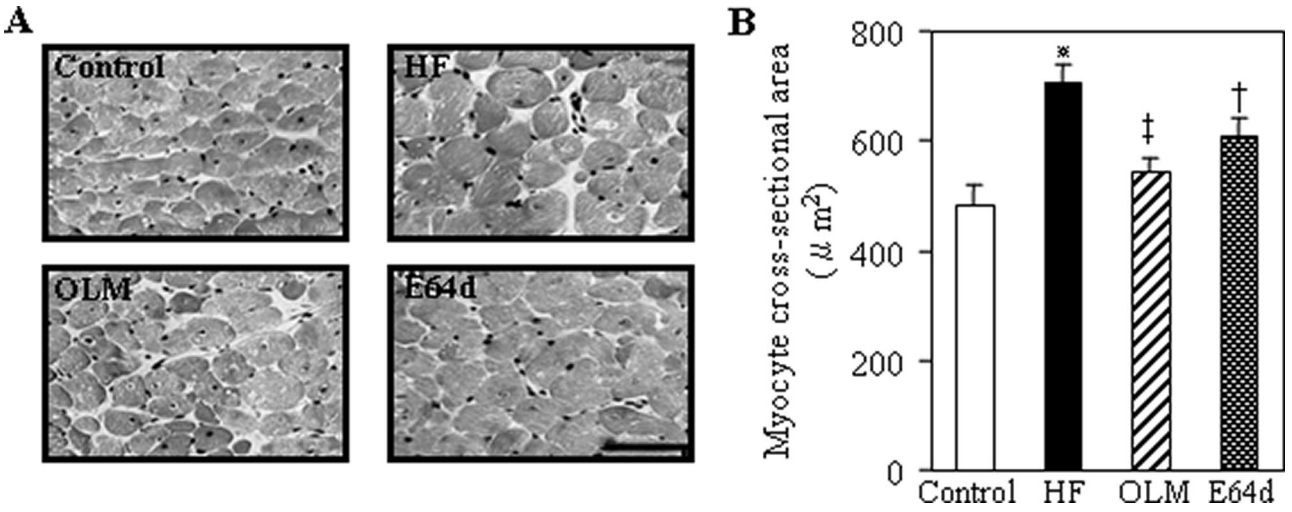


**Figure 1.** Morphological analysis of cardiac myocyte size in the LV wall of rats. **A:** Light micrographs of myocytes in H&E-stained section of the LV wall of rats in the control, HF, olmesartan (OLM), and E64d groups at 19 weeks of age. **B:** Cross-sectional area of CMCs determined from such micrographs is shown on the **right**. All quantitative data are means ± SEM ($n = 8$). $^{*}P < 0.05$ versus control group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$, versus HF group. Scale bar = 50 $\mu$m.

differences were reduced by treatment with olmesartan. The peak negative myocardial velocity gradient, an index of LV diastolic function, was reduced, whereas LV end-diastolic pressure and the pressure half-time ($T_{1/2}$) were higher in HF rats than in control rats, and these differences were attenuated by olmesartan. All of these beneficial effects were mimicked by E64d.

Histological analysis revealed that hemodynamic overload had increased the cross-sectional area of CMCs in HF rats by 40% compared with that in control rats (Figure 1, A and B). The extent of load-induced CMC hypertrophy was reduced by treatment with olmesartan or E64d. As shown in Figure 2, A, C, and D, marked perivascular and interstitial fibrosis was also detected in the LV tissues of

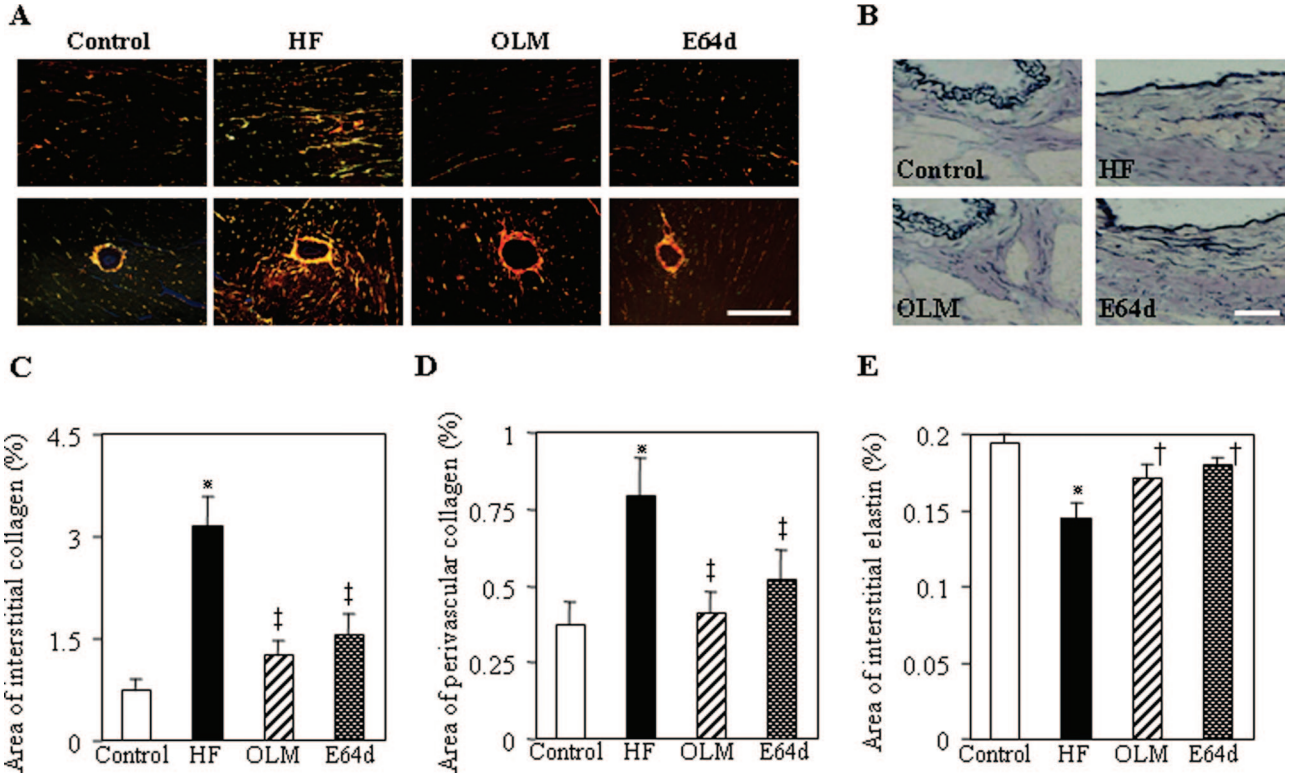


**Figure 2.** Morphological analysis of collagen and elastin deposition in the left ventricle of DS rats. **A:** Picrosirius red staining of transverse sections of the left ventricle. Interstitial and perivascular fields are shown in the **top** and **bottom** panels, respectively. **B:** Van Gieson's staining of transverse sections of the left ventricle including coronary arteries. **C** and **D:** Relative areas of interstitial and perivascular collagen determined from sections similar to those shown in **A**. **E:** Relative area of interstitial elastin determined from the transverse sections. All quantitative data are means ± SEM ($n = 8$). $^{*}P < 0.05$ versus control group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ versus HF group. Scale bar = 100 $\mu$m (**A**). Scale bar = 50 $\mu$m (**B**).

**Table 2.** Quantitative RT-PCR Analysis of the Expression of Protease and ECM Protein Genes in the Left Ventricle of DS Rats

| Parameter | Control | HF | HF + OLM | HF + E64d |
|---|---|---|---|---|
| Cathepsin S | 45.3 ± 4.0 | 210.1 ± 24.3* | 125.4 ± 21.0† | 168.4 ± 18.2† |
| Cathepsin K | 65.3 ± 10.5 | 175.6 ± 25.0* | 87.9 ± 11.2† | 127.4 ± 18.0† |
| Cathepsin B | 20.4 ± 3.0 | 54.1 ± 7.8* | 28.5 ± 4.2† | 39.9 ± 9.0† |
| Cathepsin L | 21.9 ± 5.6 | 30.4 ± 5.2 | 26.9 ± 7.9 | 27.4 ± 4.0 |
| Cathepsin D | 42.9 ± 8.8 | 52.8 ± 10.9 | 39.9 ± 9.2 | 54.1 ± 10.6 |
| Cystatin C | 790.8 ± 78.0 | 916.3 ± 182 | 715.4 ± 81 | 906.5 ± 101 |
| Type I collagen | 13.1 ± 2.3 | 35.6 ± 7.4* | 15.8 ± 2.2‡ | 13.5 ± 2.5‡ |
| Type III collagen | 25.3 ± 4.5 | 60.4 ± 9.1* | 38.2 ± 6.2† | 45.9 ± 10.2† |
| Fibronectin | 31.0 ± 7.1 | 105.5 ± 20.1* | 45.9 ± 7.0‡ | 56.4 ± 11.3† |
| Elastin | 190.4 ± 25.9 | 330.7 ± 37.5* | 278.4 ± 0.4† | 289.9 ± 47.7† |

Data are means ± SEM ($n = 8$). *$P < 0.05$ versus control group; †$P < 0.05$ and ‡$P < 0.01$ versus HF group.

HF rats as compared with controls; these changes were also attenuated by E64d and olmesartan. Quantitative PCR analysis revealed that the expression of ECM protein genes, including those for types I and III collagen, fibronectin, and elastin, was higher in HF rats than in control animals (Table 2); all of these differences were again reduced by E64d or olmesartan. Fragmentation of the elastic lamina of coronary arteries in the LV myocardium was increased in HF rats in a manner sensitive to treatment with E64d or olmesartan (Figure 2B). Furthermore, the area of interstitial elastin was smaller in HF rats than in control rats, and this difference was reduced by E64d or olmesartan (Figure 2E). The ratio of the area of interstitial elastin to that of interstitial collagen was lower in HF rats than in controls (4.6 ± 0.9% versus 22.0 ± 2.1%, $P = 0.0008$) but was increased by E64d (13.0 ± 2.8%, $P = 0.015$) or olmesartan (15.5 ± 1.9%, $P = 0.0036$).

## *Renin-Angiotensin System and NADPH Oxidase Activity and Expression*

Renin activity in plasma was lower in HF rats than in control rats and was not affected by treatment with E64d or olmesartan (data not shown). Quantitative PCR analysis revealed that hemodynamic overload increased the expression of ACE and $AT_1R$ genes in HF rats (Figure 3A) as well as in humans with HF (Figure 3, B and C). These changes were attenuated by olmesartan treatment in both rats and humans, and E64d inhibited the increases in the expression of $AT_{1A}R$ and ACE genes in rats. Furthermore, the local tissue Ang II level was higher in the LV myocardium of DS HF rats than in control rats, and this difference was reduced by olmesartan and or E64d (Supplemental Table 2, see *http://ajp.amjpathol.org*).

The NADPH oxidase activity (Figure 4A) as well as the amounts of the $p22^{phox}$, $gp91^{phox}$, and $p47^{phox}$ subunits of this enzyme (Figure 4, B and C), were increased in HF rats, and these increases were attenuated by olmesartan or E64d. Similarly, the amounts of mRNAs for NADPH oxidase subunits were increased in the LV myocardium of HF patients, and these increases were reduced by olmesartan treatment (Figure 3, D–F). Dihydroethidium staining revealed that superoxide production in the myocardial tissue sections was increased in HF rats (Figure 4D), and these increases were also attenuated in olmesartan- or E64d-treated rats. Both diphenyliodonium and PEG-SOD have been shown to reduce superoxide production in the myocardial sections of LV from HF rats, whereas no effect has been observed with native SOD (Supplemental Figure S2, see *http://ajp.amjpathol.org*). Interestingly, administration of olmesartan or E64d reduced the increase in the superoxide production of the intramyocardial coronary arteries in HF rats (Figure 4E). On the other hand, neither total SOD activity nor catalase activity differed significantly among the four groups of rats (see Supplemental Table 2 at *http://ajp.amjpathol.org*).

## *Cathepsin Expression and Activity*

The amounts of cathepsins S, K, and B mRNAs in the left ventricle were greater in HF rats than in controls (Table 2). The amounts of mRNAs for cathepsin S (340.2 ± 102.5 versus 94.1 ± 15.3, $P < 0.05$) and cathepsin K (175.9 ± 56.0 versus 85.4 ± 10.1, $P < 0.05$) were also higher in the left ventricle of humans with HF than in those of control patients. These changes in the abundance of cathepsins S, K, and B mRNA in HF rats were inhibited by olmesartan or E64d (Table 2), whereas olmesartan inhibited those changes in cathepsins S and K mRNA abundance in HF humans by 46.3% ($P < 0.05$) and 32.0% ($P < 0.05$), respectively. The amounts of cathepsin D, cathepsin L, and cystatin C mRNAs did not differ among the four groups of rats. *In situ* hybridization also revealed that the increased signal intensity for cathepsins S and K mRNAs in CMCs and for cathepsin S in smooth muscle cells of intramyocardial coronary arteries in the left ventricle of HF rats was reduced by olmesartan or E64d (Supplemental Figure S3, see *http://ajp.amjpathol.org*).

Immunohistochemical staining showed that the expression of cathepsin S protein was markedly increased throughout the myocardium of HF rats, with staining apparent in both CMCs and smooth muscle cells of coronary arteries (Figure 5 A). Cathespin K expression was also increased in CMCs of HF rats. These changes in cathepsins S and K expression in HF rats were greatly inhibited by olmesartan. Similarly, olmesartan reduced the increased staining intensity for cathepsins S and K in the LV myocardium of HF patients (Figure 5B). The elastolytic activity of LV homogenates was 4.2-fold greater for

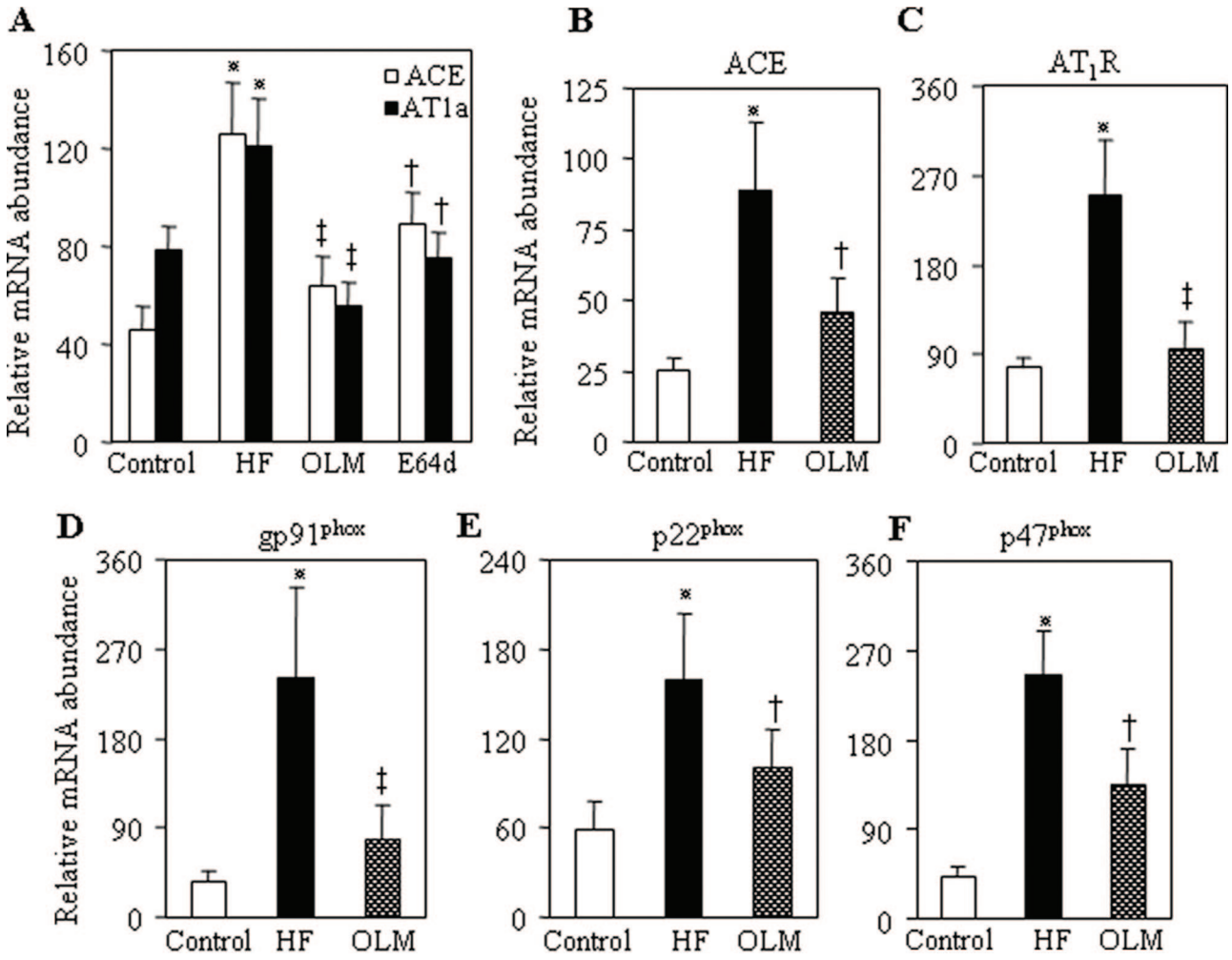


**Figure 3.** Expressions of ACE, $AT_1R$, and NADPH oxidase subunit genes in the left ventricle of rats and humans. **A:** Quantitative RT-PCR analysis of ACE and $AT_{1A}R$ mRNAs, respectively, in the left ventricle of rats; results are expressed relative to the abundance of GAPDH mRNA ($n = 8$). The abundance of ACE (**B**), $AT_1R$ (**C**), $gp91^{phox}$ (**D**), $p22^{phox}$ (**E**), and $p47^{phox}$ (**F**) mRNAs in the left ventricle of control humans ($n = 7$), patients with HF ($n = 4$), and HF patients treated with olmesartan (OLM, $n = 6$). Data are means ± SEM. $^*P < 0.05$ versus responding controls; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ versus untreated HF rats or HF patients.

HF rats than for control rats, and this increase was inhibited by E64d or olmesartan (Figure 5C).

### *Regulation of Cathepsin Expression and Activity in Cultured CMCs*

As shown in Figure 6A, a strong signal for cathepsin S was observed in CMCs stimulated with $H_2O_2$ or XO/xanthine, and the $H_2O_2$-induced staining signal was reduced by APO, PEG-SOD, or NAC. It is consistent with real-time PCR data that the amount of cathepsin S mRNA was increased by exposure to $H_2O_2$ or XO/xanthine, and this effect was inhibited again by APO or PEG-SOD (Figure 6B). Exposure of CMCs to Ang II also induced marked increases in cathepsin S mRNA and elastolytic activity, and these effects were inhibited by APO, PEG-SOD, olmesartan, or/and ALL (Figure 6, C and D). Neither olmesartan nor E64d affected blood pressure or heart rate in DS control rats at 19 weeks of age (see Supplemental Table 3 at *http://ajp.amjpathol.org*). Similarly, there were no differences in targeting gene expressions (cathepsins S and K, collagen types I and III, elastin) in control DS rats treated with or without olmesartan and E64d.

## *Discussion*

We have unveiled an important role for the elastolytic cathepsin activation system in cardiac remodeling during the development of hypertensive HF in DS rats. Furthermore, both NADPH oxidase-dependent superoxide production and cathepsin activation during this process represent targets for ARB treatment. Our study has produced several important findings. First, the increases in cathepsin expression and elastolytic activity in the LV myocardium of rats and/or humans with HF were reduced by olmesartan or E64d. Second, these drugs also downregulated ACE and $AT_1R$ mRNAs, as well as the expression of NADPH oxidase components and superoxide production in the left ventricle of rats and humans with HF. Third, the beneficial effects of olmesartan on LV hyper-

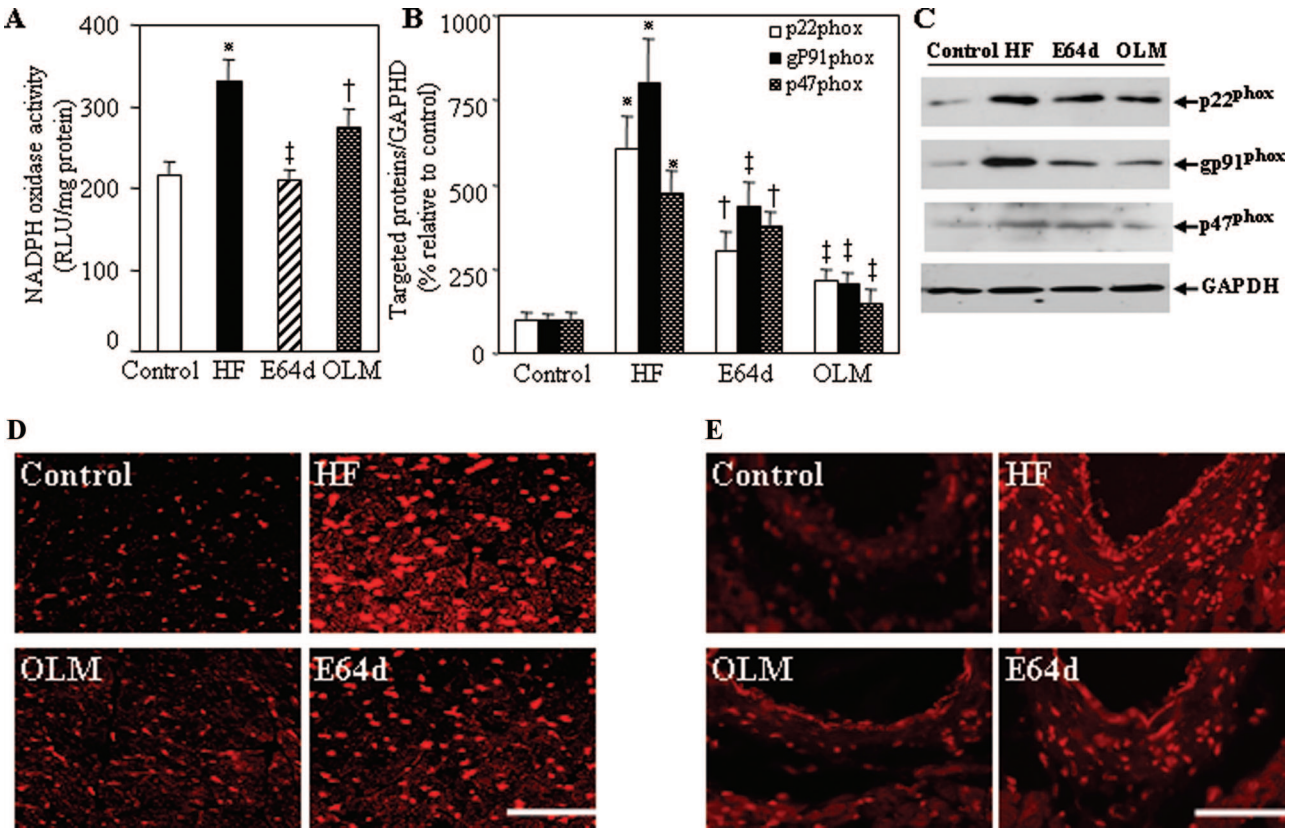


**Figure 4.** Activity and expression of NADPH oxidase in the left ventricle of DS rats. **A:** NADPH oxidase activity in homogenates of LV tissue. Data are expressed as relative light units (RLU) per mg of protein. Immunoblot analysis of NADPH oxidase subunits in the membrane fractions of the left ventricle. Representative blots are shown in **C**, and quantitative data are shown in **B**; band intensity was normalized by that for GAPDH. Dihydroethidium staining for superoxide in transverse sections of the LV myocardium **D** and intramyocoronary arteries in DS rats **E**. All quantitative data are means ± SEM ($n = 6$). $^{*}P < 0.05$ versus control group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ versus HF group. Scale bars = 50 $\mu$m.

trophy, fibrosis, and dysfunction as well as on remodeling of coronary artery were mimicked by treatment with E64d in DS rats. Finally, the Ang II signal cascade enhanced cathepsin expression and activity in cultured CMCs via NADPH oxidase-derived superoxide production. The mechanisms underlying the prevention of cardiac remodeling and dysfunction associated with hypertensive HF in olmesartan- and E64d-treated rats are schematically represented in Supplemental Figure S4 (see *http://ajp.amjpathol.org*).

E64d is a broad-spectrum inhibitor of cysteine proteases that inhibits the activity of several cathepsins (including S, K, B, and L).[11] E64d has been shown to reduce the extent of both LV interstitial and LV perivascular fibrosis with the decreased elastolytic activity in HF rats. E64d suppressed the degradation of the intramyocardial coronary elastin lamina in HF rats. However, E64d had no significant effects on MMP-2 and MMP-9 expression and activation (data not shown). These findings, coupled with previous findings that cathepsins are secreted into the extracellular space, showing potent collagenolytic and elastolytic activities,[10–12] indicate that E64d prevents LV fibrosis and intramyocardial coronary elastin lamina metabolism through a mechanism that could be associated with the reduction of cathepsin-dependent ECM degradation. It is noteworthy that E64d has also been shown to reduce the expression of ECM component mRNAs in the LV tissues of HF rats. This effect would further contribute to the prevention of LV remodeling. Moreover, E64d restored the interstitial ratio of elastin to collagen, a contributing factor to LV stiffness.[30] One of the major interesting and novel outcomes of the present study is that the inhibition of the cathepsin activation system improved LV systolic and diastolic dysfunction accompanied by positive changes in certain indices of LV performance and stiffness. E64d also downregulated mRNAs for $AT_1R$, ACE, and NADPH oxidase subunits (including $p22^{phox}$, $gp91^{phox}$, and $p47^{phox}$) as well as reduced NADPH oxidase-dependent superoxide production in the left ventricle; these effects are likely related to the inhibition of cathepsin activation, the loss of ECM components, and the consequent attenuation of LV hypertrophy and fibrosis. E64d, a cell-membrane-permeable drug, is highly selective for proteases and four times more potent the other E64 forms. It has been reported that E64d penetrated the cell membrane and inhibited lysosomal proteases, resulting in prevention of ischemia-induced neuronal apoptosis. Recently, cathepsin inhibition has been shown to prevent free radical-induced cardiomyocyte apoptosis, which has been associated with NADPH oxidase-dependent superoxide production.[3,25,31] These findings raise the possibility that E64-induced effects on NADPH oxidase-dependent superoxide production are secondary to E64's reduction of cell

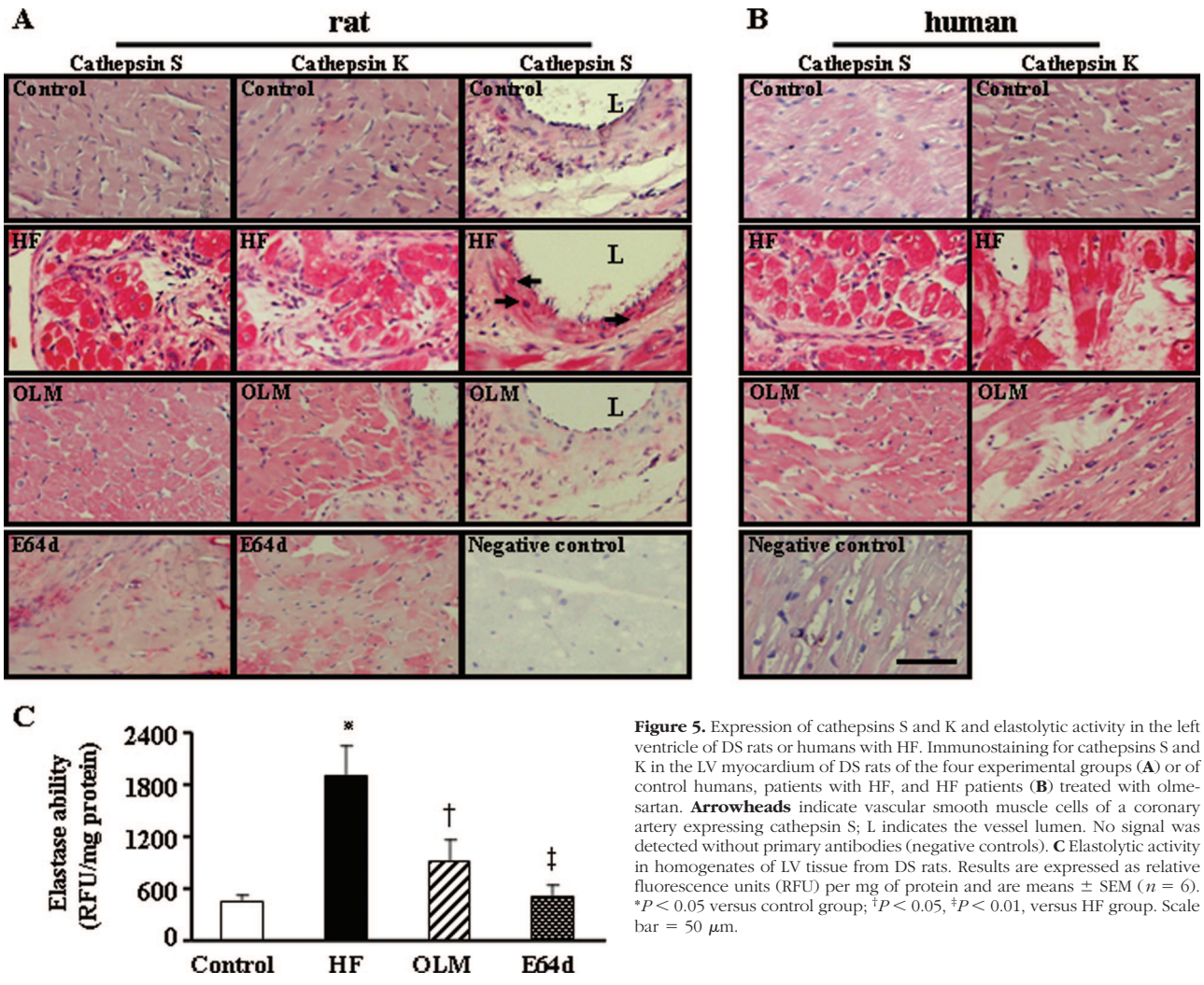


**Figure 5.** Expression of cathepsins S and K and elastolytic activity in the left ventricle of DS rats or humans with HF. Immunostaining for cathepsins S and K in the LV myocardium of DS rats of the four experimental groups (**A**) or of control humans, patients with HF, and HF patients (**B**) treated with olmesartan. **Arrowheads** indicate vascular smooth muscle cells of a coronary artery expressing cathepsin S; L indicates the vessel lumen. No signal was detected without primary antibodies (negative controls). **C** Elastolytic activity in homogenates of LV tissue from DS rats. Results are expressed as relative fluorescence units (RFU) per mg of protein and are means ± SEM ($n = 6$). $^{*}P < 0.05$ versus control group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$, versus HF group. Scale bar = 50 $\mu$m.

apoptosis via the inhibition of lysosomal protease, thereby contributing to the prevention of LV remodeling. Additionally, the fact that E64d had virtually the same beneficial effects as olmesartan on the measured antioxidative and Ang II system activation parameters suggests that there may be some feed-forward mechanism. Further investigations into these issues are required.

A phagocyte-type NADPH oxidase was reportedly expressed in human myocardium, specifically in CMCs, and its activity increased in association with the development of dilated cardiomyopathy.[4,32,33] We have now confirmed and extended these observations by showing that the abundance of mRNAs for the $p22^{phox}$, $gp91^{phox}$, and $p47^{phox}$ subunits of this enzyme were increased in patients with hypertensive HF. Similarly, we found that NADPH oxidase activity and the abundance of its subunits were increased in the left ventricle of DS HF rats. Increases in the expression of individual components of NADPH oxidase were previously shown to result in an increase in NADPH oxidase activity both *in vivo* and *in vitro*.[34,35] Additionally, we observed that neither total SOD activity nor catalase activity differed significantly among the four groups of rats. These findings, taken together with previous findings that NADPH oxidase was the major source of superoxide in the LV myocardium of HF DS rats,[19] suggest that the increase in superoxide generation in the LV myocardium is not attributable to the decreases in SOD and catalase activities but rather to the increase in NADPH oxidase activity in this rat model.

Plasma renin and Ang II levels were reportedly reduced in hypertensive DS rats.[36] However, we showed that local ACE and $AT_1R$ mRNA levels were up-regulated in the left ventricle of DS rats with HF, although the plasma level of renin was also lower in HF rats than in age-matched controls. Similarly, the abundance of ACE and $AT_1R$ mRNAs was also increased in the failing human heart. These findings suggested that the local Ang system in the LV myocardium may thus be activated in this low-renin hypertensive rat model. This notion is supported by our observation that local tissue Ang II level in the LV myocardium was higher in DS HF rats than in control rats. Olmesartan markedly reduced the amounts of ACE and $AT_1R$ mRNAs in both DS rats and humans with HF. NADPH oxidase activity and superoxide production in coronary arteries were previously shown to be reduced by $AT_1R$ blockade.[37] Olmesartan's inhibitory effects on NADPH oxidase activity and expression may thus contribute to the down-regulation of ACE and $AT_1R$

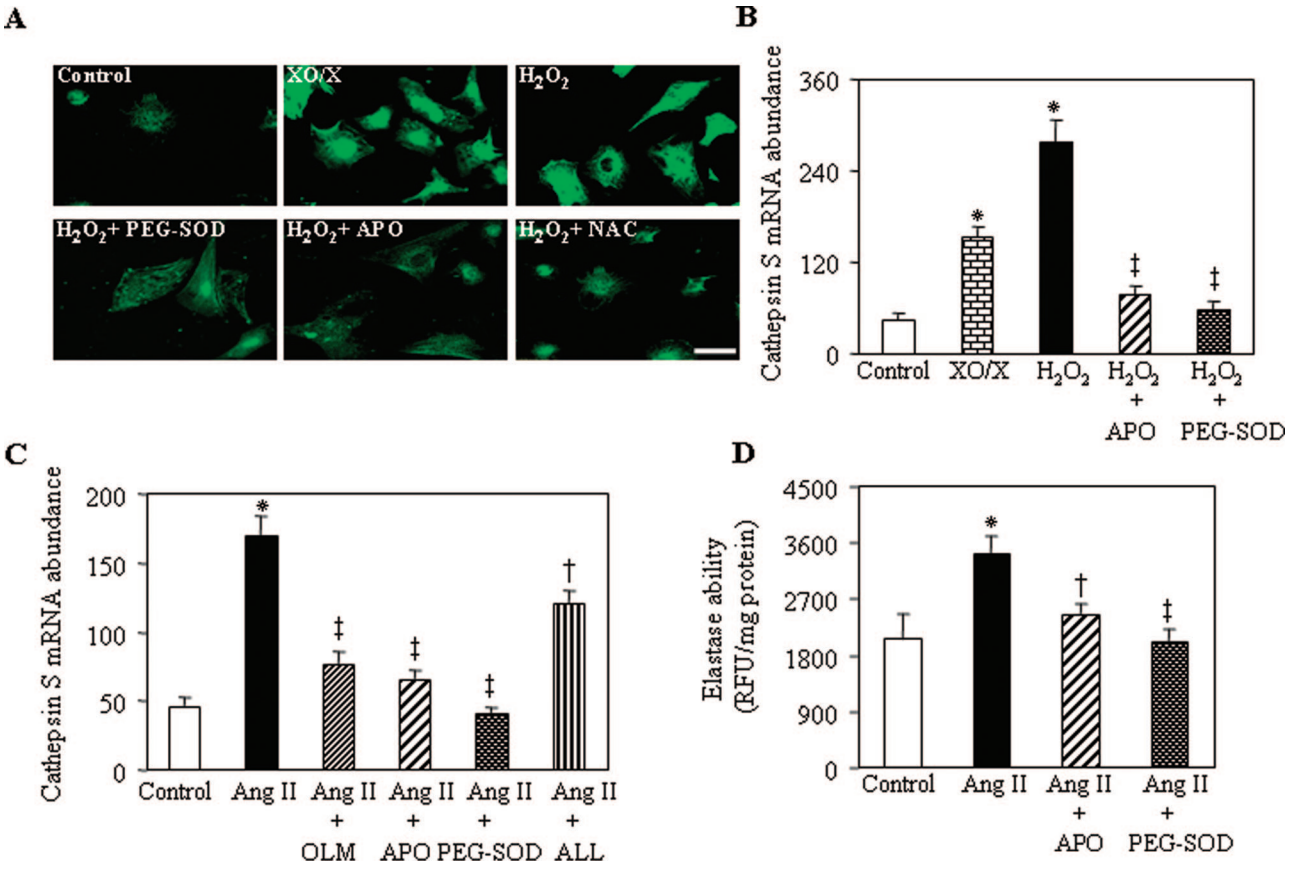


**Figure 6.** Regulation of cathepsin expression and elastolytic activity in cultured rat CMCs. After pretreatment with olmesartan (OLM, 1 $\mu$mol/L), APO (100 $\mu$mol/L), polyethylene glycol-superoxide dismutase (PEG-SOD), *N*-acetly-L-cysteine (NAC, 5 mmol/L), and allopurinol (ALL, 10 $\mu$mol/L), respectively, for 30 minutes, the CMCs were cultured in the presence or absence of angiotensin (Ang) II, $H_2O_2$, or xanthine oxidase/xanthine (XO/X) for 24 hours. They were then subjected to immunofluorescence staining for cathepsin S (**A**), to quantitative real-time PCR analysis of cathepsin S mRNA (**B** and **C**), or to assay of elastolytic activity (**D**). Quantitative data are means ± SEM ($n = 6$). $^*P < 0.05$ versus corresponding controls; $^\dagger P < 0.05$, $^\ddagger P < 0.01$ versus cells treated with Ang II or $H_2O_2$ alone.

mRNAs that this drug induces. NADPH oxidase-derived ROS play an important role in cardiac hypertrophy, fibrosis, and dysfunction in animals and humans.[3,4,25] The reduction in NADPH oxidase-dependent superoxide production by olmesartan-mediated $AT_1R$ blockade might therefore contribute to this drug's beneficial effects on LV remodeling in rats or humans with HF.

Olmesartan has been shown to reduce the increases in the expression of cathepsin mRNAs (including those for cathepsins S, K, and B) and proteins (cathepsins S and K) as well as elastolytic activity in LV tissues of HF rats and humans. *In vitro* and *in vivo* studies have shown that NADPH oxidase-derived ROS contribute to the activation of various proteases.[3,25] These findings, together with our present data showing that olmesartan inhibited NADPH oxidase activation and superoxide production, suggest that this drug's inhibitory effects on cathepsin expression and activity may result from the reduction in NADPH oxidase-derived superoxide production attributable to the blockade of $AT_1R$ signaling cascade *in vivo* in rats and humans. This notion is further supported by our finding that Ang II as well as $H_2O_2$ induced cathepsin S mRNA and protein expression as well as elastolytic activity in cultured CMCs, and that these effects were inhibited by olmesartan, APO, or PEG-SOD. As known, APO inhibits NADPH oxidase whereas PEG-SOD removes superoxide. It is noteworthy that not only APO but also PEG-SOD have been shown to inhibit the hydrogen peroxide-induced cathepsin expression. It seems to be that there is a ROS-induced ROS mechanism. Further study will be required to understand this possible mechanism in ROS generation system in the failing myocardium. On other hand, it should be recognized that other enzymes have also been implicated as possible sources of elevated oxygen-free radicals in hypertension, such as the XO system. However, previous studies have reported that the XO inhibitor allopurinol had a minimal effect on vascular $O_2^-$ production in human diabetes mellitus.[38] Recently, Picchi and colleague[39] demonstrated that the XO system was not the source of vascular endothelial $O_2^-$ production in diabetic and nondiabetic rats. Furthermore, it has been reported that xanthine oxidoreductase activity was much lower than NADPH oxidase activity in the failing myocardium of salt-loaded DS rats.[40] Interestingly, Doerries and colleagues[3] demonstrated that XO activation depended on NADPH oxidase in a mouse myocardial infarction model. These findings suggested that the XO system may thus be activated in the cardiac tissue of this DS rat model but might not be a major independent player in $O_2^-$ production. Finally, our observation that olmesartan suppressed elastic lamina degradation together with local superoxide generation and cathepsin S

expression in intramyocardial coronary arteries of HF rats suggests that this drug might prevent coronary arterial remodeling during the development of HF by the same mechanism.[41]

These data suggest that cathepsins are likely to trigger and promote cardiac remodeling, and the blockade of angiotenin II type 1 receptor attenuates such cathepsin expression and activity by inhibiting NADPH oxidase from producing superoxide, thereby attenuating cardiac remodeling. In summary, we believe that our experiments show for the first time that E64d-mediated cathepsin inhibition suppressed LV remodeling and dysfunction during the development of HF associated with hypertension in DS rats. $AT_1R$ blockade also suppressed cathepsin activation and NADPH oxidase-derived superoxide production, and attenuated LV remodeling and dysfunction in DS rats with HF as well as in HF patients. Cathepsins thus likely contribute to ECM metabolism, and $AT_1R$ blockade likely inhibits cathepsin activation by suppressing NADPH oxidase-dependent superoxide production, thereby preventing LV remodeling and dysfunction.

## *Acknowledgment*

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