Differential Subcellular Actions of ACE Inhibitors and AT\textsubscript{1} Receptor Antagonists on Cardiac Remodeling Induced by Chronic Inhibition of NO Synthesis in Rats

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Abstract—Chronic inhibition of NO synthesis induces cardiac hypertrophy independent of systemic blood pressure (SBP) by increasing protein synthesis in vivo. We examined whether ACE inhibitors (ACEIs) enalapril and temocapril and angiotensin II type-I receptor antagonists (angiotensin receptor blockers [ARBs]) losartan and CS-866 can block cardiac hypertrophy and whether changes in activation of 70-kDa S6 kinase (p70S6K) or extracellular signal–regulated protein kinase (ERK) are involved. The following 13 groups were studied: untreated Wistar-Kyoto rats and rats treated with NO synthase inhibitor N\textsubscript{o}-nitro-L-arginine methyl ester (L-NAME), D-NAME (the inactive isomer of L-NAME), L-NAME plus hydralazine, L-NAME plus enalapril (3 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1}) or temocapril (1 or 10 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1}), L-NAME plus losartan (10 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1}) or CS-866 (1 or 10 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1}), L-NAME plus temocapril-CS866 in combination (1 or 10 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1}), and L-NAME plus rapamycin (0.5 mg \cdot kg\textsuperscript{-1} \cdot d\textsuperscript{-1}). After 8 weeks of each experiment, ratios of coronary wall to lumen (wall/lumen) and left ventricular weight to body weight (LVW/BW) were quantified. L-NAME increased SBP, wall/lumen, and LVW/BW compared with that of control. ACEIs, ARBs, and hydralazine equally canceled the increase in SBP induced by L-NAME. However, ACEIs and ARBs equally (but not hydralazine) attenuated increase in wall/lumen and LVW/BW induced by L-NAME. The L-NAME group showed both p70S6K and ERK activation in myocardium (2.2-fold and 1.8-fold versus control, respectively). ACEIs inactivated p70S6K and ARBs inactivated ERK in myocardium, but hydralazine did not change activation of either kinase. Thus, ACEIs and ARBs modulate different intracellular signaling pathways, inhibiting p70S6K or ERK, respectively, to elicit equal reduction of cardiac hypertrophy induced by chronic inhibition of NO synthesis in vivo. *(Hypertension. 2001;38:404-411.)*

Key Words: remodeling ■ angiotensin-converting enzyme inhibitor ■ receptors, angiotensin ■ kinase

Many recent reports have shown that angiotensin (Ang) II, the key product of the renin-angiotensin system (RAS), is one of the critical factors for myocardial hypertrophy\textsuperscript{1,2} and fibrosis\textsuperscript{3} as well as other stimuli,\textsuperscript{4-6} such as catecholamines, endothelin-1, and peptide growth factors. These changes are accepted to be the main features of cardiac “remodeling.”\textsuperscript{7} In addition, either ACE inhibitors (ACEIs) or Ang II type-I receptor (AT\textsubscript{1}) antagonists (angiotensin receptor blockers [ARBs]) can potently reduce these changes.\textsuperscript{8,9} Because ACE and AT\textsubscript{1}, the main targets of ACEIs and ARBs, respectively, are on successive pathways in the RAS, the main effect of these agents and the underlying mechanism were believed to be identical.\textsuperscript{10} However, we reported that cardioprotection by ACEIs include different cascades from Ang II reduction\textsuperscript{11} or AT\textsubscript{1} blockade,\textsuperscript{12} which implies that ACEIs and ARBs might regulate cardiac remodeling through different mechanisms in the ischemic model. Many studies clarified that extracellular signal–regulated kinase (ERK)\textsuperscript{13} is one of the major factors mediating hypertrophy afforded by Ang II. Other studies have proposed 70-kDa S6 kinase (p70S6K),\textsuperscript{14,15} which phosphorylates the 40S ribosomal protein S6 that regulates initiation of mRNA translation,\textsuperscript{16} to be the key mediator of protein synthesis for hypertrophic changes, in addition to 90-KDa ribosomal S6 kinase (p90RSK),\textsuperscript{7,13} afforded by Ang II. p70S6K activation is related to cardiac hypertrophy\textsuperscript{15,17} and growth and proliferation of endothelial cells,\textsuperscript{18} smooth muscle cells,\textsuperscript{14} and fibroblasts.\textsuperscript{19} Although the previous report clarified that ERK pathways are distinct from the p70S6K-mediated one,\textsuperscript{20} effects of ACEIs and ARBs on the intracellular signals and cardiac hypertrophy are not clarified.

To test this hypothesis, we used the in vivo Wistar-Kyoto rat model with chronic inhibition of NO synthesis by N\textsubscript{o}-nitro-L-arginine methyl ester (L-NAME). The main features of this model in cardiovascular system are myocardial remodeling (hypertrophy/fibrosis), cardiovascular remodeling (me-
dial thickening/perivascular fibrosis), and hypertension afforded by RAS activation.\textsuperscript{10,21–23} We evaluated (1) myocardial/cardiovascular structural changes, (2) tissue p70S6K/ERK activity, and (3) effects of ACEIs (enalapril or temocapril), ARBs (losartan or CS-866), or both on each parameter.

Methods

Materials
S6 peptide (RRRLSSLRA, No. 12-124) was from Upstate Biotechnology. Rapamycin was a generous gift from Dr S. Seghal (Bar Ilan University, Ramat Gan, Israel). Temocapril and CS-866 were from Sankyo Co Ltd. Enalapril and losartan were from Banyu. The other drugs were from Sigma Chemical Co. All procedures were performed in careful conformance to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 93-23, revised 1985).

Instrumentation
Eight-week-old male Wistar-Kyoto rats (Charles River, Kanagawa, Japan) were randomly divided into 13 groups. The control group (n=8) received no treatment. The L-NAME group (n=8) received L-NAME (1 g/L in drinking water). The D-NAME group (n=7) received D-NAME (1 g/L in drinking water), the inactive isomer of L-NAME. The L-NAME+Hyd group (n=7) received L-NAME and hydralazine (120 mg/L in drinking water). The L-NAME+Ens group (n=10) received L-NAME and enalapril (10 mg · kg\(^{-1}\) · d\(^{-1}\)) orally (by gavage). The L-NAME+Lsr group (n=10) received L-NAME and losartan (10 mg · kg\(^{-1}\) · d\(^{-1}\)) orally. The L-NAME+Tmc1 and L-NAME+Tmc10 groups (n=10 each) received L-NAME and temocapril (1 or 10 mg · kg\(^{-1}\) · d\(^{-1}\), respectively) orally. The L-NAME+CS1 and L-NAME+CS10 groups (n=10 each) received L-NAME and CS-866 (1 or 10 mg · kg\(^{-1}\) · d\(^{-1}\), respectively) orally. The L-NAME+Tmc1+CS1 (n=10) and L-NAME+Tmc10+CS10 (n=6) groups received L-NAME and an equal quantity of temocapril/CS-866 (1 or 10 mg · kg\(^{-1}\) · d\(^{-1}\), respectively) orally. The L-NAME+Rap group (n=5) received L-NAME and rapamycin (0.5 mg · kg\(^{-1}\) · d\(^{-1}\)) orally. We decided dosage levels of ACEIs, ARBs, and rapamycin according to preliminary tests (data not shown) and a previous report.\textsuperscript{10} All rats were housed, treated, and euthanatized as described previously.\textsuperscript{24}

Histological Examination
Excised hearts were weighed, cut, and carefully scanned as described previously.\textsuperscript{24} Thickening of coronary arterial walls and morphometry of left ventricular (LV) myocytes were assessed, and the wall-to-lumen ratio (wall/lumen) and the cross-sectional area were calculated according to the previous report.\textsuperscript{21} As a parameter of inflammatory changes, polymorphonuclear neutrophil (PMN) infiltration into the myocardium was counted according to the previous report.\textsuperscript{23}

Assay for P70S6K and ERK Activities
Specific activity of p70S6K was determined by \(^{32}\)P incorporation into S6 peptide in the immune complex as described.\textsuperscript{26,27} Specific activity of ERK was determined as described previously.\textsuperscript{23} The experiment was repeated twice for each sample.

Statistical Analysis
Data are expressed as mean±SEM. Paired data were compared by Student’s \(t\) test. Comparisons of p70S6K and ERK activity, body weight, LV weight, ratio of LV weight to body weight (LVW/BW), coronary wall/lumen ratio (wall/lumen), and cardiomyocyte cross-sectional area were performed by ANOVA with modified Bonferroni’s multiple-comparison \(t\) test. Comparisons of time-course changes in SBP and heart rate were performed by 2-way repeated measures ANOVA with multiple-comparison test. \(P<0.05\) was considered statistically significant.

Results

Arterial Pressure, Heart Rate, Body Weight, and LV Weight
SBP (Figure 1A) was comparable among the 13 groups studied initially. After 8 weeks, LNAME and LNAME+Rap groups showed higher SBP (\(P<0.01\)) than control. SBP in LNAME+Tmc1 and LNAME+CS1 groups was higher (\(P<0.05\)) than control, but lower (\(P<0.05\)) than in the LNAME and LNAME+Rap groups. LNAME+Tmc10+CS10 group showed lower SBP (\(P<0.01\)) than the control group. Other groups showed no significant difference from the control level. Heart rate (Figure 1B) was comparable among all groups and did not change significantly throughout the study. Body and LV weights in each group after 8 weeks are indicated in the Table. ACEIs, ARBs, and rapamycin but not hydralazine reversed the increase in LVW/BW induced by L-NAME (Figure 2). Either lower or higher doses of temocapril and CS-866 in combination tended to reduce further the LVW/BW afforded by each agent alone but did not reach significance (\(P<0.10\) each).

![Figure 1. Sequential changes in systemic BP (A) and heart rate (B) in the groups studied. See text for detail. Hyd indicates hydralazine; Enr, enalapril; Tmc, temocapril; Lsr, losartan; CS, CS-866; and Rap, rapamycin. *\(P<0.05\), **\(P<0.01\) vs control.](http://hyper.ahajournals.org/)

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Body weight, g

Cross-sectional areas in the L-NAME and L-NAME

cross-sectional areas in the groups studied. Cardiomyocyte

Figure 3B shows representative examples of cardiomyocyte

Myocardial Hypertrophy

ntion, in either high or low doses, significantly (P<0.05)

but not hydralazine or lower doses of ACEIs or ARBs alone,

ACEIs and ARBs (in a dose-dependent manner) or rapamycin

myocardium was higher than the activity in the control group

P70S6K and ERK Activity in Myocardium

In the L-NAME group, P70S6K and ERK activity in the

myocardium was higher than the activity in the control group

(Figure 6). ACEIs and rapamycin but not ARBs or hydral-

azine prevented p70S6K activation (Figure 6A). Only ARBs

and not ACEIs, rapamycin, or hydralazine prevented ERK

activation induced by L-NAME (Figure 6B). Temocapril and

CS-866 in combination tended to enhance further any reduction in the

extent of PMN infiltration afforded by each agent alone but
did not reach significance (P<0.10 each).

PMN Infiltration Into Myocardium

L-NAME treatment increased counts of PMNs in myocardium
compared with the control group; this effect was not

reversed by hydralazine but was inhibited well by either

ACEIs or ARBs in a dose-dependent manner (Figure 5). Either low or high doses of temocapril and CS-866 in

combination tended to enhance further any reduction in the

extent of PMN infiltration afforded by each agent alone but
did not reach significance.

Coronary Vascular Remodeling

Representative examples of coronary vessels in the groups studied
are shown in Figure 3A. After 8 weeks, the wall/lumen in the L-NAME group was significantly larger than that in the control group (Figure 4A). Combined dosages of ACEIs and ARBs (in a dose-dependent manner) or rapamycin, but not hydralazine or lower doses of ACEIs or ARBs alone, significantly attenuated the increase in wall/lumen by L-NAME (Figure 4A). Temocapril and CS-866 in combination, in either high or low doses, significantly (P<0.05) enhanced wall/lumen reduction afforded by each agent alone.

Myocardial Hypertrophy

Figure 3B shows representative examples of cardiomyocyte

cross-sectional areas in the L-NAME and L-NAME+Hyd
groups were greater (P<0.01) than those in the control group
(Figure 4B). Rapamycin, ACEIs, and ARBs but not hydral-
zine reversed the increases in cardiomyocyte cross-sectional

areas induced by L-NAME. Either low or high doses of
temocapril and CS-866 in combination tended to enhance further the reduction in LVW/BW and cross-sectional area afforded by each agent alone but did not reach significance (P<0.10 each).

Discussion

We have demonstrated that both thickening of coronary
ty walls and cardiac hypertrophy induced by inhibition of

NO synthesis are reduced by coadministration of ACEIs or

ARBs but not hydralazine. In addition, we found that in rats treated with L-NAME, P70S6K activity in the myocardium increases but is inhibited by ACEIs or ARBs, respectively. These findings suggest that (1) either ACEIs or

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<td>n</td>
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<td>L-NAME+Tmc10+CS10</td>
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Left Ventricular Weight, mg

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<th>n</th>
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<td>7</td>
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<td>6</td>
<td>512±13*</td>
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<tr>
<td>L-NAME+Rap</td>
<td>5</td>
<td>702±26</td>
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Results are mean±SEM.

*P<0.05 vs control; †P<0.01 vs control.

Figure 2. LVW/BW in groups studied. **P<0.01 vs control; +P<0.05, ++P<0.01 vs L-NAME+Hyd group. Abbreviations as in Figure 1.

*P<0.05 vs control; †P<0.01 vs L-NAME.
ARBS can equally attenuate myocardial and cardiovascular hypertrophy, (2) the pathways to prevent these structural changes are different between ACEIs and ARBs, and (3) treatment with suboptimal doses of ACEIs and ARBs in combination could synergically attenuate cardiac, especially cardiovascular, remodeling in the in vivo rat model.

Cellular Mechanisms for Thickening of Coronary Arterial Wall and Cardiac Hypertrophy Induced by Inhibition of NO Synthesis

Chronic treatment with L-NAME reduces NO synthesis23 and induces cardiac hypertrophy and remodeling.21,29 Furthermore, we have reported that reduced plasma NO level is linearly correlated with severity of hypertension in patients with essential hypertension,30 which suggests that this model may represent cardiac hypertrophy associated with clinical essential hypertension in vivo. Inhibition of NO synthesis is also reported to be associated with increased plasma renin activity,22 increased local ACE activity,21 upregulation of Ang II receptors,31 plasminogen activator inhibitor-1 expression,32 reexpression of fetal skeletal α-actin isoform,22 and synthesis of growth factors in the endothelium.33 In addition, Ang II itself induces release of platelet-derived growth factors34 and involves protein kinase C (PKC) in the hypertrophic reactions.13 Because NO modifies vascular structure by modulating growth of vascular smooth muscle cells,35

Figure 3. Representative histologic findings of coronary vessels (A) and myocytes (B) in groups studied. A, Control; B, L-NAME; C, D-NAME; D, L-NAME + Hyd; E, L-NAME + Enr; F, L-NAME + Lsr; G, L-NAME + Rap.
inhibition of NO synthesis also in part might induce directly cardiovascular hypertrophy. However, we have previously demonstrated \(^3\) that inhibition of NO synthesis leads to PKC activation, which is also involved in the pathway of Ang II–induced cardiac hypertrophy \(^1\) by means of a cGMP-independent mechanism. Although several mechanisms may exist by which inhibition of NO synthesis induces coronary vascular and myocardial remodeling, activation of local/systemic RAS or associating inflammatory changes and the increase in growth factors taken together could contribute to “remodeling” in this model.

We demonstrated in the present studies that inhibition of NO synthesis induces vascular and cardiomyocyte structural changes associated with activation of p70S6K or ERK in myocardium. P70S6K is activated by cytokines and hormonal or growth factors through phosphoinositide 3-kinase (PI3-K) activation \(^3\) and phosphorylate S6. Furthermore, p70S6K phosphorylates a modulator of a transcriptional factor, cAMP response element modulator. \(^3\) Thus, p70S6K might be important in transcriptional as well as translational levels of protein synthesis. In addition, ERK is activated by Ang II \(^5\) or other growth factors through PKC \(^1\) or tyrosine kinases, \(^5\) which lead to p90RSK activation that also regulates transcription. \(^5\)

We demonstrated differential regulation by ACEIs or ARBs on p70S6K and ERK, which supports the previous report \(^2\) that these 2 signaling pathways are independently involved in cardiac hypertrophy in this model.

**Effects of ACEIs and ARBs on Signaling Pathways**

What makes the difference in cardiovascular effects between ACEIs and ARBs? Three candidates may be considered: (1) the effects of bradykinin-dependent pathways, (2) the effects of other types (probably type 2) of Ang II receptors, or (3) some unknown pathways. Because ACEIs inhibit the breakdown of bradykinin, which causes an increase in NO production independent of regulation of Ang II production, \(^1\) ACEIs may exert beneficial effects through a bradykinin-NO pathway. On the other hand, recent reports show that Ang II type-2 receptor (AT\(_2\)) stimulation mediates the inhibitory effects of AT\(_1\) blockade on cardiovascular remodeling. \(^4\) Both arguments may explain the whole mechanisms underlying the effect of these agents, especially in clinical settings \(^4\) and indicate that ACEIs and ARBs might modulate cardiac remodeling by different pathways. ACEIs are most likely to modulate
activation of neurohumoral systems and release of growth factors independent of Ang II level, which may modulate PI3-K or p70S6K.43

In the present study, rapamycin had greatest effect on inhibiting cardiac remodeling and PMN infiltration, which suggests that other important factors may be included in the effect of rapamycin apart from ACEIs and ARBs. Accordingly, we have reported that rapamycin also inhibits phosphorylation of 4E-binding protein I, another regulatory molecule for mRNA translation, independent of p70S6K modulation.12 Furthermore, because a recent in vitro study reported that neuregulin-1–induced increase in protein synthesis of cardiomyocyte is not prevented by PI3-K–inhibition but blocked by p70S6K inhibition,44 a PI3-K–independent pathway leading to p70S6K activation also could exist. In addition to PI3-K, CaMK or JNK45,46 is a candidate for mediation of these effects, given that CaMK can activate both PKC and JNK in vivo,46 which could use the P70S6K pathway eventually.

We also observed that ACEIs and ARBs dose-dependently and equally inhibited PMN infiltration into myocardial tissue. However, we do not have direct evidence that these agents regulate either cardiac structural changes or inflammatory changes by use of the common pathway. Indeed, we cannot deny the possibility that ACEIs and ARBs regulate cardiac structural changes using the same signaling pathways in part. Further investigation is needed to clarify (1) the effects of ACEIs or ARBs on modulating PI3-K, CaMK, or JNK activity and associated inflammatory changes and (2) whether selective inhibition of ERK also can exert a maximal reduction in cardiac remodeling or associated inflammatory changes in this model, for example, by use of PD 098,059.

Synergistic Effects of ACEIs and ARBs in Combination

Because hydralazine did not reduce any of the parameters despite the complete inhibition of BP increase afforded by L-NAME, we conclude that the additional effects of ACEIs and ARBs in combination are also independent of the reduction in BP. Ten milligrams per kilogram per day of temocapril and CS-866 in combination blocked either P70S6K or ERK activation by L-NAME in myocardium to the extent of that afforded by each agent alone, without further enhanced reduction of individual kinase activity. Accordingly, either 1 or 10 mg · kg⁻¹ · d⁻¹ of temocapril and CS-866 in combination further reduced the LVW/BW, cross-sectional area, wall/lumen, and PMN infiltration afforded by each agent alone, but only further reduction of wall/lumen reached significance. This may have occurred because the LVW/BW, cross-sectional area, and PMN infiltration were reduced to a control level by a low dose of either ACEIs or ARBs. An additional trial with even lower doses of these agents alone and in combination might confirm that suboptimal doses of ACEIs and ARBs in combination further reduce cardiac remodeling in this model, as clearly indicated in the case of cardiovascular remodeling (wall/lumen) in the present study.

Clinical Indications

In conclusion, the present study strongly suggests that both ACEIs and ARBs use different actions to exert a similar effect on attenuation of the cardiac hypertrophy and cardiovascular remodeling in vivo model of chronic inhibition of NO synthesis. Because cardiac remodeling is hard to reverse by either ACEIs or ARBs alone under clinical conditions, the present study proposes a good clinical indication for combination therapy with ACEIs and ARBs against cardiac, espe-
cally cardiovascular remodeling. However, continuous stud-
ies in basic and clinical phases are needed.

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