Increased Myocardial Collagen Content in Transgenic Rats Overexpressing Cardiac Angiotensin-Converting Enzyme Is Related to Enhanced Breakdown of N-Acetyl-Ser-Asp-Lys-Pro and Increased Phosphorylation of Smad2/3

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**Background**—Although increased activity of angiotensin-converting enzyme (ACE) has been associated with increased cardiac collagen, no studies to date have established a direct cause-and-effect relation between the two.

**Methods and Results**—We used transgenic rats that overexpress human ACE selectively in the myocardium. Two independent heterozygous transgenic rat lines were studied, one expressing 2 to 3 copies (L1172) and the other expressing 5 to 10 copies (L1173) of the ACE transgene. These rats were normotensive but developed a proportionate increase in myocardial collagen depending on the ACE gene dose (up to 2.5-fold, \( P<0.01 \)), but cardiac angiotensin II levels remained normal, whereas collagen content reversed to control levels on ACE inhibition. To explain these changes, we investigated N-acetyl-Ser-Asp-Lys-Pro (AcSDKP), an alternative substrate that is catabolized exclusively by ACE. Increased cardiac expression of ACE was paralleled by a reciprocal decrease in cardiac AcSDKP and a proportionate increase in phosphorylated Smad2 and Smad3, all of which normalized after both ACE inhibition and AcSDKP infusion. Furthermore, a functional link of this signaling cascade was demonstrated, because AcSDKP inhibited Smad3 phosphorylation in a dose-dependent manner in cultured cardiac fibroblasts and in vivo.

**Conclusions**—Our findings suggest that increased cardiac ACE activity can increase cardiac collagen content by degradation of AcSDKP, an inhibitor of the phosphorylation of transforming growth factor-\( \beta \) signaling molecules Smad2 and Smad3. This implies that the antifibrotic effects of ACE inhibitors are mediated in part by increasing cardiac AcSDKP, with subsequent inhibition of Smad2/3 phosphorylation. (Circulation. 2004;110:3129-3135.)

**Key Words:** enzymes ■ hypertrophy ■ myocardium ■ peptides

Induction of angiotensin-converting enzyme (ACE) has been associated with cardiac fibrosis since increased expression of ACE was reported in animal models accompanied by increased cardiac collagen, such as pressure-loading-induced hypertrophy and myocardial infarction.\(^1\)\(^-\)\(^3\) However, it is still unknown whether the increase in cardiac ACE and increased cardiac collagen deposition are functionally linked. It has been a common assumption that the profibrotic effects of ACE are mediated via increased production of angiotensin (Ang) II. However, recent reports have shown that Ang II is not always elevated when cardiac collagen content is increased\(^4\) and tissue levels of Ang II are not lowered in homozygous ACE-null mutant mice,\(^5\) shedding doubt on this assumption.

Recent observations have suggested that excess degradation of N-acetyl-Ser-Asp-Lys-Pro (AcSDKP) can mediate the profibrotic effects of ACE. AcSDKP is hydrolyzed specifically by the N-terminal domain of ACE.\(^6\) It has been shown previously that AcSDKP attenuates cardiac fibroblast proliferation and collagen synthesis both in vitro and in vivo,\(^7\)\(^,\)\(^8\) and we have subsequently shown in vitro that AcSDKP directly inhibits Smad2 phosphorylation,\(^9\) which has been confirmed in other cell types.\(^10\) Recent reports have suggested that phosphorylated Smad3 (P-Smad3) is a more potent regulator

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of collagen synthesis than P-Smad2,11,12 but a direct relation between AcSDKP and P-Smad3 has not yet been shown in vivo.

To address a causal role for ACE in cardiac collagen production, we used a transgenic rat model that expressed the full-length human ACE cDNA selectively in cardiomycocytes and tested whether myocardial changes were related to altered levels of Ang II or rather AcSDKP as an alternative substrate of ACE.

Methods

Transgenic Rat Model and Treatment Protocol
We have described this model previously.13 In brief, the full-length human somatic ACE cDNA (4.02 kb) placed under the control of a 2.1-kb rat myosin light chain-2 (MLC-2) promoter yielded 2 independent transgenic lines, the first with 2 to 3 copies (L1172) and the second with 5 to 10 copies (L1173) of the transgene. Activity of membrane-bound ACE was determined as reported previously.13 The ACE-transgenic rats were normotensive compared with the controls.

Because L1173 rats expressed the highest ACE activity, we treated 2 subgroups (n=6 to 8 each group) of male L1173 rats 12 to 15 months old either with the ACE inhibitor lisinopril (5 mg · kg⁻¹ · d⁻¹ in drinking water) for 12 weeks or with AcSDKP before they were euthanized. Nontransgenic Sprague-Dawley rats were used as controls. AcSDKP (Bachem) was infused at a rate of 1 mg · kg⁻¹ · d⁻¹ via subcutaneously placed miniosmotic pumps (Alzet, model 2 ML 4) for 3 weeks. The local institutional animal experiment committee approved all experiments.

Northern Blotting
Northern blotting was performed as reported previously.14 The atrial natriuretic factor probe was obtained from a 825-bp BamHI fragment of the atrial natriuretic factor cDNA cloned in pGEM-4Z (kindly provided by Dr Sigrid Hoffmann, University of Mannheim, Germany) and the transforming growth factor (TGF)-β probe (823-bp cDNA fragment) was generated by reverse transcription–polymerase chain reaction.

Histology
Myocardial collagen was stained with picrosirius red, as described earlier.15 Left ventricular (LV) interstitial collagen was quantified by computerized planimetry. To measure myocyte cross-sectional area, transversely cut tissue sections were stained with anti-laminin antibody (Sigma) counterstained with hematoxylin.

Measurement of Ang II Levels
For the determination of cardiac Ang II levels, heart tissue was homogenized and centrifuged, and the supernatant was diluted with methanol. The eluate was incubated with tracer and Ang II antiserum (Bachem). Captopril (10 μmol/L) was added to the medium to prevent degradation of AcSDKP by ACE present in the serum.

TGF-β1, Receptor Displacement Assay
To investigate whether AcSDKP competes with TGF-β1, for its receptor binding, synchronized cardiac fibroblasts were treated with 125I-labeled TGF-β1, in the presence and absence of AcSDKP according to a protocol published previously.17

Protein Isolation and Western Blotting
Protein isolation and Western blotting were performed as described previously.9 Anti–P-Smad3 antibody was a kind gift from Professor E. Leof and Dr M. Wilkes (Mayo Clinic Cancer Research, Rochester, Minn), anti-collagen I/III antibodies were from Abcam, and anti–TGF-β1 antibody was from Promega. All other antibodies were purchased from Cell Signaling Technologies.

Statistical Analyses
Data are shown as mean±SEM. The data were analyzed by 1-way ANOVA in combination with a Dunnett post hoc analysis to correct for multiple comparisons (SPSS 10.0). Probability values of P≤0.05 were considered statistically significant.

Results

Increased ACE Activity in the Heart
In the LV, ACE activity was increased 50-fold in L1173 and 13-fold in L1172 rats compared with controls (Figure 1). ACE activity in kidneys and skeletal musculature was only slightly and nonsignificantly increased, whereas serum ACE was not increased in transgenic rats.

Lack of Increase in Cardiac Ang II Levels in ACE-Transgenic Rats
Cardiac Ang II levels did not differ between transgenic and nontransgenic rats (Figure 2). Pentobarbital was administered to a subgroup of experimental animals to investigate whether increasing renin by pentobarbital would unmask a propensity of the ACE-transgenic rats to increase their cardiac Ang II levels beyond that in controls. Pentobarbital increased renin to a similar extent in both groups (Sprague-Dawley, 10.5±2.5; human (h) ACE-transgenics, 9.4±1.8 ng Ang I · mL⁻¹ · h⁻¹; P=0.76) and increased cardiac Ang II levels, but this increase was comparable in both transgenic and wild-type rats (Figure 2A). Because coronary vasoconstrictor response caused by intracoronary infusion of Ang I was
significantly enhanced in hACE-transgenic rats, this indicated that the transgene expression was functional.13

Decreased Cardiac AcSDKP Levels in ACE-Transgenic Rats

The myocardial concentration of AcSDKP was significantly lower in both ACE-transgenic rat lines (L1173 and L1172) compared with nontransgenic littermates. The low level of AcSDKP in L1173 normalized after lisinopril treatment (Figure 2B).

Increased Myocardial Collagen Content in ACE-Transgenic Rats

LV collagen volume fraction was significantly increased in 12-month-old ACE-transgenic rats compared with controls, which was not seen at 3 months of age.13 Furthermore, LV collagen volume fraction was also increased in the high (L1173) versus the moderate (L1172) ACE-overexpression line (data not shown). Western blotting of myocardial homogenate with anti–collagen I and anti–collagen III antibodies confirmed the proportional increase in cardiac collagen (Figure 3). Replacement of AcSDKP or treatment with lisinopril significantly decreased myocardial collagen types I and III in high-ACE-expressing (L1173) rats.

AcSDKP Decreased Smad3 Phosphorylation in Cardiac Fibroblasts

In synchronized cardiac fibroblasts, TGF-β (10 ng/mL) for 60 minutes increased Smad3 phosphorylation 5-fold, which was attenuated by AcSDKP (1 and 100 nmol/L) in a dose-dependent manner (Figure 4).
Increased Smad2 and Smad3 Phosphorylation in ACE-Transgenic Rats

Both P-Smad2 and P-Smad3 were upregulated in ACE-transgenic rats in an ACE-gene-dose–dependent manner compared with controls. P-Smad2 and P-Smad3 levels in L1173 rats normalized after lisinopril or AcSDKP treatment. The levels of total Smad2 and Smad3 (relative to GAPDH) were unaltered among all the study groups (Figure 5).

Normal TGF-β1 mRNA and Protein Expression in ACE-Transgenic Rats

Cardiac TGF-β1 mRNA and protein levels were unaltered in ACE-transgenic rats with higher ACE gene expression (L1173) (Figure 6).

AcSDKP Does Not Compete With TGF-β1 for Receptor Binding

To exclude the possibility that AcSDKP inhibits Smad signaling by competing for the TGF-β1 receptor complex, we performed a competition assay, which demonstrated that addition of AcSDKP (1 and 100 nmol/L) did not change the amount of receptor-bound 125I-TGF-β1 in cardiac fibroblasts (controls, 499±55 cpm; AcSDKP 100 nmol/L, 536±65 cpm; AcSDKP 1 nmol/L, 585±98 cpm; n=7, P=NS).

No LV Hypertrophy in ACE-Transgenic Rats

Heart weight–to–body weight ratio, myocyte cross-sectional area, and mRNA expression of atrial natriuretic factor in the highest cardiac ACE-expressing (L1173) rats did not differ from controls, as reported previously (data not shown).

Discussion

Although increased activity of ACE has been shown to be associated with cardiac fibrosis, it has remained unclear whether this association is causal. To investigate this, we characterized 2 lines of ACE-transgenic rats with either a lower or a higher copy number of the ACE transgene to assess gene-dose effects on cardiac architecture. The main finding of this study is that cardiac collagen content increased in parallel with the ACE-gene dose, which reversed on ACE inhibition. Because Ang II levels were not elevated in ACE-transgenic rats, we sought an alternative mechanism to explain the
AcSDKP Is an Antifibrotic Agent
AcSDKP is a specific substrate of ACE, and its level increases substantially after ACE inhibitor therapy. Although the functional role of exogenous AcSDKP was demonstrated in the myocardium, endogenous levels of this peptide in the myocardium had not yet been shown. We show here that cardiac AcSDKP levels in the myocardium of wild-type rats is higher than what has been reported in plasma, suggesting that the myocardium actively generates or traps AcSDKP.

The cardiac concentration of AcSDKP correlated inversely with the level of myocardial ACE overexpression. After ACE inhibitor therapy, the level of AcSDKP increased profoundly in ACE-transgenic rats, reaching the level obtained in non-transgenic controls. Previous studies have suggested an important role for AcSDKP in cardiac matrix regulation. AcSDKP was shown to reduce DNA and collagen synthesis in cardiac and renal fibroblasts. Moreover, AcSDKP decreased cardiac and renal fibrosis in 2-kidney, 1-clip hypertensive as well as aldosterone-salt hypertensive rats. We found that the levels of AcSDKP correlated inversely with cardiac collagen content in ACE-transgenic rats. Even though AcSDKP was infused for only 3 weeks, AcSDKP-treated ACE-transgenic rats had substantially lower levels of collagen I and III compared with the untreated transgenic controls. This relatively rapid decrease in collagen indicates that apart from its inhibitory role in collagen synthesis, AcSDKP may also stimulate collagenolysis, possibly via independent mechanisms, such as activating collagenases in a direct or indirect way.

Relation Between TGF-β, Smad, and AcSDKP
TGF-β stimulates the deposition of collagen, leading to an increased myocardial collagen content during cardiac hypertrophy and failure. This profibrotic effect of TGF-β is importantly conveyed by Smad proteins, which are phosphorylated and then translocate to the nucleus to initiate gene expression. We have recently shown that AcSDKP directly inhibits Smad2 phosphorylation in isolated cells. Here, we observed that AcSDKP also inhibits TGF-β-mediated phosphorylation of Smad3 in cardiac fibroblasts. Moreover, infusion of AcSDKP in ACE-transgenic L1173 rats normalized the levels of P-Smad2 and 3 in the myocardium. Taken together, AcSDKP appears to be an endogenous inhibitor of Smad signaling. Therefore, ACE may induce myocardial collagen production via breakdown of AcSDKP and the subsequent release of inhibition on Smad2/3 phosphorylation. Smad2 and 3 are reported to be elevated in various models of myocardial fibrosis, and inhibition of Smad2/3 phosphorylation by angiotensin-receptor blockers (AT1 blockers) has been shown to reduce postinfarction fibrosis. An AT1 blocker could possibly inhibit myocardial collagenesis in ACE-transgenic rats as well by this mechanism. In our study, we found upregulated levels of P-Smad2/3 in ACE-transgenic rats, whereas the mRNA expression of TGF-β was not elevated. It is striking that the direct effects of AcSDKP in vitro and in

Relation Between ACE, Ang II, and Collagen Content
An increased level of ACE has been associated with excess production of extracellular matrix in the heart, which was presumed to be mediated via increased Ang II. We did not find an increase in cardiac Ang II, which underscores that ACE is not the rate-limiting step in the formation of Ang II. In agreement, Perry et al have shown that cardiac Ang II levels remained unaltered despite differences in ACE genotype and ACE expression in mice. The lack of rise in Ang II cannot be explained by a lack of interaction between the overexpressed enzyme and intracoronary Ang I, because we have described increased ACE-dependent conversion of intracoronary Ang I in the isolated perfused hearts of ACE-transgenic rats.

Figure 6. TGF-β measurements in myocardial homogenate. A, mRNA expression of TGF-β1 in myocardium of L1173 rats compared with wild-type controls (n=7). B, protein levels in controls and transgenic rats (n=3 each). Top, Representative blot corresponding to bar. Lane 1, control; lane 2, L1172; lane 3, L1173.
vivo on both Smad2 and Smad3 are highly similar, suggesting a common mode of action of AcSDKP in inhibiting Smad2 and 3 phosphorylation. In isolated cardiac fibroblasts, AcSDKP inhibited Smad3 phosphorylation within 1 hour. This indicates that de novo synthesis of an inhibitory protein or inhibition of synthesis of an excitatory molecule of Smad phosphorylation is less likely. These findings suggested that AcSDKP may be a competitive inhibitor of the TGF-β receptor complex, but a ligand displacement assay did not support that idea. Therefore, the precise mechanism by which AcSDKP inhibits Smad phosphorylation remains elusive.

It has been shown that overexpression of Smad7, an inhibitory Smad protein, decreased collagen synthesis in adult cardiac fibroblasts and that Smad7 expression was decreased in infarct scar. However, in neonatal cardiac fibroblasts, the levels of Smad7 were undetectable, and we did not find any effect of AcSDKP on Smad7. Still, because we have not measured Smad6/7 in the myocardium of ACE-transgenic rats, the possible effects of ACE on the expression of these inhibitory proteins cannot be excluded.

Apart from Ang I and AcSDKP, ACE catalyzes the breakdown of several substrates, including Ang-(1–7) and bradykinin. Although bradykinin is shown to be related to cardiac fibrosis, a relationship between Smad2/3 phosphorylation and bradykinin is not yet established. It was recently demonstrated that Ang-(1–7) is localized selectively in the myocardium, whereas fibroblasts and other collagen-containing cells were devoid of Ang-(1–7). This suggested that the decrease in the breakdown of Ang-(1–7) may have less effect on cardiac collagen production in ACE-transgenic rats. Nevertheless, we cannot rule out the effects of excess degradation of these molecules in our transgenic model.

In summary, this study shows that AcSDKP is present in the myocardium in biologically relevant concentrations. Increased cardiac ACE decreased cardiac AcSDKP in vivo in a gene-dose–dependent manner, which was paralleled by increased phosphorylated Smad2 and Smad3 as well as increased cardiac collagen content, suggesting that increased cardiac ACE degrades AcSDKP in sufficient amounts to release the inhibition of Smad2/3 phosphorylation and induce an excess deposition of collagen. ACE inhibitors may interfere with this process by preventing the degradation of AcSDKP.

This indicates that the maintenance of adequate AcSDKP concentrations in the heart may be one of the major factors mediating the beneficial structural effects of ACE inhibition.

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