Activation and Functional Significance of the Renin-Angiotensin System in Mice With Cardiac Restricted Overexpression of Tumor Necrosis Factor

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Background—The functional significance of cross-regulation between the renin-angiotensin system (RAS) and tumor necrosis factor (TNF) has been established in nonmyocyte cell types; however, the degree and functional significance of the interaction between RAS and TNF has not been characterized in the heart.

Methods and Results—We examined the expression of components of the RAS in a line of transgenic mice (MHCsTNF) with cardiac restricted overexpression of TNF. When examined at 4, 8, and 12 weeks of age, the MHCsTNF mice had increased activation of myocardial RAS, as shown by an increase in ACE mRNA level and ACE activity and increased angiotensin II peptide levels. Furthermore, myocardial angiotensin receptor mRNA and protein levels were reduced in the MHCsTNF mice, consistent with homologous desensitization of the receptors. However, expression of renin and angiotensinogen was not increased in MHCsTNF mice compared with littermate controls. To determine the functional significance of RAS activation in the MHCsTNF mice, we treated the mice with an angiotensin type I receptor antagonist, losartan (30 mg/kg), or diluent from 4 to 8 weeks of age. Analysis of cardiac structure with MRI showed that treatment with losartan normalized left ventricular mass and wall thickness. Furthermore, treatment with losartan reduced myocardial collagen content and reduced the incidence of myocyte apoptosis.

Conclusions—Taken together, these results show that there are functionally significant interactions between RAS and TNF in the heart and that these interactions play an important role in the development and progression of left ventricular remodeling. (Circulation. 2003;108:598-604.)

Key Words: hypertrophy • genes • heart failure • remodeling • apoptosis
(TNF) in the cardiac compartment.\textsuperscript{2–4} These lines of transgenic mice share many phenotypic characteristics that have been associated with increased expression of angiotensin II and/or increased expression of the angiotensin receptor type IA (AT\textsubscript{1A}), including concentric hypertrophy, myocardial fibrosis, and cardiac myocyte apoptosis. Accordingly, in the present study, we sought to characterize the specific nature and the functional consequences of the interaction between the individual components the RAS and TNF, in a line of transgenic mice\textsuperscript{6} with cardiac restricted overexpression of TNF.

**Methods**

**Generation and Characterization of Transgenic Mice (MHCsTNF) With Cardiac Restricted Overexpression of TNF**

We have shown previously that mice (bred in our laboratory) with targeted overexpression of TNF in the cardiac compartment (referred to as MHCsTNF) develop a concentric hypertrophic phenotype at 4 weeks of age that progresses to a dilated cardiac phenotype by 12 weeks of age.\textsuperscript{4} Accordingly, we characterized the RAS in the MHCsTNF mice at 4, 8, and 12 weeks of age. These studies were carried out according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, the principles of the NIH Guide for the Care and Use of Laboratory Animals, and the policies and procedures of Baylor College of Medicine.

**Renin, Angiotensinogen, ACE, and Angiotensin Receptor mRNA Levels in MHCsTNF Mice**

Levels of renin, angiotensinogen, ACE, and AT\textsubscript{1A} and angiotensin receptor type 2 (AT\textsubscript{2}) mRNAs were determined by ribonuclease protection assay (RPA). We also used reverse transcription–polymerase chain reaction (RT-PCR) to detect the presence of renin and AT\textsubscript{2} receptor mRNA, by use of the specific mouse primers. For these studies, we generated a series of mouse-specific cDNA templates by use of RT-PCR and published primer sequences (see online Data Supplement, available at http://www.circulationaha.org).

**Angiotensinogen Protein, ACE Activity, and Angiotensin I and Angiotensin II Peptide Levels in MHCsTNF Mice**

Angiotensinogen protein levels were determined in the hearts of littermate control and MHCsTNF mice at 4, 8, and 12 weeks of age by use of Western blot analysis, as described previously (see Data Supplement).\textsuperscript{3}

**Angiotensin Receptor Binding Assays in MHCsTNF Mice**

The density of AT\textsubscript{1A} receptors was determined by radioligand binding assay. Membrane preparations were prepared as described previously\textsuperscript{6} with minor modifications (see Data Supplement). All experiments were performed in triplicate. Receptor densities are expressed as femtomoles of sites per milligram of myocardial protein.

**Functional Effects of RAS Activation in MHCsTNF Mice**

To determine the functional significance of RAS activation in the MHCsTNF mice, we examined the effects of AT\textsubscript{1} receptor blockade in the MHCsTNF mice and littermate controls. The rationale for use of an AT\textsubscript{1} receptor blocker as opposed to an ACE inhibitor stemmed from preliminary control experiments that showed that there was significant chymase activation in the hearts of the MHCsTNF mice at 4, 8, and 12 weeks of age. On the basis of losartan dosing used successfully in previous studies, the mice were treated from 4 to 8 weeks of age with 30 µg/g body wt losartan (2-n-butyl-4-chloro-5-hydroxymethyl-1-[2-(1H-tetrazol-5-yl) biphenyl-4-yl]methyl imidazole, potassium salt) or diluent, which were added to the drinking water.\textsuperscript{7} The amount of water consumption was controlled daily to ensure consistency of dosing.

**Cardiac Hypertrophy**

Cardiac hypertrophy was assessed by determining the heart weight–body weight ratio in the MHCsTNF and littermate control mice after 4 weeks of diluent or losartan treatment (ie, at 8 weeks of age) and by examining LV mass by use of multislice, multiphase, cardiac cine-MRI to determine LV mass and LV wall thickness in the MHCsTNF and littermate mice at 8 weeks of age, exactly as described previously.\textsuperscript{8}

**Myocardial Fibrillar Collagen Content**

Perfusion-fixed hearts from MHCsTNF mice and littermate controls at 8 weeks of age were embedded in paraffin and stained with the picrosirius red technique, as described previously.\textsuperscript{9} The percent area of extracellular picrosirius red staining was computed from 20 random fields within the midmyocardium to exclude large epicardial arteries and/or veins and any cutting and/or compression artifacts.

**Cardiac Myocyte Apoptosis**

The prevalence of cardiac myocyte apoptosis was determined by use of the in situ DNA ligase technique, as described previously,\textsuperscript{10} in 8-week-old littermate control and MHCsTNF mice. To determine the fraction of myocyte nuclei that were labeled, we determined the total number of myocyte nuclei per unit area of the myocardium (10 000 µm\textsuperscript{2}) by enumerating the number of DAPI-stained myocyte nuclei; final results were expressed as (number of positively labeled nuclei/total number of DAPI-stained nuclei/10 000 µm\textsuperscript{2})×100%. For these studies, we examined a total of 24 fields/heart, taken at the midpapillary muscle level.

**Hemodynamic Effects**

To determine whether the dose of losartan that was used in these studies resulted in significant hemodynamic effects, we performed Millar catheterization, exactly as described previously.\textsuperscript{11} We studied wild-type mice that were treated with diluent and MHCsTNF mice that were treated with diluent or losartan (30 µg/g body wt).

**Myocardial TNF**

To determine whether the dose of losartan that was used in these studies resulted in changes in TNF levels in the MHCsTNF mice, we measured myocardial TNF levels, as described previously.\textsuperscript{4}

**Statistical Analysis**

Data are mean±SE. One-way ANOVA was used to test for differences in group means in angiotensin I peptide levels. Two-way ANOVA was used to evaluate mean differences in the level of ACE mRNA, ACE activity, angiotensin I and II peptide levels, angiotensinogen (mRNA and protein), and angiotensin receptors (mRNA and protein) in littermate control and MHCsTNF mice. When appropriate, post hoc ANOVA testing (Tukey test) was performed to test for differences for differences between littermate controls and MHCsTNF mice. Significant differences were said to exist at a value of \( P<0.05 \).

**Results**

**Renin and Angiotensinogen Expression in MHCsTNF Mice**

Figure 1A shows that renin mRNA was not detectable in hearts from the MHCsTNF mice or in littermate control mice at 4, 8, or 12 weeks of age. In contrast, mRNA levels were easily detectable in mouse kidney, which was used as a positive control.\textsuperscript{12} In addition, we used conventional RT-PCR to detect renin mRNA in the hearts of the MHCsTNF and littermate controls. This analysis showed that renin mRNA
was not detectable after 30 cycles of amplification. Figure 1B shows that angiotensinogen mRNA levels were easily detectable by RNAse protection assay in hearts of MHCsTNF and littermate control mice at 4, 8, and 12 weeks. The results of group data show that angiotensinogen mRNA levels were significantly lower \( (P < 0.001) \) in the hearts from MHCsTNF than littermate controls at 4, 8, and 12 weeks of age. Figure 1C shows that the bands corresponding to angiotensinogen protein (58 kDa) were detectable at 4, 8, and 12 weeks of age, whereas Figure 1D summarizes the results of group data. The important finding shown by Figure 1D is that the levels of angiotensinogen protein were significantly lower \( (P < 0.001) \) by ANOVA in the hearts from MHCsTNF than littermate controls at 4, 8, and 12 weeks of age.

ACE Activity and mRNA Levels in MHCsTNF Mice

Figure 2 shows 2 important findings. First, ACE mRNA levels were easily detectable in the hearts of the MHCsTNF and littermate control mice at 4, 8, and 12 weeks (Figure 2A). As shown by the group data in Figure 2B, ACE mRNA levels were significantly greater in hearts from MHCsTNF mice than in hearts from littermate controls \( (P < 0.001) \) by ANOVA) from 4 to 12 weeks of age; post hoc ANOVA showed that these differences were significantly different \( (P < 0.025) \) from values in littermate control mice at 4 and 8 weeks of age. Second, there was a striking time-dependent increase in ACE activity in the hearts of the MHCsTNF mice from 4 to 12 weeks of age, whereas there was a relatively smaller time-dependent increase in ACE activity in the hearts of the littermate control mice at comparable time points. Figure 2C shows that ACE activity was significantly greater overall \( (P < 0.001) \) in hearts from MHCsTNF mice and was significantly different \( (P < 0.05) \) from littermate controls at 4, 8, and 12 weeks of age (Tukey test).

Angiotensin I and II Peptide Levels in MHCsTNF Mice

Figure 3A shows myocardial angiotensin I peptide levels decreased significantly in the MHCsTNF mice \( (P < 0.003) \) and the littermate control mice \( (P < 0.001) \) in a time-dependent manner. However, there was no significant overall difference \( (P = 0.085) \) in angiotensin I levels between MHCsTNF and littermate mice. Figure 3B shows that myocardial angiotensin II peptide levels were significantly \( (P < 0.001) \) greater in the MHCsTNF mice compared with littermate controls from 4 to 12 weeks of age. These differences were statistically significant at 4 and 8 weeks of age \( (P < 0.05) \) by the Tukey test) but not at 12 weeks of age \( (P = 0.09) \). To
Angiotensin Receptor mRNA and Protein Levels in MHCsTNF Mice

Figure 4A shows that AT1A receptor mRNA was detectable in the hearts from MHCsTNF and littermate control mice, albeit to a lesser extent in the MHCsTNF mice. In contrast, levels of AT2 receptor mRNA were barely detectable in hearts from MHCsTNF and littermate control hearts. Importantly, AT2 receptor levels were readily detected in mouse testis and ovary samples, which were used as positive controls. In addition, we used conventional RT-PCR to detect AT1 mRNA in the hearts of the MHCsTNF and littermate controls. This analysis showed that AT1 mRNA was not detectable after 30 cycles. As shown by the group data in Figure 4C, there was a significant overall decrease in AT1 mRNA levels that were observed in the MHCsTNF and littermate control mice at 4, 8, and 12 weeks of age (n=5 samples per time period). A, Representative ribonuclease protection assay for AT1A, AT1B, and AT2 mRNA levels in hearts from MHCsTNF and littermate control mice at 4, 8, and 12 weeks of age. B, Results of group data for AT1A receptor numbers by radioligand binding assay (n=9 hearts/group). *P<0.05 vs age-matched control group by the Tukey test.

Functional Effects of RAS Activation in MHCsTNF Mice

Cardiac Hypertrophy

Analysis of the heart weight–to–body weight ratios in the MHCsTNF and littermate controls showed that there was a significant (P<0.05) increase in this ratio in MHCsTNF mice (5.4 ± 0.2 versus 4.2 ± 0.2 mg/g; n=8 per group) compared with littermate controls. Although treatment with losartan had no effect on the heart weight–to–body weight ratio in the littermate controls, this ratio was normalized (4.4 ± 0.1 mg/g) in the losartan-treated MHCsTNF mice and was not significantly (P<0.05) different from littermate controls. Analysis of LV structure by cardiac MRI yielded similar results. That is, treatment with losartan for 4 weeks resulted in a significant (P<0.05) decrease in the LV mass/body weight ratio in the MHCsTNF mice (3.33 ± 0.3) compared with diluent-treated animals (2.68 ± 0.12) and a significant decrease (P<0.05) in LV wall thickness in the MHCsTNF mice (1.22 ± 0.05 mm) compared with diluent-treated MHCsTNF mice (0.83 ± 0.03 mm). Interestingly, LV mass and LV wall thickness were completely normalized in the losartan-treated MHCsTNF mice compared with the diluent-treated littermate controls (P=0.96).

Collagen Content

To determine whether the RAS activation contributed to the myocardial fibrosis that we have observed in the MHCsTNF mice, we assessed total collagen content in MHCsTNF mice in the presence and absence of losartan treatment. The
Cardiac Myocyte Apoptosis

Figure 6, A–C, shows representative examples of ligase staining in the littermate (6A) and MHCsTNF mice treated with diluent (6B) or with losartan (6C). As shown, there was no detectable apoptosis in the littermate control mice, whereas there was detectable apoptosis in the diluent- and losartan-treated MHCsTNF mice. Figure 6G summarizes the results of group data and shows that treatment with losartan for 4 weeks reduced the prevalence of apoptosis in the MHCsTNF mice ($P<0.05$). However, the prevalence of cardiac apoptosis (0.6±0.1%) was still significantly higher in the losartan-treated MHCsTNF mice than in littermate controls.

Hemodynamic Effects

The Table shows that 30 μg/g body wt losartan had no significant effect on heart rate, arterial blood pressure, or LV pressures in the MHCsTNF mice compared with diluent-treated MHCsTNF mice and diluent-treated littermate controls.

Discussion

This study, in which we examined activation of the RAS in mice with targeted cardiac overexpression of TNF, shows for the first time that there is functionally significant cross-talk between the RAS and proinflammatory cytokines in the heart. Three major lines of information support this statement. First, there was a progressive time-dependent increase in myocardial ACE activity in the MHCsTNF transgenic mice compared with littermate controls (Figure 2). Importantly, the observed increase in ACE activity in the MHCsTNF mice was accompanied by a significant increase in myocardial angiotensin II peptide levels (Figure 3). Second, AT1 receptor mRNA and angiotensin receptor levels were downregulated in the MHCsTNF mice compared with littermate controls, consistent with homologous desensitization of the angiotensin receptors (Figure 4). Third, AT1 receptor blockade with losartan prevented the development of concentric hypertrophy and myocardial fibrosis in the MHCsTNF mice (Figure 5). Taken together, these observations suggest that sustained TNF signaling leads to progressive time-dependent activation of RAS and that RAS activation contributes importantly to the observed hypertrophic phenotype that has been observed consistently in transgenic mice with targeted overexpression of TNF.2–4

Cross-Talk Between the RAS and Proinflammatory Cytokines

Although important interactions between proinflammatory cytokines and the adrenergic system have been recognized in the heart for more than a decade,16 the interaction between proinflammatory cytokines and the RAS has not been examined formally in the heart. In nonmyocyte cell types, the interaction between TNF and RAS has not been examined formally in the heart. However, the observed hypertrophic phenotype that has been observed consistently in transgenic mice with targeted overexpression of TNF.2–4

Figure 5. Effect of angiotensin receptor antagonism on myocardial fibrillar collagen content in MHCsTNF and littermate control mice. A, Representative picrosirius red staining of myocardial samples from littermate controls, diluent-treated MHCsTNF mice (Dil), and losartan-treated (Los) (4 weeks) MHCsTNF mice. In these sections, myocardial collagen appears red, and myocardium appears green. B, Summary of results of group data for fibrillar collagen content (expressed as percentage of myocardial area), diluent-treated MHCsTNF mice, and losartan-treated MHCsTNF mice (n=10 hearts/group). *$P<0.05$ vs littermate control, †$P<0.05$ vs MHCsTNF control.

Figure 6. Effect of angiotensin receptor antagonism on prevalence of apoptosis in MHCsTNF and littermate control mice. Cardiac myocyte apoptosis was detected by in situ DNA ligase technique. Total number of cardiac myocyte nuclei per section was determined by DAPI counter staining. A–C, Representative examples of ligase staining in littermate and MHCsTNF mice treated with diluent (Dil) or with losartan (Los), respectively; D–F, corresponding DAPI staining in littermate and MHCsTNF mice treated with diluent or with losartan, respectively. Ligase-positive nuclei appear bright green; cardiac myocyte nuclei appear blue. G, Results of group data in MHCsTNF and littermate control mice (n=5/group) depicting prevalence of cardiac myocyte apoptosis, determined as ratio of ligase-positive cardiac myocytes/total number of myocyte nuclei per unit area (10 000 μm) of myocardium. *$P<0.05$ vs littermate control, †$P<0.05$ vs diluent-treated MHCsTNF mice.
and adrenal gland in the rat. Conversely, TNF inhibits the synthesis and release of renin in human decidual cells and downregulates ACE activity in isolated endothelial cells. Thus, the nature of the interaction between RAS and proinflammatory cytokines is cell- and tissue type–dependent.

Here, we show that targeted overexpression of TNF in the cardiac compartment leads to selective activation of components of the myocardial RAS system. That is, the increase in RAS activity in the MHCsTNF mice was principally the result of increased ACE activity, as opposed to increased activation of the more proximal components of RAS, namely renin and angiotensinogen. Indeed, renin was not detectable in the hearts of the MHCsTNF mice either by RPA or by PCR. In contrast to studies in which acute TNF stimulation has been shown to activate angiotensinogen, we observed a decrease in angiotensinogen mRNA and protein after chronic stimulation with TNF (Figure 1B and 1C). Finally, we cannot exclude the possibility that there was increased myocardial scavenging of renin and/or angiotensin I in the hearts of the MHCsTNF mice, insofar as the angiotensin I peptide levels were similar in the MHCsTNF and littermate control mice (Figure 2), whereas the levels of angiotensinogen were lower in the MHCsTNF mice.

The results of this study also show that activation of the RAS in the MHCsTNF mice is functionally significant. Although we and others have reported that acute stimulation with TNF provokes a modest increase in overall protein synthesis in isolated cardiac myocytes, the results of the present study suggest that selective AT1 receptor blockade completely abrogates the concentric hypertrophy phenotype in the MHCsTNF mice in vivo. These findings were not secondary to changes in hemodynamic loading conditions, which were not significantly different in the losartan- and diluent-treated MHCsTNF mice. Nonetheless, because we examined the effects of selective AT1 receptor blockade at a single time point (8 weeks), we cannot exclude the possibility that TNF may have contributed independently to the development of cardiac hypertrophy at earlier or later time points. Indeed, given that both TNF and angiotensin II signal through unique and common mitogen-activated signaling pathways, it is likely that these 2 molecules act synergistically during the process of LV remodeling. In this regard, it was interesting to note that AT1 receptor blockade only partially abrogated the development of cardiac myocyte apoptosis in the MHCsTNF mice (Figure 6), suggesting that not all aspects of the cardiac phenotype in the MHCsTNF mice were mediated by crosstalk between the RAS and proinflammatory cytokines. Finally, the results of these studies must be viewed with the understanding that although transgenic systems offer advantages in terms of studying longitudinal changes in cardiac phenotype, transgenic model systems may increase the expression of proteins beyond those that are found in pathophysiological contexts and thus may not necessarily reflect the biological response observed in clinical pathological contexts.

### Conclusions

As noted at the outset, the activation of proinflammatory cytokines and the RAS represents a teleologically conserved acute-phase response that is intended to maintain critical homeostatic responses in the host. An extension of the foregoing argument is that this type of cross-regulation may lead to deleterious forward-feed back loops that can accentuate the development of disease progression within a given tissue, particularly in the setting of chronic activation. And indeed, the results of this study in cardiac tissue and other studies in the kidney suggest that the interaction between these 2 systems plays an important role in the resulting disease phenotype. Accordingly, one of the important unanswered questions that arises from the present study will be to delineate the downstream signaling pathways that are both unique and common to RAS and inflammatory cytokines, with the intent of better understanding the important homeostatic and the deleterious interactions that occur between these 2 teleologically conserved biological systems.

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### References


### Table: Effect of AT1 Receptor Blockade With Losartan on Hemodynamics

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<th>Wild-Type (Diluent; n=6)</th>
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<th>MHCsTNF (Losartan; n=9)</th>
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LVESE indicates LV end-systolic pressure; LVESD, LV end-diastolic pressure. All data are mean±SEM. Wild-type mice were treated with diluent, and MHCsTNF transgenic mice were treated with diluent or losartan (30 μg/kg), from 4 to 6 weeks of age. Measurements of arterial blood pressure and LV pressures were performed with Millar catheters. All data were analyzed by 1-way ANOVA.
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