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

Markus Flesch, MD; Anje Höper, MD; Louis Dell’Italia, MD; Kenda Evans, PhD; Richard Bond, PhD; Ronald Peshock, MD; Abhinav Diwan, MBBS; Theresa A. Brinsa, BS; Chih-Chang Wei, PhD; Natarajan Sivasubramanian, PhD; Francis G. Spinale, MD, PhD; Douglas L. Mann, MD

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

One of the recent conceptual advances in our understanding of the pathogenesis of left ventricular (LV) remodeling has been the insight that the remodeling process is driven by the overexpression of portfolios of biologically active molecules, including (but not limited to) norepinephrine, angiotensin II, and proinflammatory cytokines. When these molecules are expressed at sufficiently high concentrations and/or for prolonged periods of time, they contribute directly to the remodeling process by virtue of their direct pathophysiological effects on cardiac myocytes, noncardiac myocytes, and the extracellular matrix (reviewed by Mann1). Whereas the classic neurohumoral and cytokine systems have heretofore been regarded as functionally distinct biological systems, there is growing evidence in noncardiac cell types that these 2 systems are not independent but rather represent integral components of a tightly coordinated and teleologically conserved “acute-phase” host response. For example, in systemic sepsis, the elaboration of proinflammatory cytokines would be beneficial to the host by virtue of activating components of the innate and adaptive immune system, whereas activation of the renin-angiotensin system (RAS) would be beneficial to the host by offsetting the vasodilatory actions of inflammatory mediators and thereby maintaining cardiovascular homeostasis. Thus, it is likely that nature has developed and retained mechanisms that would allow proinflammatory cytokines and RAS to cross-regulate each other.

Recently, we and others have developed lines of transgenic mice with targeted overexpression of tumor necrosis factor...
(TNF) in the cardiac compartment. 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 1A (AT1A), 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 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. 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 AT1A and angiotensin receptor type 2 (AT2) 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 AT2 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).

Angiotensin Receptor Binding Assays in MHCsTNF Mice

The density of AT1A receptors was determined by radioligand binding assay. Membrane preparations were prepared as described previously, 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 AT1 receptor blockade in the MHCsTNF mice and littermate controls. The rationale for use of an AT1 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/kg body wt losartan (2-n-butyl-4-chloro-5-hydroxymethyl-1-[2-{'1H-tetrazol-5-yl}bibiphenyl-4-yl]methyl)imidazole, potassium salt) or diluent, which were added to the drinking water. The amount of water consumption was controlled daily to ensure consistency of dosing.

Cardiac Hypertrophy

Cardiac hypertrophy was assessed by determining the heart weight–to–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.

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. 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 ligation technique, as described previously. 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.

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. 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
determine whether the decrease in angiotensin II peptide levels that were observed in the MHCsTNF and littermate control mice was related to the observed decrease in angiotensin I peptide levels, we performed a linear regression analysis for angiotensin I and II levels at 4, 8, and 12 weeks of age. This analysis showed that there was a significant linear relationship between angiotensin II and angiotensin I peptide levels in the hearts from the MHCsTNF (r = -0.77, P < 0.01) and littermate control mice (r = -0.70, P < 0.01), suggesting that the formation of angiotensin II is related to the amount of ACE substrate, as has been reported previously.13

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.14 In addition, we used conventional RT-PCR to detect AT2 mRNA in the hearts of the MHCsTNF and littermate controls. This analysis showed that AT2 mRNA was not detectable after 30 cycles. As shown by the group data in Figure 4C, there was a significant overall decrease (P = 0.001) in AT1A receptor mRNA levels in the MHCsTNF mice compared with littermate controls at 4, 8, and 12 weeks of age. In addition, we measured angiotensin receptor binding levels in the MHCsTNF and littermate control mice at 8 weeks of age. Although the absolute levels of specific binding were relatively low, consistent with what other investigators have reported in murine hearts,15 there was a significant (P = 0.04) decrease in [125I] (Sar-1, Ile-8) angiotensin II receptor binding in the MHCsTNF mice compared with littermate controls (Figure 4D).

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). Importantly, treatment with losartan had no effect on LV mass or wall thickness in the littermate control mice (data not shown).

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. In nonmyocyte cell types, the interaction between TNF and 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. In nonmyocyte cell types, the interaction between TNF and RAS has not been examined formally in the heart.

References

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 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 cross-talk 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.

**Acknowledgments**

This research was supported by research funds from the National Institutes of Health (P50-HL-O6H, RO1-HL-58081-01, RO1-HL-61543-01, and HL-42250-10/10). Dr Flesch was supported by a personal grant from the Deutsche Forschungsgemeinschaft (FL 338/1-1). We gratefully acknowledge Dorellyn Lee-Jackson and Stacey Walker for their indefatigable technical assistance and Mary Helen Soliz for secretarial assistance. We thank Gilberto DeFreitas and Dr Masakuni Ishiyama for performing the hemodynamic studies.

**References**

Activation and Functional Significance of the Renin-Angiotensin System in Mice With Cardiac Restricted Overexpression of Tumor Necrosis Factor
Markus Flesch, Anje Höper, Louis Dell'Italia, Kenda Evans, Richard Bond, Ronald Peshock, Abhinav Diwan, Theresa A. Brinsa, Chih-Chang Wei, Natarajan Sivasubramanian, Francis G. Spinale and Douglas L. Mann

Circulation. 2003;108:598-604; originally published online July 21, 2003;
doi: 10.1161/01.CIR.0000081768.13378.BF
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/108/5/598

Data Supplement (unedited) at:
http://circ.ahajournals.org/content/suppl/2003/08/04/01.CIR.0000081768.13378.BF.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/