β-Blockade Prevents Sustained Metalloproteinase Activation and Diastolic Stiffening Induced by Angiotensin II Combined With Evolving Cardiac Dysfunction

Hideaki Senzaki, Nazareno Paolocci, Yehezkiel A. Gluzband, Merry L. Lindsey, Joseph S. Janicki, Michael T. Crow, David A. Kass

Abstract—Angiotensin II (Ang II)–mediated sympathostimulation may worsen the progression of cardiac failure, although the nature and mechanisms of such interactions are largely unknown. We previously demonstrated that Ang II combined with evolving cardiodepression (48-hour tachycardia pacing, 48hP) induces marked chamber stiffening and increases metalloproteinases (MMPs). Here, we test the hypothesis that both abnormalities stem from sympathostimulatory effects of Ang II. Forty-eight dogs were instrumented to serially assess conscious ventricular mechanics, MMP abundance and activity, and myocardial histopathology. 48hP combined with 5 days of Ang II (15±5 ng·kg⁻¹·min⁻¹ IV) more than doubled chamber stiffness (end-diastolic pressure >25 mm Hg, *P*<0.001), whereas stiffness was unchanged by Ang II or 48hP alone. In vitro and in situ zymography revealed increased MMP abundance and activity (principally 92-kDa gelatinase) from Ang II+48hP. Both stiffening and MMP changes were prevented by cotreatment with high-dose atenolol (which nearly fully inhibited isoproterenol-induced inotropy) but not partial β-blockade. Myocellular damage with fibroblast/neutrophil infiltration from Ang II+48hP was also inhibited by high- but not low-dose atenolol, whereas collagen content was not elevated with either dose. These data support a role of sympathostimulation by Ang II in modulating myocardial MMP abundance and activity and diastolic stiffening in evolving heart failure and suggest a novel mechanism by which β-blockade may limit chamber remodeling and diastolic dysfunction. (Circ Res. 2000;86:807-815.)

Key Words: angiotensin II • heart failure • metalloproteinase • diastole • β-receptor blocker

Dilated cardiomyopathy remains a leading cause of morbidity and mortality, resulting in nearly 1 million hospitalizations per year in the United States alone.1 The disease is initiated by a myocardial insult resulting in a sustained incapacity to deliver adequate cardiac output and systolic pressure. However, it is the complex interplay of primary dysfunction with reactive neurohumoral stimulation and molecular signaling that ultimately worsens function and leads to progressive remodeling and the induction of counterproductive molecular responses.2,3 An appreciation for such interactions has refocused therapeutic efforts over the past decade from agents targeting hemodynamics to those inhibiting sympathostimulatory effects on the myocardium. Direct effects in normal tissue include positive inotropic and hypertrophic signaling,4−11 whereas in failing hearts, the response reportedly switches to negative inotropy and lusitropy.12 Sympathostimulatory effects stem from presynaptic and postsynaptic modulation of norepinephrine (NE) and baroreflex modulation.13−16 This pathway may also be important, because previous studies have shown that Ang II–mediated myocardial tissue damage in rats is inhibited by propranolol.17,18

Ang II also influences the extracellular matrix by altering collagen19−21 and the abundance and activity of metalloproteinases (MMPs).19−22 Increased MMPs are reported in late-stage experimental and human heart failure23−25 and may play a role in chamber remodeling and diastolic decompensation. In this regard, we recently reported that combining exogenously administered Ang II with evolving cardiodepression induced by 48 hours of tachycardia pacing (Ang II+48hP) stimulated MMPs and also markedly exacerbated diastolic stiffening.22 Whether this synergistic interaction and MMP

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807
change were a result of direct Ang II–mediated effects or of toxicity related to sympathostimulation remains unknown. Accordingly, the present study tested the hypothesis that β-blockade can offset both diastolic stiffening and increased MMP abundance and tissue activity from Ang II + 48hP. The results reveal substantial interplay between Ang II, β-adrenergic activation, cardiodepression, and MMP activity and support a major role of adrenergic signaling in Ang II–mediated diastolic dysfunction with evolving heart failure. They further support a novel mechanism by which β-blockade may ameliorate chamber remodeling and improve diastolic function in heart failure by countering Ang II–modulated sympathostimulation.

Materials and Methods

Animal Preparation

Forty-eight adult mongrel dogs of either sex (45 to 65 lb) were studied. The protocol and procedures were approved by the Animal Care and Use Committee of the Johns Hopkins Medical Institutions. Dogs were chronically instrumented to measure right atrial and left ventricular cavity pressure and left ventricular anteroposterior dimension and to perform transient inferior vena cava occlusion to assess left ventricular pressure-dimension relations. Rapid right ventricular pacing was achieved with a programmable stimulator (Spectrax, Medtronics). Exogenous intravenous Ang II was provided by osmotic pump (Alzet 2 ML1). Details of the preparation have been reported.

Protocol

Five animal groups were studied. Group 1 animals (n = 5) were exposed to 6 to 7 days of Ang II infusion (15.3 ± 4.5 ng·kg⁻¹·min⁻¹ in 0.01N acetic acid), with right ventricular tachypacing (240 bpm) superimposed during the final 48 hours. The Ang II dose yields plasma levels of 150 to 200 pg/mL, similar to human and experimental heart failure. Group 2 animals received 1 week of oral atenolol before and during the Ang II infusion at a total magnification of 400×. Myocyte necrosis and PSR staining for collagen content were graded qualitatively by an individual blinded to the conditions underlying the biopsy.

Statistical Analysis

Data are presented as mean ± SEM. Within-group comparisons were made by paired t test. Between-group analysis was performed by ANOVA with a post hoc Tukey test. Histopathology scores were assessed by Kruskal-Wallis test.

Results

Baseline Data and Dose-Dependent Effects of Chronic β-Adrenergic Blockade

Baseline hemodynamic parameters were very similar among the 5 study groups (Table 1). To assess the efficacy of β-receptor blockade, the inotropic response to isoproterenol was evaluated in each dog. In low-dose atenolol–treated animals, there was a 41% decline in maximal isoproterenol (0.80 ± 0.09 μg·kg⁻¹·min⁻¹ IV) inotropic response, assessed by a dp/dtmax normalized to EDD (to adjust for preload change) (Figure 1). Inotropic stimulation was nearly totally blocked in animals receiving the higher dose. Figure 1 also shows that baseline dp/dtmax/EDD rose modestly after 1 week of low-dose atenolol and declined to a similar extent in high-dose–treated animals (both P < 0.05). The latter was accompanied by a ≈ 23± 4.8% decline in basal HR.

![Figure 1. Effect of 1 week of low-dose (left) versus high-dose (right) atenolol on isoproterenol-induced inotropic reserve. With low dose, the response declined partially, whereas it was essentially fully blocked at the higher dose. *P < 0.005 vs control response; #P < 0.005 vs baseline response; ∆P < 0.05 vs low-dose response; †P < 0.05 vs pre–β-blocker control.](http://circres.ahajournals.org)
Synergy Between Ang II and 48hP

Figure 2A displays example pressure-dimension relations for animals exposed to Ang II combined with 48hP. This interaction resulted in systolic depression similar to that induced by 48hP alone but markedly increased diastolic stiffening (elevated chamber stiffness \([\text{ESP}, \%]\)) and EDP (Figure 2B) once 48hP was superimposed. Lysis declined to near control levels in high-dose-atenolol–treated hearts (3 right lanes). In contrast, hearts treated with low-dose atenolol revealed persistent lysis in the MMP-9 region and in some instances, in the region in which human MMP-2 migrated (also identified in positive control lane), similar to that observed without \(\beta\)-blockade \(^{22}\) (\(P=0.03\) versus high-dose, \(n=7\) in each group).

In Situ Zymography

Figure 4 displays typical results of in situ zymography. Control tissue (Figure 4a) displayed minimal gelatin digestion, resulting in a uniform dark background with blue-stained nuclei. In contrast, Ang II+48hP tissue (Figure 4b) showed substantial digestion, evidenced by the appearance of green fluorescence. Positive staining was blocked by coinuculation of the same tissue with EDTA (Figure 4c), a nonspecific inhibitor of MMPs, and also was substantially reduced by coincubation with MMP-9–blocking antibody (Figure 4d). Together with the in vitro analyses, these data support increased MMP abundance and tissue activity, and in particular, activity from MMP-9 in this model. Results for hearts exposed to low- or high-dose atenolol are also shown. High-dose \(\beta\)-blockade (Figure 4e) inhibited in situ gelatin lysis, whereas low-dose \(\beta\)-blockade did not (Figure 4f).

### TABLE 1. Baseline Data for 5 Animal Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>HR, bpm</th>
<th>ESP, mm Hg</th>
<th>FS, %</th>
<th>(dP/dt_{max}), mm Hg/s</th>
<th>MSW, mm Hg</th>
<th>EDP, mm Hg</th>
<th>Tau, ms</th>
<th>(\beta), mm Hg/mm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>125.8±5.1</td>
<td>96.4±7.2</td>
<td>24.0±1.1</td>
<td>2339±131</td>
<td>76.9±3.4</td>
<td>11.3±1.2</td>
<td>32.7±1.1</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Group 2A</td>
<td>122.9±5.0</td>
<td>125.1±5.0</td>
<td>20.1±2.0</td>
<td>2760±277</td>
<td>75.1±4.5</td>
<td>14.3±1.7</td>
<td>31.4±2.4</td>
<td>0.20±0.03</td>
</tr>
<tr>
<td>Group 2B</td>
<td>124.0±6.7</td>
<td>121.7±3.7</td>
<td>25.1±1.9</td>
<td>2930±188</td>
<td>75.7±7.1</td>
<td>14.2±1.3</td>
<td>27.5±3.3</td>
<td>0.18±0.02</td>
</tr>
<tr>
<td>Group 3</td>
<td>119.2±4.6</td>
<td>121.7±3.7</td>
<td>24.8±1.9</td>
<td>2984±128</td>
<td>84.3±4.8</td>
<td>15.1±1.8</td>
<td>33.1±1.7</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>Group 4</td>
<td>119.2±4.6</td>
<td>121.7±3.7</td>
<td>25.1±1.9</td>
<td>2830±150</td>
<td>73.4±3.9</td>
<td>12.6±1.1</td>
<td>32.1±2.2</td>
<td>0.22±0.02</td>
</tr>
</tbody>
</table>

HR indicates heart rate; ESP, end-systolic pressure; FS, fractional shortening; \(M_{\text{sw}}\), slope of stroke work–EDD relation; \(\beta\), chamber stiffening; EDP, end-diastolic pressure; and tau, isovolumic relaxation time constant.

### In Vitro Zymography

Figure 3 shows gelatin zymograms from atenolol-treated animals. Baseline tissue (B) displayed minimal gelatin lysis, indicating low levels of MMP expression and activation in normal canine hearts. The upper gel shows typical changes after 1 week of low- or high-dose atenolol before and after the addition of 4 days of Ang II infusion. Gelatin lysis in the regions comigrating with human MMP-9 (indicated by positive controls) was consistently observed in each \(\beta\)-blocker+Ang II lane and appeared as a doublet, consistent with zymogen activation (lower-molecular-weight activated enzyme). This was not present in control or \(\beta\)-blocker-only–treated tissue (\(P<0.01\) for both dose groups, total \(n=21\)). In separate analysis, we compared gel lysis with \(\beta\)-blocker+Ang II to that with Ang II only \(^{22}\) and found no significant change with \(\beta\)-blockade.

In contrast to the results with Ang II alone, high-dose atenolol had a marked inhibitory effect on gelatin lysis (Figure 3, bottom) once 48hP was superimposed. Lysis declined to near control levels in high-dose-atenolol–treated hearts (3 right lanes). In contrast, hearts treated with low-dose atenolol revealed persistent lysis in the MMP-9 region and in some instances, in the region in which human MMP-2 migrated (also identified in positive control lane), similar to that observed without \(\beta\)-blockade \(^{22}\) (\(P=0.03\) versus high-dose, \(n=7\) in each group).

### TABLE 2. Change in Systolic and Diastolic Left Ventricular Function From Combined Ang II+48hP

<table>
<thead>
<tr>
<th></th>
<th>Ang II+48hP</th>
<th>Ang II (1 wk)</th>
<th>48hP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESP, mm Hg</td>
<td>-4.8±4.7</td>
<td>20.9±5.3*</td>
<td>-25.5±5.0*</td>
</tr>
<tr>
<td>FS, %</td>
<td>-7.8±0.6*</td>
<td>-0.8±0.9</td>
<td>-5.47±1.1*</td>
</tr>
<tr>
<td>(dP/dt_{max}), mm Hg/s</td>
<td>-564±136*</td>
<td>168±198</td>
<td>-1066±133*</td>
</tr>
<tr>
<td>(M_{\text{sw}}), mm Hg</td>
<td>-22.3±4.5*</td>
<td>3.2±6.8</td>
<td>-23.9±4.3*</td>
</tr>
<tr>
<td>Diastolic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDP, mm Hg</td>
<td>10.6±1.9*</td>
<td>2.8±0.5*</td>
<td>-0.17±1.5</td>
</tr>
<tr>
<td>EDD, mm</td>
<td>0.8±3.2</td>
<td>0.1±0.7</td>
<td>-0.9±0.9</td>
</tr>
<tr>
<td>(\beta), mm Hg/mm</td>
<td>0.16±0.03*</td>
<td>0.03±0.03</td>
<td>0.01±0.02</td>
</tr>
<tr>
<td>Tau, ms</td>
<td>12.3±2.4*</td>
<td>3.7±2.8</td>
<td>7.0±1.2*</td>
</tr>
</tbody>
</table>

Abbreviations as in Table 1. Data are presented as absolute change from baseline (compare Table 1). Comparison results from animals exposed solely to Ang II or 48hP are also provided.

*\(P<0.05\) vs baseline.
Similar results were confirmed in 3 to 4 samples for each condition. In situ zymography of biopsies exposed to Ang II and atenolol (ie, before 48hP) were also positive, commensurate with the in vitro assay (data not shown). Thus, gel lysis observed by in vitro zymography correlated with MMP activity in tissues examined by in situ assay.

**Collagen Staining and Cellular Histology**

Both Ang II alone and Ang II + 48hP induced myocardial tissue damage, characterized by patchy myocyte necrosis with neutrophil and/or fibroblast infiltration,22 similar to that reported in rat hearts.17 This damage was significantly inhibited by high-dose but not low-dose β-blockade. Figure 5A displays tissue from a low-dose group after 4 days of Ang II exposure, revealing necrotic damage (arrow) and corresponding fibrosis (Figure 5B). Myocardial damage persisted with superimposition of tachypacing (P=0.02, data not shown); however, collagen staining consistently declined (Figure 5C). This is intriguing, given that diastolic stiffening was observed principally during this period. Figure 5D through 5F displays analogous data from a heart treated with high-dose atenolol. There was generally less myocardial damage with Ang II and Ang II + 48hP (Figure 5D) and less fibrosis. Summary histology and collagen scores are provided in Figure 5G. Fibrosis was greatest in group 2A, exposed to Ang II before the onset of tachypacing (and diastolic stiffening), with less collagen observed in both low- and high-dose-atenolol–treated tissue after superimposition of 48hP.

**Discussion**

Adverse consequences from adrenergic and renin-angiotensin stimulation in heart failure are well recognized, yet the mechanisms for this interaction and cross-talk between the systems remain less well understood. The present study
provides novel information in this regard. We found that marked exacerbation of diastolic dysfunction induced by combining Ang II infusion with 48hP was prevented by β-receptor blockade. This normalization of chamber stiffness was accompanied by inhibition of myocardial MMP abundance and activation. Use of a blocking antibody to MMP-9 in situ zymography highlighted this protein in particular, although other MMP species are likely also involved. In contrast, net collagen deposition correlated poorly with diastolic stiffening. These are the first data to confirm MMP activation in situ with Ang II–modulated cardiac dysfunction, and they support a novel link between this change and Ang II–mediated sympathostimulation. These results suggest a novel mechanism by which β-blockade may limit remodeling and improve function of the failing heart.

Myocardial Effects of Ang II
Elevation of plasma and myocardial Ang II is a common feature of severe, late-stage cardiac failure. All the necessary enzymes for generating Ang II exist in the myocardium and appear enhanced in heart failure. Chamber distension may be important in this regard, because cell stretch can itself increase the expression of a broad range of renin-angiotensin system genes in neonatal myocytes. Ang II has potent effects on normal myocytes that acutely enhance contractile function. These include phospholipase C–mediated Ca2+ release and myofilament Ca2+ sensitization and sympathomimetic effects via AT1 receptor binding to presynaptic nerve terminals. The latter enhances NE release relative to efferent nerve activity and lowers NE reuptake. Ang II can also modulate the baroreflex and thus trigger sympathostimulation. Sustained Ang II activates protein kinase C and cellular alkalization, increasing Ca2+ by Na+-Ca2+ exchange.

In normal hearts, the net result of acute or 4- to 7-day Ang II exposure is an increase in systolic function, with little to no change in diastolic properties. However, Ang II induces quite different responses in hearts with established or early-evolving cardiac dysfunction. Cheng et al reported that both failing hearts and myocytes exposed to markedly elevated Ang II levels develop systolic depression and worsened diastolic function, in contrast to normal tissues. Furthermore, we recently reported that whereas 1 week of Ang II had negligible effects on diastolic properties of normal hearts, when combined with 48hP, the result was marked synergistic exacerbation of chamber stiffness, with EDPs often exceeding 30 mm Hg.

The present study demonstrates that sympathostimulation is central in modulating the Ang II–48hP synergy. The ability of high-dose but not low-dose atenolol to inhibit this synergy may have related to incomplete blockade by the latter and/or to loss of β1 versus β2 selectivity and thus more comprehensive antagonism with the higher dose. A key element of this synergy was the superimposition of 48hP. Cardiac failure is associated with reduced efficiency of NE reuptake and increased neuronal release, and both contribute to higher NE drive and gradual depletion of myocardial NE stores. Under these conditions, AT1 receptor binding might further elevate NE, exacerbating catecholamine myotoxicity. Even 1 day of tachypacing has been shown to influence myocardial adrenergic signaling, reducing high-affinity binding receptors and lowering adenylyl cyclase while increasing NE stimulatory drive. Although further reduction of adrenergic signaling by more advanced failure might be anticipated to limit sympathotoxicity, we found that near-total β-blockade was necessary (ie, high-dose atenolol) to inhibit it. Even in severe heart failure, downregulation more compatible with low-dose atenolol data is generally observed.

Myocardial tissue is induced by Ang II infusion alone in normal rat hearts, and this is inhibited by β-adrenergic blockade. However, as shown in this study, this combination did not correlate with systolic or diastolic dysfunction in otherwise normal hearts. However, once 48hP was instituted, persistence of these changes in the low-dose group did correlate with worsened diastolic dysfunction. This probably reflects additional sympathetic-mediated myotoxicity.

Effects of Ang II on the Interstitium
In addition to myocyte effects, Ang II has potent influences on the cardiac interstitium mediated principally via the AT1 receptor on fibroblasts. Ang II stimulates fibroblasts in culture to increase types I and III collagen synthesis and

![Gelatin zymography in vitro assay. Top, Effect of Ang II infusion superimposed on pretreatment with low- and high-dose atenolol. M indicates molecular weight marker lane; +, positive control lane for human MMP-9 and MMP-2. Baseline (B) and β-blocker–treated tissues (β) displayed minimal lysis. In contrast, Ang II–atenolol–treated hearts (β+A) displayed increased lysis, consistent with a 92-kDa gelatinase (MMP-9). This was observed similarly in both atenolol dose groups. Bottom, Effect of Ang II–48hP on gelatinases. Substantial gel lysis was observed in all low-dose group tissues [Ang II–48hP+β(L)], whereas this was inhibited with high-dose atenolol [Ang II–48hP+β(H)].](http://circres.ahajournals.org/)

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reduce MMP1 (interstitial collagenase) activity. AT2-receptor binding may inhibit this cascade, because Ang II–mediated collagen synthesis nearly doubles in the presence of AT2-receptor blockade. The present data also revealed Ang II–mediated fibrosis, primarily in the low-dose atenolol group, and similar to data in nontreated animals, but this was not observed when chamber stiffening was most marked. Rather, collagen deposition declined with superimposition of 48hP despite chamber stiffening. This suggests that changes in the tertiary structure and/or extracellular environment (ie, collagen turnover, MMP activation) may be more important than absolute collagen content.

Only a few recent studies have examined the role of MMPs in cardiac failure, and little is currently known about the mechanisms or physiological consequences of their activation. Elevated MMP expression in human failure was reported by Gunja-Smith et al, who found increases in association with reduced TIMP-1, and by Thomas et al, who reported marked increases in MMP-3 and MMP-9 with increases in TIMP-1. Experimental models of failure, including the tachypacing model, have also reported increased MMP abundance by zymography and immunoblot. Such MMP activation may play a role in cardiac remodeling, as recently suggested by the ability of an MMP inhibitor to limit murine infarct dilation. MMP activation had minimal impact by itself on global chamber function. However, the persistence of activity during Ang II+48hP was associated with diastolic stiffening. The present data are consistent with a linkage between these behaviors, in that high-dose atenolol substantially inhibited both. Ang II also enhances coronary vascular permeability associated with increased gelatinase and thus could potentially contribute to myocardial edema. Only small increases of interstitial water content can greatly increase chamber stiffness. Furthermore, MMPs can degrade proteoglycans and mucopolysaccharides (such as hyaluronidate), molecules that become highly hydrophilic when structurally uncoiled. This could serve as an interstitial sponge contributing to water retention and diastolic stiffening. Altered collagen cross-linking might also play a role. Sustained MMP activation during 48hP might therefore influence diastolic properties by providing an abnormal extracellular environment with which the myocytes interact, and as myocyte function declined, this could play a greater role.
Experimental Limitations
Given the complex chronic preparation involved in these studies, we did not perform catecholamine spillover studies with radiolabeled tracers and coronary sinus and arterial blood sampling. One would predict a substantial rise in spillover associated with Ang II, and even more so with Ang II superimposed with cardiac depression from pacing. As noted earlier, heart failure and Ang II both enhance NE release and diminish neuronal uptake,13–15,36,37 so their interaction may be particularly potent.

In vitro zymography was useful for identifying the presence of MMPs but was not ideal for determining their activation or the precise species involved. Although immunoblotting can resolve the latter issue, we instead performed in situ zymography to identify particular species (ie, MMP-9) with blocking antibody and to yield key information regarding tissue activation. However, some other MMPs, such as membrane-bound species, and extracellular matrix inducer protein57 might be upregulated in this model, and these changes would not be assayed by either approach. Clarifications of these issues await further study.

Conclusions
In conclusion, we have shown that synergistic antagonism of diastolic chamber dysfunction and activation of myocardial MMPs from Ang II combined with evolving cardiac depression are due to sympathostimulation. The data further suggest a novel mechanism by which β-blockade may limit chamber remodeling and improve diastolic dysfunction by offsetting Ang II–mediated toxicity.

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