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. Myocardial 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. 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. 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, whereas in failing hearts, the response reportedly switches to negative inotropy and lusitropy. Sympathostimulatory effects stem from presynaptic and postsynaptic modulation of norepinephrine (NE) and baroreflex modulation. This pathway may also be important, because previous studies have shown that Ang II–mediated myocardial tissue damage in rats is inhibited by propranolol.

Ang II also influences the extracellular matrix by altering collagen and the abundance and activity of metalloproteinases (MMPs). Increased MMPs are reported in late-state experimental and human heart failure 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. Whether this synergistic interaction and MMP...
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=13) 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 supine levels of 150 to 200 pg/mL,22 similar to human and experimental heart failure.23 Group 2 animals received 1 week of oral atenolol before and during the Ang II + 48hP protocol at 2 doses: 0.18±0.01 g/d atenolol (low-dose group 2A, n=8) and 2.8±0.5 g/d (high-dose group 2B, n=9). Additional control groups included dogs undergoing 48-hour tachycardia pacing (48hP) only (group 3, n=12) and those exposed only to 7 days of Ang II (group 4, n=6). Data from groups 1, 3, and 4 have been reported previously.22 Hemodynamics were recorded in conscious animals, with pacing suspended at least 30 minutes before study. Left ventricular endomyocardial biopsies were obtained serially for tissue analysis.

Statistical Analysis

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/dt max 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/dt max/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.

Results

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

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Histological Analysis

Formalin-fixed endocardial biopsies were embedded in paraffin, and 5-μm serial sections were stained with hematoxylin-eosin and the collagen-specific stain picrosiris red F3BA (PSR). Histological examination was performed blinded to protocol as described in Henegar et al.21 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.

In Vitro and In Situ Zymography

MMP abundance was assessed by in vitro gelatin zymography as previously described. Ten to 40 μg total protein was loaded onto 10% polyacrylamide gels containing 0.1% gelatin (Novex Chemical), and after electrophoresis, gels were stained with 0.5% Coomassie blue R250. Metalloproteinases produced gelatin lysis (semiquantified by reverse-image densitometry), andzymogen activation was suggested by doublet band formation.

In vivo MMP activation is often accompanied by coexpression of tissue inhibitors of metalloproteinases (TIMPs), and this interaction is lost with in vitro zymography, limiting its use for determining net activity. We therefore also performed in situ zymography by incubating fresh-frozen 4-μm slices with 0.1 mg/mL gelatin–oregon green (Molecular Probes) in 1× developing buffer (see online supplementary information, http://www.circresaha.org). Gelatin lysis was visualized by fluorescence microscopy. Coincubation with 50 mmol/L EDTA or MMP-9 antibody confirmed MMP and MMP-9 activity, respectively.

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.
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 [\(P<0.01\) for both dose groups, total \(n=21\)]. In separate analysis, we compared gel lysis with \(-\)blocker-only–Ang II to that with Ang II only \(22\) and found no significant change with \(-\)blocker.

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 in cardiac systolic and diastolic parameters versus baseline for the Ang II+48hP, 48hP-only, and Ang II-only groups. Heart rate was not significantly altered in any of these groups.

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 coinubcation of the same tissue with EDTA (Figure 4c), a non-speciﬁc inhibitor of MMPs, and also was substantially reduced by coinubcation 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 \(-\)blockade (Figure 4e) inhibited in situ gelatin lysis, whereas low-dose \(-\)blockade did not (Figure 4f).

Influence of \(-\)-Adrenergic Blockade on Ang II–48hP Effects

Figure 2B displays pressure-dimension data for representa-
tive animals treated concomitantly with atenolol. Regardless of dose, atenolol treatment had no signiﬁcant effect on systolic cardiodepression associated with subsequent Ang II+48hP; however, high-dose \(-\)-blockade (group 2B) pre-
vented the synergistic exacerbation of diastolic stiffening. In contrast, there was no inhibitory effect from partial blockade (low-dose atenolol). Neither dose altered the delay of pressure relaxation (tau) induced by 48hP, suggesting that the ameliorative effect of high-dose atenolol was targeted to passive diastolic properties.

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><strong>Systolic</strong></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>(\text{dP/dt}_{\text{max}}, \text{mm Hg/s})</td>
<td>-564±136*</td>
<td>168±198</td>
<td>-1066±133*</td>
</tr>
<tr>
<td>(M_{\text{sw}}, \text{mm Hg})</td>
<td>-22.3±4.5*</td>
<td>3.2±6.8</td>
<td>-23.9±4.3*</td>
</tr>
<tr>
<td><strong>Diastolic</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ESP, mm Hg</td>
<td>10.6±1.9*</td>
<td>2.8±0.5*</td>
<td>-0.17±1.5</td>
</tr>
<tr>
<td>EDP, mm</td>
<td>0.8±3.2</td>
<td>0.1±0.7</td>
<td>-0.9±0.9</td>
</tr>
<tr>
<td>(\beta, \text{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 Ca\(^{2+}\) release and myofilament Ca\(^{2+}\) sensitization and sympathetic effects via AT\(_1\) 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.

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. 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 β\(_1\) versus β\(_2\) 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, AT\(_1\) 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 AT\(_1\) receptor on fibroblasts. Ang II stimulates fibroblasts in culture to increase types I and III collagen synthesis and
reduce MMP1 (interstitial collagenase) activity. \textsuperscript{19,21} AT\textsubscript{2}-receptor binding may inhibit this cascade, because Ang II–mediated collagen synthesis nearly doubles in the presence of AT\textsubscript{2}-receptor blockade. \textsuperscript{20} The present data also revealed Ang II–mediated fibrosis, primarily in the low-dose atenolol group, and similar to data in nontreated animals,\textsuperscript{22} 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\textsuperscript{42} 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.,\textsuperscript{43} who found increases in association with reduced TIMP-1, and by Thomas et al.,\textsuperscript{25} who reported marked increases in MMP-3 and MMP-9 with increases in TIMP-1. Experimental models of failure, including the tachypacing model,\textsuperscript{23,24} 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.\textsuperscript{44}

MMPs are normally present in the extracellular matrix in an inactive form, resulting from noncovalent interactions between coordinated Zn\textsuperscript{2+} at the active site and Cys in the prodomain. In vivo activation occurs via a broad range of serine proteases, cytokines, and reactive oxygen species.\textsuperscript{45,46} In cardiac tissue, we have consistently observed minimal MMP synthesis by in vitro zymography under baseline conditions, and the present study extends this to tissue activity by in situ assay. However, exposure to only 4 days of Ang II infusion results in marked increases in both MMP abundance and activity. The in situ zymography identified a role for MMP-9, and Ang II could mediate its expression via linkage to the AP-1 transcription factor.\textsuperscript{47–49} Generation of reactive oxygen species by Ang II and sympathostimulatory toxicity and inflammation could contribute to its activation.\textsuperscript{46,49–51} In this regard, it is worth noting that MMP activation was also observed with Ang II+high-dose atenolol, despite minimal tissue damage or inflammation, indicating that alternative pathways also existed.

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.\textsuperscript{22} 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\textsuperscript{52} and thus could potentially contribute to myocardial edema. Only small increases of interstitial water content can greatly increase chamber stiffness.\textsuperscript{53,54} Furthermore, MMPs can degrade proteoglycans and mucopolysaccharides (such as hyaluronidate), molecules that become highly hydrophilic when structurally uncoiled.\textsuperscript{55,56} This could serve as an interstitial sponge contributing to water retention and diastolic stiffening. Altered collagen cross-linking might also play a role.\textsuperscript{42} 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, and 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 protein 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 from pacing is 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.

Acknowledgments

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