Metalloproteinase Inhibitor Counts High-Energy Phosphate Depletion and AMP Deaminase Activity Enhancing Ventricular Diastolic Compliance in Subacute Heart Failure

Nazareno Paolocci, Barbara Tavazzi, Roberto Biondi, Yehezkel A. Gluzband, Angela Maria Amorini, Carlo G. Tocchetti, Mehrdad Hejazi, Patrizio M. Caturegli, Jan Kajstura, Giuseppe Lazzarino, and David A. Kass

Division of Cardiology, Department of Medicine (N.P., C.G.T., D.A.K.) and Department of Pathology (M.H., P.M.C.), Johns Hopkins Medical Institutions, Baltimore, Maryland; Department of Clinical Medicine, Section of General Pathology, University of Perugia, Perugia, Italy (N.P., R.B.); Institute of Biochemistry and Clinical Biochemistry, Catholic University of Rome, Rome, Italy (B.T.); Laboratory of Cardiovascular Science, Gerontology Research Center, National Institute of Aging, National Institutes of Health, Baltimore, Maryland (Y.A.G.); Department of Chemical Sciences, Laboratory of Biochemistry, University of Catania, Catania, Italy (A.M.A., G.L.); and Department of Medicine, New York Medical College, Valhalla, New York (J.K.)

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ABSTRACT

Cardiac matrix metalloproteinases (MMPs) stimulated by the sympathomimetic action of angiotensin II (AII) exacerbate chamber diastolic stiffening in models of subacute heart failure. Here we tested the hypothesis that MMP inhibition prevents such stiffening by favorably modulating high-energy phosphate (HEP) stores more than by effects on matrix remodeling. Dogs were administered AII i.v. for 1 week with tachypacing superimposed in the last two days (AII+P; n = 8). A second group (n = 9) underwent the same AII+P protocol but was preceded by oral treatment with an MMP inhibitor PD166793 [(S)-2-(4-bromo-biphenyl-4-sulfonylamino-3-methyl butyric acid] 1 week before and during the AII+P period. Pressure-volume analysis was performed in conscious animals, and myocardial tissue was subjected to in vitro and in situ zymography, collagen content, and HEP analysis (high-performance liquid chromatography). As reported previously, AII+P activated MMP9 and MMP2 and specifically exacerbated diastolic stiffening (+130% in chamber stiffness). PD166793 cotreatment prevented these changes, although myocardial collagen content, subtype, and cross-linking were unaltered. AII+P also reduced ATP, free energy of ATP hydrolysis (ΔGATP), and phosphocreatine while increasing free [ADP], AMP catabolites (nucleoside-total purines), and lactate. PD166793 reversed most of these changes, in part due to its inhibition of AMP deaminase. MMP activation may influence cardiac diastolic function by mechanisms beyond modulation of extracellular matrix. Interaction between MMP activation and HEP metabolism may play an important role in mediating diastolic dysfunction. Furthermore, these data highlight a potential major role for increased AMP deaminase activity in diastolic dysfunction.

Diastolic chamber stiffening is a prominent feature of congestive heart failure (CHF) and a major contributor to the morbidity and mortality of this disease (Kass et al., 2004). Such stiffening can result from alterations in the extracellular matrix and matrix turnover (Norton et al., 1997), the latter controlled by metalloproteinases (MMP) (Thomas et al., 1998). MMP activation is often more prominent in late stage CHF and accompanied by oxidative stress and activation of the renin-angiotensin (AII) simulation (McElmurray et al., 1999). We have reported that AII infusion combined with short-term rapid ventricular pacing in the dog (AII+P) induces systolic dysfunction with exacerbated diastolic stiffening and marked activation of cardiac MMPs (Senzaki et al., 1998, 2000). The latter two appeared coupled, but not via changes in cardiac interstitial fibrosis (Senzaki et al., 2000).

ABBREVIATIONS: CHF, congestive heart failure; AII, angiotensin II; AMPD, AMP deaminase; HEP, high-energy phosphates; MMP, metalloproteinase; P, tachypacing; LV, left ventricular; CNBr, cyanogen bromide; PD166793, (S)-2-(4-bromo-biphenyl-4-sulfonylamino-3-methyl butyric acid; HPLC, high-performance liquid chromatography.
This is consistent with other studies where MMP inhibition improved compliance without lowering collagen content (Brower and Janicki, 2001) or in which increased myocardial stiffness was not associated with changes in collagen volume fraction (Norton et al., 1996). These results suggest that other mechanisms may link MMP activation and diastolic compliance.

A potential alternative mechanism is that MMP activation influences HEP metabolism (Iwaki-Egawa et al., 2001), increasing free cytosolic ADP that could exacerbate diastolic stiffness (Tian et al., 1997a,b). HEP stores decline with CHF (Ingwall and Weiss, 2004) often coupled to oxidative stress from neurohumoral stimulation. Increased Mg\(^{2+}\)-ADP competes with ATP-myosin binding to favor strongly bound cross-bridges that increase stiffness and slow motor velocity. Cross-activity of MMP9 on AMP catabolism (Iwaki-Egawa et al., 2001) could contribute to the formation of oxypurines, such as uric acid, which itself is correlated with diastolic dysfunction in heart-failure patients (Ciocria et al., 2002). Accordingly, the present study examined the effect of a broad MMP inhibitor on cardiac function, the extracellular matrix, and HEP stores and enzymatic activities involved in AMP catabolism in a canine model of subacute heart failure (AII+P) exhibiting prominent diastolic dysfunction.

## Materials and Methods

### Chemicals

Angiotensin II was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were obtained from commercial suppliers and were of the highest purity available.

### Animal Preparation

Studies employed the AII+P model as described previously (Senzaki et al., 1998, 2000). In brief, adult mongrel dogs received angiotensin II (AII, 15 ± 4 ng·kg\(^{-1}\)·min\(^{-1}\) i.v. x 7 days) superimposed with right ventricular tachypacing (240 base beats/min) during the final 48 h. In group 1 (n = 8), no further interventions were made, whereas group 2 animals (n = 9) received the broad-spectrum MMP inhibitor PD166793 (S)-2-(4-bromo-biphenyl-4-sulfonylamino-3-methyl butyric acid; 5 mg/kg/day p.o.) (Spanel et al., 1999; Peterson et al., 2001) for 2 weeks, starting 1 week before and continuing during the AII+P protocol. This PD166793 dose yields plasma levels well below the IC\(_{50}\) for neutral endopeptidase, angiotensin-converting enzyme, or other protease inhibition (Ye et al., 1994). Animals were instrumented with indwelling high-fidelity left ventricular (LV) pressure transducers, sonomicrometers for LV cross-sectional area, inferior vena cava cuff occluders, right atrial and aortic indwelling catheters, and pacing leads as described previously (Senzaki et al., 1998, 2000). At terminal study, all animals were euthanized, hearts were excised, and tissue was stored in liquid N\(_{2}\), optimal cutting temperature compound, or formalin for analysis.

### In Vitro and In Situ Zymography

MMP abundance was assessed by in vitro gelatin zymography, with 10 to 40 µg of protein/lane loaded onto 10% polyacrylamide gels containing 0.1% gelatin (Novex, San Diego, CA), and gels were stained with 0.5% Coomassie Blue. In situ MMP activation was assessed in freshly frozen 5-µm myocardial slices incubated with 0.1 mg/ml gelatin-Oregon green (Invitrogen, Carlsbad, CA). Gelatin lysis was visualized by emitted fluorescence, and co-incubation with 50 mmol/EDTA or anti-MMP9 antibody (Calbiochem, San Diego, CA) was used to confirm general and specific activity.

### Collagen Evaluation

Collagen subtypes and content were assessed by quantitative immunohistochemistry on left ventricle sections (4 µm each), fixed in 10% phosphate-buffered formalin, and paraffin-embedded (Cesselli et al., 2001). Collagen cross-linking was determined by cyanogen bromide (CNBr) digestion (Vasan et al., 1996), with final aliquots of CNBr digests equivalent to 4 µg of hydroxyproline analyzed by SDS-polyacrylamide gel electrophoresis under reducing conditions using 7.5% separating gel/5% stacking gel and visualized by silver stain.

### Assessment of Redox and Energy Metabolism

At the time of sacrifice, animals underwent thoracotomy and hearts from both study groups and a separate set of normal controls (n = 7) were removed under ice-cold cardioplegia (100 mEq K\(^{+}\); Plegisol; Abbott Laboratories, Abbott Park, IL). Transmural samples from the LV (100–200 mg each) were harvested from the left ventricle far from the K\(^{+}\)-injected region, immediately frozen in liquid nitrogen, and stored at −80°C until use. At the time of redox and energy status assessment, each heart specimen was processed according to a recent organic deproteinization procedure suitable for the determination of water-soluble low-molecular weight compounds and representative of both tissue oxidoreductive and energy status (Lazzarino et al., 2003).

High-energy phosphates, oxypurines (hypoxanthine, xanthine, and uric acid), nucleosides (inosine and adenosine), ascorbic acid, malondialdehyde, and reduced and oxidized glutathione were measured by ion-pairing HPLC (Lazzarino et al., 2003) using a Kromasil C-18, 250 × 4.6-mm, 5-µm particle size column, provided with its own guard column (Eka Chemicals AB, Bohus, Sweden). The HPLC apparatus consisted of a SpectraSystem P2000 pump system and highly sensitive UV6000LP diode array detector (both instruments from ThermoFinnigan Italia, Rodano, Milan, Italy) equipped with a 5-cm light path flow cell and set up between 200 and 300 nm wavelength. Data acquisition and analysis were performed using the ChromQuest software package (Thermo Electron Corporation, Waltham, MA) (Lazzarino et al., 2003). Pi was determined colorimetrically from the same extracts, and phosphocreatine (PCr) and creatine were measured enzymatically using a Jasco-650 UV-visible double-beam spectrophotometer (Jasco, Tokyo, Japan). PCr can rapidly degrade during the biopsy/freezing process, sometimes rendering lower polymerase chain reaction/ATP values than those derived from noninvasive 31P magnetic resonance measures. The biopsy approach was nonetheless used here because the biopsies provide samples to assess both energetics and oxidative stress (by HPLC) and, more importantly, because NMR could not have easily been performed in these animals with chronically implanted electronic pacemakers. Moreover, such a systematic effect, if present, would similarly affect biopsies from control and PD16673 hearts. However, the polymerase chain reaction/ATP ratio we found (≈1) was not that dissimilar from that reported in some NMR studies (≈1.3) (Horn et al., 1998).

Free cytosolic [ADP] was calculated from the creatine kinase equilibrium reaction: free [ADP] = [ATP] [free creatine]/[Pc] [H\(^{+}\) \(1.66 \times 10^{9}\) (Lawson and Veech, 1979). The free energy available (\(\Delta G_{\text{ATP}}\)) from the hydrolysis of ATP was calculated as follows: \(\Delta G_{\text{ATP}} = \Delta G_{0} + RT \log[\text{ADP}]/[\text{ATP}]\), where \(\Delta G_{0}\) is free energy of ATP, \(\Delta G_{0}\) is standard free energy change, R is the universal gas constant, and T is absolute temperature (Gibbs, 1985).

### Assessment of PD166793 Effects on AMP Deaminase Activity

To determine whether PD166793 had direct effects on enzymes involved with AMP and purine catabolism, 20% tissue homogenates from control canine myocardium (n = 3) were prepared in 150 mM KCl and centrifuged at 20,600g for 15 min at 4°C, and aliquots (20 µl) were incubated for 45 min in 100 mM KCl, 20 mM NH\(_{4}\)\(^{+}\) COOH, and 2 mM AMP (250 µl of final volume), with PD166793 added at 0 to 10 µM concentrations. Incubation was stopped by the addition of 750 µl of HPLC-grade acetonitrile and deproteinized, and AMP was assessed by HPLC to index AMP deaminase activity, as described previously (Tavazzi et al., 2000).

### Statistical Analysis

Data are presented as mean ± S.E.M. unless otherwise specified. Between- and within-group analysis was performed by repeated-measures analysis of variance, with a Tukey test for multiple comparisons. In addition, specific within-group-paired data were analyzed by nonpaired Student’s t test for between-group treatment effect determination.
Results

Ventricular Diastolic Stiffening Induced by AII+P Is Inhibited by PD166793. As reported previously, AII+P resulted in both depression of systolic function and exacerbated diastolic chamber stiffening. Figure 1A displays example LV pressure-area relations at baseline and following AII+P, showing both features, i.e., reduced slope of end-systolic pressure-area relation and steeper diastolic relation (the latter in a magnified display is shown in Fig. 1C). The systolic changes are similar to 48-h tachypacing alone, whereas diastolic stiffening is amplified specifically (Senzaki et al., 1998, 2000). In group 2 animals, diastolic stiffening was largely prevented (see Fig. 1, B and D), whereas systolic function and active relaxation were depressed similar to group 1 dogs (Table 1).

PD166793 Reduces Cardiac MMP Abundance and Activation. In situ myocardial tissue analysis of MMP activity revealed minimal basal activation but marked stimulation by AII+P (Fig. 2, A and B). MMP activation was substantially inhibited by coinubcation with an MMP9-blocking antibody (Fig. 2C), consistent with prior reports (Senzaki et al., 1998, 2000). Similar results were obtained using an MMP2-blocking antibody (data not shown), supporting the involvement of this species as well. MMP activation was also absent in chronic PD166793-treated animals (Fig. 2D). In vitro zymography (Fig. 2E) yielded marked gelatin-lysis at 92 and 72 kDa (MMP9 and MMP2, respectively) induced by AII+P, with double-banding patterns consistent with activation. This was also prevented by PD166793 treatment, indicating reduced MMP abundance and activity.

MMP Inhibition by PD166793 Does Not Reduce Collagen or Collagen Cross-linking. Both type I and III collagen increased with AII+P, with collagen I/III ratio declining (all \( p < 0.001 \); Fig. 2, F–G; Table 2). Collagen cross-linking also increased as revealed by reduced soluble collagen by CNBr assay. As shown in Fig. 2J, the percentage of collagen soluble after CNBr digestion decreased from 15.8 ± 1.65 in controls to 10.16 ± 1.3 in AII+P hearts (\( p = 0.01 \), \( n = 5 \) for each group), indicating increased cross-linked collagen. However, this was not further altered by PD166793 treatment (10.68 ± 1.19, \( n = 5 \), \( p = \) not significant versus AII+P). Although these changes would be consistent with diastolic stiffening, they were not reversed by PD166793 treatment (Fig. 2, H–I; Table 2) despite reduced chamber stiffness. An alternative cause of chamber stiffness is tissue edema, and it was possible that MMP inhibition reduced myocardial water content. This was assessed by wet-dry weight ratio, but these ratios were nearly identical between group 1 (3.57 ± 0.15) and group 2 (3.55 ± 0.12) animals (\( n = 4–5 \)).

PD166793 Restores PCR and ΔG_ATP while Reducing Elevated Free ADP from AII+P. AII+P induced marked abnormalities in HEP metabolism, including a ~50% decline in myocardial ATP, and a 4-fold increase in AMP: 22.5 ± 2.3

![Fig. 1. Hemodynamic influence of MMP inhibition with PD166973 in AII+P model. A, pressure-area (P-A) loops and relations obtained in AII+P model without MMP inhibition. Control end-systolic and end-diastolic relations are shown (dashed lines). The combined effect of AII and 48-h rapid pacing markedly increased diastolic stiffness (arrow) and depressed systolic function (right-shift of end-systolic pressure-area relation). In B, P-A loops show the dramatic EDPVR amelioration but not in systolic dysfunction obtained after chronic administration of PD166973. C and D show data on an expanded pressure-scale to highlight changes in the end-diastolic pressure-area relation. Base is baseline before initiating AII infusion; AII is data after 4 days of AII infusion; AII+48hrP is after 1 week of AII superimposed with tachypacing during the last 48 h.](image-url)
creased oxypurines (hypoxanthine, xanthine, and uric acid) because of increased AMP catabolism as reflected by in-
revealed significant blunting of diastolic chamber stiffness (β) and diastolic pressure rise. Active pressure relaxation (τ) as well as systolic function was similarly reduced.

TABLE 1
Hemodynamic changes due to angiotensin II infusion combined with 48-h tachypacing in dogs with and without concurrent treatment with the MMP inhibitor PD166793
Baseline data are provided for both dog groups and were similar between them. The percentage change for each variable after AII + 48 h of tachypacing is provided and revealed significant blunting of diastolic chamber stiffness (β) and diastolic pressure rise. Active pressure relaxation (τ) as well as systolic function was similarly reduced.

<table>
<thead>
<tr>
<th>Group 1 (No MMP Inhibitor)</th>
<th>Group 2 (+PD166793)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVEDP mm Hg</td>
<td>10.6 ± 3.1</td>
</tr>
<tr>
<td>β mm⁻²</td>
<td>0.031 ± 0.02</td>
</tr>
<tr>
<td>LVEDD mm²</td>
<td>99.13 ± 9.6</td>
</tr>
<tr>
<td>FS %</td>
<td>46.9 ± 19</td>
</tr>
<tr>
<td>ESP mm Hg</td>
<td>117 ± 14.6</td>
</tr>
<tr>
<td>Msw mm Hg</td>
<td>72.5 ± 7.1</td>
</tr>
<tr>
<td>τ ms</td>
<td>34.2 ± 4.3</td>
</tr>
</tbody>
</table>

Baseline %Δ (+48 h of Pacing)

LVEDP, left ventricular end-diastolic pressure; β, chamber elastic stiffness coefficient; FS, percentage of fractional shortening; NS, not significant; ESP, end-systolic pressure; Msw, preload recruitable stroke work; τ, isovolumic relaxation time constant.

Fig. 2. Evaluation of MMP expression/activity and collagen amount and cross-linking in normal, AII+P, and MMPi hearts. In situ zymography: normal control myocardium (A); AII+P myocardium, with marked diffuse green fluorescence indicating MMP-gelatin digestion (B); same tissue coincubated with MMP9-blocking antibody (C), which shows primary involvement of MMP9 to this response, consistent with in vitro zymography; and AII+P cotreated with PD166793 MMP inhibitor showing minimal MMP activation (D). In vitro gelatin zymography. E, ST, standard lanes showing active MMP9 (upper band) and MP-2 (lower band). B, baseline (normal myocardium); AII+P, four different dog hearts treated with MMP inhibitor; AII+P, five different hearts exposed to AII+P without MMP inhibition, revealing marked gelatin lysis. In contrast, there was minimal gelatinolytic activity on the zymograms in myocardium from MMP inhibitor-treated hearts. Confocal analysis of collagen content and subtype. F and G, AII+P showing enhanced collagen type I (F) and type III (G). H and I, similar enhanced collagen was observed in hearts exposed to AII+P with MMP inhibition. Collagen cross-linking evaluation. J, percentage of myocardial soluble collagen to CNBr digestion in normal (Con), AII+P, and MMPi hearts (n = 5 for each group).

TABLE 2
Myocardial collagen I, III, total, and I/III ratio in control hearts, hearts treated with AII+P, and hearts treated with AII+P and MMP inhibition
Collagen data are expressed as percentage volume fraction (mean ± S.E.M.). p value is for three-way Kruskal Wallis test.

<table>
<thead>
<tr>
<th>n</th>
<th>Collagen I</th>
<th>Collagen III</th>
<th>Ratio I/III</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5</td>
<td>0.70 ± 0.15</td>
<td>0.27 ± 0.06</td>
<td>3.20 ± 0.52</td>
</tr>
<tr>
<td>AII+P</td>
<td>7</td>
<td>2.81 ± 0.20</td>
<td>1.81 ± 0.25</td>
<td>1.72 ± 0.16</td>
</tr>
<tr>
<td>AII+P + MMPi</td>
<td>8</td>
<td>2.79 ± 1.61</td>
<td>1.87 ± 0.41</td>
<td>2.02 ± 0.35</td>
</tr>
<tr>
<td>p value</td>
<td>0.007</td>
<td>0.008</td>
<td>0.056</td>
<td>0.005</td>
</tr>
</tbody>
</table>

ñ p < 0.02 versus control (Bonferroni correction).

in controls (n = 5) to 97 ± 16 nmol/g wet weight in AII+P (n = 8, p < 0.001; Fig. 3). Total adenine nucleotides declined because of increased AMP catabolism as reflected by increased oxypurines (hypoxanthine, xanthine, and uric acid) and nucleosides (inosine and adenosine). IMP nearly doubled: from 32.02 ± 2.3 in controls to 57 ± 5 nmol/g wet weight in AII+P hearts (p < 0.005). The sum of oxypurines/nucleosides and adenine nucleotides (ATP + ADP + AMP) re-
mained unchanged. Thus, HEP depletion was paralleled by catabolite accumulation, indicating an altered phosphorylation-dephosphorylation balance.

In group 2 PD166793-treated dogs (n = 9), total adenine nucleotide levels remained near control levels. Whereas ATP levels remained reduced (Fig. 3), total ADP and AMP increased (p < 0.001 versus group 1) and AMP catabolites and IMP declined (p < 0.001 versus group 1). Thus, one particular target of PD166793 effects was AMP catabolism. The sum of adenine nucleotides, oxypurines, and nucleosides remained unaltered.

It is noteworthy that, from the perspective of diastolic stiffening, group 2 animals had reduced estimated levels of free ADP. The majority of ADP is protein-bound in myocytes, with the biologically relevant form in the free cytosolic fraction. Calculated free ADP increased nearly 80% (0.34 ± 0.01 versus 0.54 ± 0.14 μM, p < 0.01), with AII+P paralleled by a 150% rise in Pi, PCR and ΔG_2-ATP declined while lactate and lactate/pyruvate ratio increased (Fig. 4), the latter supporting reduced oxidative glycolysis. These changes were absent in MMP inhibitor-treated hearts.

**Myocardial Redox Conditions.** AII+P stimulated myocardial oxidative stress, as reflected by a >20-fold rise in malondialdehyde and decline in nonenzymatic antioxidants ascorbate and reduced glutathione (Table 3). MMP inhibition (group 2) did not ameliorate these changes, indicating that improved cardiac energetics and diastolic mechanics were unlikely due to altered redox state.

**Dose-Dependent Inhibition of AMP Deaminase by PD166793.** Given the inhibition of myocardial AMP catabolism in group 2 dogs, we tested whether PD166793 had any direct, previously unknown action on purine catabolism. AMP deaminase activity was 0.139 ± 0.028 IU/g wet weight in control canine myocardium (n = 3). PD166793 modestly and dose-dependently inhibited this activity, with ∼20% inhibition at 0.1 μM. This was specific, because PD166793 had no effect on adenosine-deaminase, 5’-nucleotidase, purine-nucleoside-phosphorylase, or xanthine oxidoreductase activities (data not shown).

**Discussion**

The current study reveals a novel effect of MMP inhibition treatment on HEP, oxidative glycolysis, and AMP catabolism in hearts subjected to subacute cardiac dysfunction (AII+P). AII+P itself altered collagen matrix content and characteristics, reduced ATP, and increased free ADP accompanied by oxidative stress. Although these factors could have potentially contributed to diastolic stiffening, only energetic and HEP changes were prevented by the MMP inhibitor PD166793 along with enhanced diastolic compliance. These findings reveal a novel feature of PD166793, an MMP inhib-
itor employed in many prior studies, and support the role of altered increased free [ADP], reduced ATP, and AMP catabolism to in vivo diastolic stiffening. Lastly, our study suggests that targeting AMP deaminase activity to reduce adenine nucleotide catabolism, responsible for the increase in nucleosides/oxypurines and decrease in myocardial energy state, is a potential new strategy to counter heart-failure features, particularly diastolic stiffening.

Collagen, MMPs, and Diastolic Dysfunction. Prior studies have tended to focus on changes in matrix proteins and tertiary structure related to MMP activation in explaining alterations in myocardial geometry and function. MMPs degrade matrix, although their activation also reflects remodeling and matrix turnover, and net increases in matrix have been observed. Likewise, variability in matrix change with MMP inhibition has been reported. In some models, increased collagen content from MMP inhibition worsened diastolic stiffening (Spinale et al., 1999), whereas transgenic models targeting specific MMPs (e.g., MMP9) reported reduced collagen accumulation (Ducharme et al., 2000), although compensatory changes in other MMPs (e.g., MMP13) may explain this observation. It is noteworthy that PD166793 has itself been shown previously to reduce LV dilation in aged spontaneously hypertensive heart failure (SHHF) rats while improving diastolic compliance (Peterson et al., 2001). In the AII-P canine model, expression and activity of myocardial MMPs (notably MMP9, MMP2, and interstitial collagenase MMP1) increase due to sympatho-stimulation, because they are prevented by β-blockade (Senzaki et al., 2000). However, suppression of MMP activity by β-blockade did not lower collagen content, although it prevented diastolic stiffening. This finding hinted that MMP activation may be central to the behavior but work by an alternative mechanism.

High-Energy Phosphate and AMP Deaminase Contributions to Diastolic Dysfunction. Abnormal HEP metabolism is thought to contribute to myocardial dysfunction in CHF (Ingwall and Weiss, 2004). This is characterized by reduced total ATP and free energy of ATP hydrolysis (Ingwall and Weiss, 2004), a fall in PCr or energy storage (Shen et al., 1999), and shift from oxidative glycolysis. ATP-dependent processes may influence systolic function and active diastolic

![Graphs and tables]

**Fig. 4.** Calculated free cytosolic ADP ([ADP]) and free-energy change during ATP hydrolysis (ΔG_{ATP}) and spectrophotometric measurements of PCr, inorganic phosphate (free Pi), and lactate and lactate/pyruvate ratio in normal hearts (Con, n = 5), angiotensin II + pacing treated hearts (AII + P, n = 8), and AII + P hearts treated with the MMP inhibitor PD166793 (n = 9). Data are expressed in micromolars per liter and presented as mean ± S.E. *p < 0.001 versus Con; †p < 0.05 versus Con; ‡p < 0.03 versus MMPi; #p < 0.01 versus both.

**TABLE 3**

Effects of AII + P on tissue markers of myocardial redox conditions in the absence and presence of MMP inhibition

<table>
<thead>
<tr>
<th></th>
<th>Controls (n = 5)</th>
<th>AII + P (n = 8)</th>
<th>AII + P + PD166793 (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA</td>
<td>0.31 ± 0.11</td>
<td>6.73 ± 2.33*</td>
<td>6.1 ± 1.34*†</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>254 ± 12.5</td>
<td>145 ± 16.5†</td>
<td>132 ± 12††</td>
</tr>
<tr>
<td>GSH</td>
<td>4265 ± 450</td>
<td>2092 ± 181†</td>
<td>1279 ± 99†</td>
</tr>
<tr>
<td>GSSG</td>
<td>69 ± 9.5</td>
<td>55 ± 4</td>
<td>153 ± 9††</td>
</tr>
</tbody>
</table>

GSH, reduced glutathione; GSSG, oxidized glutathione; MDA, malondialdehyde.

* p < 0.05 vs. control.
†p < 0.001 vs. control.
‡p < 0.001 vs. AII + P.
relaxation, and it is intriguing in this regard that these functional abnormalities were similar in group 1 and group 2 hearts, along with ATP depletion levels. However, other abnormalities, such as free ADP levels, increased AMP catabolism and increased lactate, and lactate/pyruvate were nearly fully prevented by MMP inhibition; these latter abnormalities may play a more important role in diastolic stiffening.

Most ADP is bound in myocytes, but the smaller pool of free ADP (≈3–4% total ADP) is the one that is metabolically active and coupled to diastolic function (Tian et al., 1997a,b). ADP release is a rate-limiting step in the actomyosin ATPase reaction. Increases in free [ADP] compete for ATP binding to myosin, reducing cross-bridge cycling rate, enhancing the formation of strongly bound cross-bridges, and lowering the free energy due to ATP hydrolysis ($\Delta G_{\text{ATP}}$) (Senzaki et al., 1998). Our data support a role for free ADP on diastolic stiffening and, importantly, provide the first demonstration of this relationship in vivo, with increases in cytosolic free [ADP] in the current model indeed comparable with those observed previously in vitro (Tian et al., 1997a,b).

Elevated free [ADP] reduced $\Delta G_{\text{ATP}}$, which also can contribute to diastolic dysfunction (Katz, 1998). The decline in $\Delta G_{\text{ATP}}$, observed with All+P (58 to 53 kJ/mol) is probably significant, because it is in the range required by ATPases, such as sarcoplasmic reticulum Ca$^{2+}$ ATPase 2a and maximal contractile function in isolated hearts (Tian and Ingwall, 1996), although its restoration appeared only to improve diastolic stiffening in the present model. Because adaptive responses moderate energetically inefficient changes in cytosolic free [ADP] and $\Delta G_{\text{ATP}}$ over time, the subacuity of the present model probably explains, at least partially, the magnitude of the bioenergetic abnormalities. With more chronic tachypacing, a gradual loss of ATP/total purines occurred over 7 to 9 weeks with an early loss of creatine (Shen et al., 1999). The latter is likely a compensatory mechanism to minimize the increase in free [ADP] and reduced $\Delta G_{\text{ATP}}$ with sustained ATP loss. The current acute model allowed for less compensation, enabling more direct evaluation of the mechanical consequences of energetic abnormalities before adaptive processes intervened.

AMP deaminase (AMPD) is a ubiquitous AMP-catabolizing enzyme constitutively active in cardiac muscle (Barsacchi et al., 1979). Cardiac AMPD may help regulate adenine nucleotide catabolism during myocardial ischemia (Thakkar et al., 1994) under oxidative stress conditions (Tavazzi et al., 2001), increased ADP (Chung and Bridger, 1976), and sustained $\beta$-stimulation (Hohl, 1999). In skeletal muscle in vivo, AMPD activity increase during muscular work is mediated by ADP, AMP, and pH (Wheeler and Lowenstein, 1979). Neurohumoral/mechanical stimulation with All+P probably activated AMPD as reflected by increased adenine nucleotide catabolism and loss in phosphorylation potential. Intriguingly, AMP catabolites, particularly uric acid, are associated with worsened CHF prognosis and independent predictors of elevated diastolic pressure (Cicoira et al., 2002). Among the proposed mechanisms for this are increases in xanthine oxidase expression and activity that enhance purine catabolism and urate synthesis and serve as a source for oxidative stress (Doehner et al., 2002). Although oxidative stress was clearly induced by All+P, it was not prevented by MMP inhibition, whereas AMP catabolism largely was. This suggested a more direct effect of the MMP inhibitor on AMPD, and modest direct effects were demonstrated.

PD166793 partially inhibited AMPD directly and, although the exact magnitude of inhibition in vivo remains unclear, even partial decreases could have played an important role in AMP reaccumulation. The latter combined with higher total ADP could lead to an improved phosphorylation potential and $\Delta G_{\text{ATP}}$. Furthermore, although not directly tested in our study, higher concentrations of AMP might also increase AMP kinase activity, an enzyme up-regulated in several conditions with potential benefits in the energy-stressed heart (Young et al., 2005).

Still, it seems unlikely that this mechanism fully explains all of the observed changes, for example, the restoration of normal lactate and lactate/pyruvate ratios and recovery of PCr. Cross-reactivity of a MMP inhibitor on AMPD is consistent with the latter being a zinc metalloenzyme (Ranieri-Raggi et al., 2003), and PD166793 like other MMP inhibitors act as zinc chelators in the catalytic site. Further studies are needed to establish whether this is a feature common to all MMP inhibitors or extends to other zinc metalloenzymes. Likewise, it remains to be determined whether PD166793 displays a dual dose-dependent action, whereby at low doses, it may chiefly target AMPD activity, although at higher doses, it may interfere with other enzymatic activities along the same AMP-catabolic pathway.

Experimental Limitations and Future Directions. We used HPLC rather than NMR approaches to assess HEP metabolism. This was due in part given the complexity of the chronic instrumentation that precluded NMR studies and our desire to assess both redox conditions and HEP in the same tissue. PCr/ATP ratios by HPLC were somewhat lower than those reported by NMR (Kantor et al., 1986), perhaps reflecting PCr decline during tissue procurement (flash-freezing not employed). However, values were consistent, and all of the tissues were processed identically, reducing bias.

The present study does not establish a causal link between the energetic changes and diastolic stiffening with All+P, nor does it prove that this was due to MMP inhibition or effects from an MMP inhibitor. Even accepting the latter, the results are pharmacologically important because much of our understanding of MMPs and their inhibition was derived from data employing this agent. The results certainly highlight another pathway that may be profoundly influenced by MMP inhibition and play an important role in regulating cardiac function. Ongoing efforts are testing whether more selective MMP inhibitors (Peterson, 2004) are endowed with the same or superior AMPD inhibitory activity. This is pharmacologically and functionally relevant in the light of current lack of “selective” (Kasibhatla et al., 2001) and cell-permeable AMPD inhibitors and AMPD-proposed role in CHF unfolding (Kalsi et al., 2003).

Conclusions

Diastolic chamber stiffening remains among the more difficult features of cardiac failure to treat, yet it is a major determinant of clinical symptoms and prognosis. The current study indicates that altered HEP plays a role and can be modulated by MMP-inhibiting agents, in part through modulation of AMPD activity. These data further provide impor-
tant in vivo support for a role of altered free ADP and HEP in the generation of diastolic stiffening while showing a novel role for AMPD to this respect. Lastly, our data demonstrate the reversibility of diastolic stiffening if such energetic abnormalities can be prevented.

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Address correspondence to: Dr. Nazareno Paolocci, Ross 835, Division of Cardiology, Johns Hopkins University School of Medicine, 720 Rutland Avenue, Baltimore, MD 21205. E-mail: npaoloc1@jhmi.edu

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