Downregulation of Matrix Metalloproteinases and Collagens and Suppression of Cardiac Fibrosis by Inhibition of the Proteasome

Silke Meiners, Berthold Hocher, Andrea Weller, Michael Laule, Verena Stangl, Christoph Guenther, Michael Godes, Alexander Mrozikiewicz, Gert Baumann, Karl Stangl

Abstract—Myocardial remodeling is an adaptive response of the myocardium to several forms of stress culminating in cardiac fibrosis, left ventricular dilation, and loss of contractility. The remodeling processes of the extracellular matrix are controlled by matrix metalloproteinases, which are in turn regulated by growth factors and inflammatory cytokines. The inflammatory transcription factor nuclear factor κB has been implicated in the transcriptional regulation of several matrix metalloproteinases. Because activation of nuclear factor κB in turn is essentially controlled by the ubiquitin-proteasome system, we investigated the hypothesis that inhibition of the proteasome may prevent activation of matrix metalloproteinases. We demonstrate here that inhibition of the proteasome in rat cardiac fibroblasts suppressed not only expression of matrix metalloproteinases 2 and 9, but also expression of collagen Iα1, Iα2, and IIIα1 as determined by in-gel zymography and real-time reverse transcription–polymerase chain reaction. Moreover, myocardial expression of matrix metalloproteinases and collagens was effectively suppressed by systemic treatment of spontaneously hypertensive rats over 12 weeks with the proteasome inhibitor MG132, which resulted in a marked reduction of cardiac fibrosis (~38%) compared with control animals. We conclude that inhibition of the ubiquitin-proteasome system may provide a new and attractive tool to interfere with collagen and matrix metalloproteinase expression, and therefore might be of possible use in the therapy of myocardial remodeling. (Hypertension. 2004;44:471-477.)

Key Words: myocardial remodeling • collagen • fibrosis

The extracellular matrix (ECM) of the myocardium can be regarded as a structural scaffold for alignment of myofibrils. In response to hormonal activation or hemodynamic overload, the ECM undergoes structural remodeling, which involves production of new collagens, alterations in collagen structure, and matrix degradation leading to progressive ventricular dilation, and eventual congestive heart failure. ECM remodeling is regulated by matrix metalloproteinases (MMPs). Activity of MMPs is regulated on a post-translational and transcriptional level. Several inflammatory cytokines and growth factors upregulate MMP mRNA expression in cardiac fibroblasts, which has been correlated with heart failure. Correspondingly, a number of studies have shown that inhibition or knockout of MMPs suppresses ventricular remodeling, myocardial dysfunction, and progression of heart failure. Among others, the inflammatory transcription factor nuclear factor κB (NFκB) has been implicated in transcriptional regulation of several MMPs. NFκB forms a cytoplasmic complex with its inhibitory κB proteins (IκB). On cell stimulation, IκB is phosphorylated and degraded, allowing NFκB to activate genes involved in response to infection, inflammation, and stress.

Activation of NFκB is essentially controlled by the ubiquitin-proteasome system (UPS). The UPS is the major pathway for intracellular protein degradation in eukaryotic cells. Substrates are targeted for proteasomal degradation by covalent attachment of multi-ubiquitin chains. Activation of NFκB is regulated by signal-induced degradation of IκB by the proteasome. Because studies have shown that inhibition of the proteasome suppresses NFκB-mediated transcriptional activation in various cell types, proteasome inhibition has been proposed as a new anti-inflammatory strategy.

In the present study, we investigate the hypothesis that inhibition of the proteasome may suppress expression of MMPs in cardiac fibroblasts. We furthermore analyzed the effects of proteasome inhibition on myocardial remodeling in spontaneously hypertensive rats (SHRs).

Materials and Methods

Cell Culture

Cardiac fibroblasts were prepared from neonatal Wistar rat hearts by plating the nonmyocyte fraction. Cardiac myocytes were prepared as described previously. After preplating for 2 hours, the adherent cardiac fibroblasts were cultivated under standard conditions in

Received March 18, 2004; first decision April 8, 2004; revision accepted August 10, 2004.

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Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000142772.71367.65
M199 medium containing 10% fetal calf serum, l-glutamine, and penicillin-streptomycin (Invitrogen). Fibroblasts from passages 3 to 7 were used.

Reagents
MG132, clasto-Lactacystin-β-lactone, and ALLM were purchased from Calbiochem and dissolved in DMSO (10 mmol/L). Interleukin 1β (IL-1β) was obtained from Biomol. Antibodies against IκBα (C21) and β-actin were obtained from Santa Cruz and Chemicon International, respectively.

Proliferation Assay
Fibroblasts were treated with DMSO (0.1%) or MG132 (0.1, 1 μmol/L) for 24 hours, and proliferation of cells was determined as described elsewhere.14

Cell Viability Assay
Cell viability was assessed after stimulation of cells with DMSO or MG132 for 24 hours under serum-free conditions using XTT assay.13

Measurement of Proteolytic Activities in Cell Lysates
Cells were treated for 24 hours under serum-free conditions, lysed, and the chymotrypsin-like, caspase-like, and trypsin-like activities of the proteasome were determined using the peptide substrates Succinyl-Leu-Leu-Val-Tyr-AMC (SLLVY), Benzoylcarbonyl-Leu-Leu-Gly-AMC (ZLLE), and Benzoyl-Val-Gly-Arg-AMC (BZVGR) from Bachem, respectively, as described previously.15

Preparation of RNA and Reverse Transcription–Polymerase Chain Reaction Analysis
Total RNA extraction and reverse transcription (RT) was performed as described.15 The polymerase chain reaction (PCR) primers for rat collagen Iα1, Iα2, IIIα1, MMP2, MMP9, and the housekeeping gene hypoxanthine phosphoribosyl transferase cDNAs were purchased from TIB MOLBIOL and cDNAs were quantitatively amplified by real-time PCR (SYBR Green method; Applied Biosystems) as described.15

Western Blot and Bandshift Analysis
Cytoplasmic and nuclear extracts were prepared as described using 0.5% Triton X-100 as a nonionic detergent.16 Western Blot analysis and NFκB bandshifts were performed as described previously.14

Gelatin Zymography
Cells were treated for 24 hours under serum-free conditions and analyzed for gelatin-degrading activities by zymography. Samples were diluted in sample buffer (63 mmol/L Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.0025% bromphenol blue) and separated on 10% SDS-polyacrylamide gels containing 0.1% gelatin (Invitrogen). After removal of SDS, gelatinase activity was assessed by overnight incubation in developing buffer (50 mmol/L Tris, 40 mmol/L HCl, 200 mmol/L NaCl, 5 mmol/L CaCl2, 2 H2O, 0.02% Brij 35) and staining with 0.25% Coomassie R250. Zymograms were destained in 40% methanol and 10% acetic acid until the bands of lysis became clear.

Animals and Study Design
Age-matched male SHRs were obtained from Jackson Laboratory (Bar Harbor, Me). Animals were housed conforming to the international guidelines for care and use of laboratory animals. Rats that were 6 weeks old were treated with MG132 (0.1 mg/kg body weight), 0.1% DMSO, or with saline (n=10 per group, daily intraperitoneal injections) for 12 weeks. Blood pressure was measured weekly by standard tail-cuff method. At the end of the study, animals were euthanized (6 mL intraperitoneal of 25 mg/mL Trapanal; Byk Gulden), and organs were either shock-frozen or paraffin-embedded. Blood counts and laboratory markers of renal and hepatic function were determined by standard methods. Because no difference between DMSO and saline treatment of SHRs was observed with respect to the aforementioned parameters, the DMSO-treated animals served as control for MG132 treatment.

Histological Determination of Fibrosis
Paraffin-embedded left ventricular tissue was cut into 3-μm sections, subjected to collagen-specific Sirius-red staining, and the relationship of red-stained area to the total area of the whole heart section was analyzed as recently described.17

Statistics
We calculated significance by 1-way ANOVA (Dunnett method) or Mann–Whitney U test (SPSS 11.0). An error of P<0.05 was regarded as significant.
Results

Inhibition of MMP2 and MMP9 Activity in Rat Cardiac Fibroblasts

Because IL-1β has been shown to stimulate MMP activity in an NFκB-dependent manner in several cell types, we used IL-1β to investigate the effects of proteasome inhibition on MMP activity in rat cardiac fibroblasts. Cells were treated with IL-1β (4 ng/mL) and proteasome inhibitors for 24 hours, and MMP activity was determined by in-gel zymography. Positive controls confirmed that the specific bands at 72/66 kDa and 95/88 kDa corresponded to MMP2 and 9, respectively (data not shown). We were unable to detect MMP13 in supernatants under the conditions used. Whereas IL-1β treatment resulted in increased activity of MMP2 and 9, cotreatment with 0.1 and 0.5 μmol/L MG132 dose-dependently inhibited both activities (Figure 1A and 1B). Similarly, 10 μmol/L c-lactacystin, an irreversible inhibitor of the proteasome, reduced IL-1β-stimulated activity of MMP2 and 9 (Figure 1A and 1B). Interestingly, when cardiac fibroblasts were treated with proteasome inhibitors in the absence of IL-1β, MMP2 activity also appeared to be reduced by treatment with 0.5 μmol/L MG132 (Figure 1C and 1D). MMP9 activity was barely detectable in unstimulated cardiac fibroblasts.

These data indicate that inhibition of the proteasome suppresses IL-1β-stimulated MMP2 and 9 activities. Proteasome inhibition also appears to affect basal MMP2 activity in unstimulated cardiac fibroblasts.

Inhibition of Collagen and MMP Expression

To investigate whether the observed reduction in MMP2 and 9 activities is caused by reduced expression of MMP, we analyzed RNA expression of MMP2 and 9 by real-time RT-PCR. Treatment of cardiac fibroblasts with IL-1β induced expression of MMP2 and 9 as described (Figure 2A). Cotreatment with proteasome inhibitors significantly inhibited IL-1β-mediated expression of MMP9 and, to a lesser extent, of MMP2. This inhibition was already evident with 0.05 μmol/L MG132. It was also observed with c-lactacystin and MG262, a boronate derivative of MG132 (data not shown). Similar effects were observed for MMP13 expression, although overall expression levels were very low (data not shown). Because expression of MMPs was reduced below unstimulated control levels, we analyzed the effects of proteasome inhibitors in unstimulated cardiac fibroblasts; basal expression of MMP2 was significantly reduced by MG132 (0.05 to 0.5 μmol/L) by ∼30%, and expression of MMP9 by >70% (Figure 2B).

To investigate whether inhibition of the proteasome generally counteracts the IL-1β-mediated net effect on ECM turnover, we analyzed the effects of proteasome inhibitors on the expression of collagen RNAs. As shown previously by Siwik et al, we observed that treatment of cardiac fibroblasts with IL-1β reduced expression of collagen Iα1, Iα2, and IIIα1 (Figure 2C). Cotreatment with proteasome inhibitors did not alleviate IL-1β-mediated suppression of collagens but rather enhanced suppression of collagen Iα2 and IIIα1 expression. MG132, furthermore, dose-dependently inhibited basal expression of collagens in the absence of IL-1β (Figure 2D). Whereas expression of collagen Iα1 was reduced by 40% to 60% by 0.05 to 0.5 μmol/L MG132, expression of collagen Iα2 and IIIα1 was downregulated by 55% to 75% by 0.05 and 0.5 μmol/L MG132, respectively. Treatment with c-lactacystin was less effective, but MG262 showed similar suppressive effects (data not shown).

These data show that inhibition of the proteasome results in reduced RNA expression of collagens and MMPs in IL-1β–stimulated and unstimulated cardiac fibroblasts.

Suppression of MMP and Collagen Expression Is Observed with Nontoxic Doses of Proteasome Inhibitor That Inhibit Proliferation of Cardiac Fibroblasts

To exclude that these suppressive effects of proteasome inhibitors are caused by cytotoxic side effects, we analyzed viability of cardiac fibroblasts by XTT assays. As depicted in Figure 3A, treatment with 0.05 to 0.5 μmol/L MG132 for 24 hours did not significantly affect cell viability, whereas doses of 1 μmol/L MG132 resulted in ∼30% cell death. Addition of 10 μmol/L c-lactacystin did not affect viability of cardiac fibroblasts.
fibroblasts over 24 hours (data not shown). Moreover, 0.1 μmol/L MG132 also inhibited proliferation of cardiac fibroblasts without apparent cytotoxicity, whereas 1 μmol/L MG132 induced severe cell loss and growth arrest (Figure 3B). The nontoxic inhibitor doses specifically inhibited the proteasome. The 3 main proteolytic activities of the proteasome—i.e., chymotrypsin-like (Ch-TL), trypsin-like (TL), and caspase-like (Casp-L) activities were dose-dependently inhibited by MG132 (0.05 to 1 μmol/L; Figure 3C).

Inhibition of IL-1β–Mediated NFκB Activation

We next investigated activation of NFκB by Western blot analysis of its inhibitor IkBα, and by bandshift analysis with NFκB-specific oligonucleotides (Figure 4).

Stimulation of cardiac fibroblasts with IL-1β for 30 minutes induced nearly complete degradation of IkBα in cytoplasmic extracts (Figure 4A). Concomitantly, NFκB-mediated DNA binding activity was increased in corresponding nuclear extracts (Figure 4B). Pretreatment with proteasome inhibitors not only prevented degradation of IkBα but also dose-dependently inhibited activation of NFκB. The cathepsin- and calpain-specific inhibitor ALLM had no effect. Competition of NFκB-dependent DNA binding with excess unlabeled oligonucleotide verified the specificity of our NFκB bandshift analysis (Figure 4B, lane 1). These data indicate that inhibition of the proteasome effectively prevents IL-1β–induced activation of NFκB.

Inhibition of Cardiac Fibrosis In Vivo

The observed suppressive effects of proteasome inhibitors on the expression of MMPs and collagens led us to investigate the effects of proteasome inhibition on the development of myocardial remodeling in an animal model of SHRs. Six-week-old SHRs were treated daily with MG132 (0.1 mg/kg) or 0.1% DMSO for 12 weeks. Treatment of SHRs with MG132 was generally well-tolerated over this long period: 2 animals died in the DMSO-treated group, but none in the MG132-treated group. In blood samples taken after 6 weeks and at the end of the study, we observed no changes in differential blood counts and no abnormalities in laboratory markers that would indicate renal or hepatic side effects (data not shown). MG132 treatment did not significantly alter blood pressure, body weight, and heart weight (Table). Myocardial remodeling was assessed by quantitative morphometry of Sirius red-stained sections of left ventricles.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n=8)</th>
<th>MG132 (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP, mm Hg</td>
<td>196.7±9.1</td>
<td>192.2±10.4</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>286±36.6</td>
<td>272±39.7</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>1.28±0.07</td>
<td>1.32±0.24</td>
</tr>
<tr>
<td>HW/BW, mg/g</td>
<td>4.55±0.62</td>
<td>4.85±0.49</td>
</tr>
</tbody>
</table>

BP indicates blood pressure; BW, body weight; HW, heart weight. Values are expressed as mean±SD.
Treatment with MG132 resulted in pronounced reduction (−38%) of left ventricular collagen deposition in SHRs compared with controls (Figure 5A, 5B; for quantification, 5C). These findings correlated well with significantly reduced levels of collagen Iα2 and Iλ1 in the left ventricles as determined by real-time RT-PCR analysis (Figure 5D). Moreover, expression of MMP2 RNA was also significantly reduced in MG132-treated animals (Figure 5D). Expression of MMP9 was below detection level in all animals, and expression of MMP13 was very low but also significantly reduced (data not shown).

Discussion

Our study shows for the first time that inhibition of the proteasome suppresses expression of MMPs and collagens in rat cardiac fibroblasts and effectively prevents myocardial remodeling in spontaneously hypertensive rats. These in vivo effects are independent of blood pressure and involve reduced expression of collagens and MMPs. Importantly, systemic long-term treatment with MG132 was apparently well-tolerated, which indicates that the observed reduction in cardiac fibrosis is not caused by cytotoxic side effects.

Inhibition of MMP Expression

NFκB-dependent expression has been observed for several MMPs,5-7 In particular, MMP9 is a well-known cytokine-inducible MMP whose expression is stimulated via NFκB-regulatory elements in the promoter of the MMP9 gene.20 In the present study, we show that proteasome inhibitors effectively prevent cytokine-mediated and basal expression of MMP2 and 9 in cardiac fibroblasts (Figures 1 and 2A, 2B). We also demonstrate that cytokine-mediated stimulation of MMPs was associated with activation of NFκB, which is effectively prevented by pretreatment of cells with proteasome inhibitors (Figure 4). Suppression of MMPs was more pronounced on the RNA level than observed with zymography. Because MMP RNAs have been shown to be very stable, suppression of MMP expression by proteasome inhibition is probably caused by transcriptional downregulation.21 The observed suppressive effects accord with other studies of different cell types, in which MMP expression was inhibited either by addition of proteasome inhibitors22-23 or by overexpression of IxBα.24

Inhibition of Collagen Expression

To our knowledge, this is the first study that shows downregulation of collagen expression on proteasome inhibition. Interestingly, IL-1β-mediated suppression of collagen expression was not alleviated but rather enhanced by proteasome inhibition (Figure 2C and 2D), indicating that inhibition of NFκB by MG132 is not sufficient to overcome the suppressive effects of IL-1β. This suggests the possible involvement of additional signaling mediators in the transcriptional regulation of collagen expression. A complex interplay of transcription factors has been implicated in the regulation of collagen promoters involving several substrates of proteasomal protein degradation such as NFκB, AP-1, SP-1, and p53.25 The exact mechanisms, which underlie the suppression of collagen expression by proteasome inhibition, remain to be elucidated and are subject of further studies in our laboratory.

Inhibition of Cardiac Remodeling

The suppressive effects of proteasome inhibition on MMP and collagen expression observed in vitro prompted us to further study the effects of proteasome inhibition on myocardial remodeling in vivo. Brilla et al26 had previously shown that pronounced hypertension develops in SHRs within only 14 weeks, and was accompanied by enhanced collagen deposition and myocardial fibrosis compared with controls. To analyze the effects of proteasome inhibitors on the development of myocardial remodeling, we treated young SHRs with MG132 for 12 weeks and assessed cardiac remodeling at the end of the study. MG132 treatment significantly reduced deposition of interstitial collagens and expression levels of MMPs in the myocardium, compared with controls. Perivascular fibrosis was only nonsignificantly affected. The antifibrotic effects are possibly mediated by diminished expression of MMPs and collagens and reduced proliferation of cardiac fibroblasts, as suggested by our in vitro results. Reduced proliferation might be caused by inappropriate accumulation of cyclins and cdk inhibitors on...
proteasome inhibition as described for other cell types.\textsuperscript{9,14} The nearly 40\% reduction of cardiac fibrosis by proteasome inhibition is highly encouraging, because it lies well within the range of established antifibrotic agents such as inhibitors of the renin-angiotensin-aldosterone system (RAAS) and endothelin-1 antagonists.\textsuperscript{27}

Inhibition of cardiac fibrosis has been associated not only with reduced levels of collagens and alterations in collagen structure but also with decreased activity of MMPs.\textsuperscript{2} This apparent paradox is because of the fact that the total collagen content of the myocardium is a function of both synthesis and degradation. Increased fibrosis has thus been shown to be accompanied by increased MMPs and reduced fibrosis by decreased MMP activity.\textsuperscript{28,29} These studies are in accord with our observation that proteasome inhibition reduces MMP expression and deposition of collagens in the myocardium of SHRs.

Interestingly, we did not observe any effects of MG132 treatment of SHRs on left ventricular hypertrophy (Table). The explanation here may lie in the fact that we treated young SHRs for 12 weeks and euthanized the animals at the age of 18 weeks. At this time point, the elevation of blood pressure in the MG132-treated and control groups was only moderate in comparison to other studies.\textsuperscript{26,30} This moderate increase in blood pressure correlated with only weak left ventricular hypertrophy, whereas cardiac fibrosis was already well-established. The animal model chosen is therefore primarily suited for investigation of the effects of proteasome inhibition on cardiac fibrosis, but not on hypertrophy. We are currently analyzing whether inhibition of the proteasome also has an effect on cardiomyocyte hypertrophy and left ventricular remodeling.

Expression array analysis (BDTransduction Laboratories) of single DMSO-treated and MG132-treated animals revealed that proteasome inhibition resulted in a complex change of RNA expression of various genes (data not shown), as also observed by Mitsiades on tumor cells.\textsuperscript{31} These data suggest that suppression of collagens and MMPs might not be pinpointed to one specific pathway, such as inhibition of NF\textsuperscript{k}B activation, but appear to be the result of a complex interplay of numerous signaling pathways that are affected by proteasome inhibition. The complex transcriptional effects of proteasome inhibition on several signaling mediators may also affect specific fibrosis-related signaling pathways. Studies have shown that interfering with downstream effectors of these signaling pathways—e.g., by blocking the Na\textsuperscript{+}/H\textsuperscript{+} exchanger—not only effectively prevents the development of cardiac fibrosis in \(\beta(1)\)-receptor transgenic mice\textsuperscript{32} but also induces reduction of hypertensive myocardial fibrosis in SHRs.\textsuperscript{33} It is also feasible that inhibition of the proteasome may interfere with reactive cardiac fibrosis after myocardial infarction, as shown for the anti-inflammatory peptide AcSDKP.\textsuperscript{34} Pye et al have recently demonstrated that the proteasome inhibitor PS-519 reduces NF\textsuperscript{k}B-mediated myocardial reperfusion injuries due to its anti-inflammatory features.\textsuperscript{35}

**Perspectives**

Inhibition of the UPS may represent a new and attractive approach to interfere with collagen and MMP expression. It might therefore be of possible use in the therapy of myocardial fibrosis. Moreover, because reduction of MMP activity previously has been shown to offer a promising strategy for preventing heart failure,\textsuperscript{4} inhibition of the proteasome might possibly affect progressive ventricular dilation and eventual congestive heart failure. Systemic treatment with low doses of proteasome inhibitors may therefore provide possible benefit in treatment of myocardial remodeling beyond local delivery of these inhibitors as proposed for vascular restenosis,\textsuperscript{14} and beyond acute treatment of myocardial reperfusion injury.\textsuperscript{35}

**Acknowledgments**

This study was partly supported by a grant from the Deutsche Forschungsgemeinschaft (DFG HO 1665/5-1) to Berthold Hocher.

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Hypertension. 2004;44:471-477; originally published online August 30, 2004;
doi: 10.1161/01.HYP.0000142772.71367.65
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
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