Ubiquitin-Proteasome Pathway as a New Target for the Prevention of Restenosis

Silke Meiners, PhD; Michael Laule, MD; Wim Rother, MD; Christoph Guenther, MD; Ines Prauka, MD; Peter Muschick, PhD; Gert Baumann, MD; Peter-Michael Kloetzel, PhD; Karl Stangl, MD

Background—The ubiquitin-proteasome system is the major intracellular protein degradation pathway in eucaryotic cells. It regulates central mediators of proliferation, inflammation, and apoptosis that are fundamental pathomechanisms in the development of vascular restenosis.

Methods and Results—Effects of proteasome inhibition on neointima formation were studied in a balloon injury model in the rat carotid artery. Local application of the proteasome inhibitor MG132 (1 mmol/L) resulted in significant inhibition of intimal hyperplasia, that is, by 74% (P=0.008). This effect was accompanied by decreased proliferation, reduced infiltration of macrophages, and prolonged apoptosis, as determined by immunohistochemical and TUNEL analyses. Functional effects of proteasome inhibition on proliferation, activation of nuclear factor kappa B, and apoptosis were further characterized in rat primary vascular smooth muscle cells. MG132 dose-dependently inhibited vascular smooth muscle cell proliferation with 50% inhibition at 10 μmol/L. TNFα-induced degradation of IκBα and β was blocked, and activation of nuclear factor kappa B was suppressed in a concentration-dependent manner in bandshift assays. Moreover, proteasome inhibition (1 to 50 μmol/L MG132) induced apoptotic cell death up to 80%, as confirmed by DNA/Histone-ELISA and TUNEL-FACS analysis. Specificity of proteasome inhibition was shown by accumulation of multiubiquitylated proteins and accumulation of specific proteasomal substrates.

Conclusions—These proof-of-principle experiments demonstrate that inhibition of the ubiquitin-proteasome system effectively reduces neointima formation in vivo, which corresponds to strong antiproliferative, anti-inflammatory, and proapoptotic effects in vitro and in vivo. Our data suggest the ubiquitin-proteasome system as a new target in the prevention of vascular restenosis. (Circulation. 2002;105:483-489.)

Key Words: restenosis • apoptosis • inhibitors • inflammation

Despite remarkable advances in interventional cardiology, restenosis remains a major problem. A number of therapeutic approaches have failed—with the exception of paclitaxel and brachytherapy with intravascular irradiation—to demonstrate effectiveness in higher-animal models or in clinical trials. Further, the proteasome is required for activation of nuclear factor kappa B (NFκB), a central transcription factor that regulates inflammatory genes by degradation of its inhibitory IκB proteins. In addition, mediators of apoptosis such as p53, c-myc, and bcl2 are substrates of the proteasome. There is emerging evidence that inhibition of the proteasome leads to cell-cycle arrest, inhibition of NFκB activation, and possibly to induction of apoptosis. Because proliferation, inflammation, and apoptosis also play a crucial role in neointima formation, we hypothesized that proteasome inhibition may exert antiproliferative, anti-inflammatory, and proapoptotic effects and that it may consequently represent a potent, broad-spectrum tool in the prevention of restenosis.

Methods

Cell Culture

Vascular smooth muscle cells (VSMCs) were prepared from carotid arteries of Wistar rats and cultivated as described elsewhere. Purity

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From Medizinische Klinik und Poliklinik (Kardiologie, Angiologie, Pneumologie), Charité, Campus Mitte, Humboldt-Universität zu Berlin, Germany (S.M., M.L., W.R., C.G., I.P., G.B., K.S.); Institut für Biochemie, Charité, Humboldt-Universität zu Berlin, Germany (P.-M.K.); and Schering AG, Berlin, Germany (P.M.).

Correspondence to Karl Stangl, MD, Medizinische Klinik und Poliklinik (Kardiologie, Angiologie, Pneumologie), Charité, Campus Mitte, Schumannstrasse 20/21, D-10117 Berlin, Germany. E-mail karl.stangl@charite.de

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of VSMC preparations was confirmed to be 95% by immunostaining with anti-smooth muscle α-actin antibodies (Sigma).

**Reagents**

MG132, MG262, ALLM, and clasto-Lactacystin β-Lactone were purchased from Calbiochem and prepared as 10 mmol/L stock solutions in DMSO. Human tumor necrosis factor (TNF) was obtained from PharMingen (Becton, Dickinson). Antibodies against PCNA (PC10), p53 (Pab 240), p21 (C19), and IκBα (C21), β (C20) were obtained from Santa Cruz Biotechnology. Anti-Ki67 antibody was purchased from Dianova, the anti-ED-1 antibody from Serotec, and the anti-ubiquitin antibody from DAKO. Antibodies against c-jun were kindly provided by W. Dubiel.

**VSMC Proliferation Assay**

Primary VSMCs were seeded onto 24-well plates. Cells were incubated for 5 minutes with MG132 (1, 10, and 100 μmol/L) or DMSO (0.1%), washed twice in PBS, and further cultivated. Proliferation was determined by daily counting of viable cells (trypan blue exclusion).

**Preparation of Cellular Extracts**

Cytoplasmic and nuclear extracts were prepared as described elsewhere.17 Total cellular extracts were prepared in RIPA buffer containing a protease-inhibitor cocktail (Complete, Roche Molecular Biochemicals). Protein content was determined with the use of the Bradford method (Bio-Rad).

**Western Blot Analysis**

Cytoplasmic or cellular VSMC extracts were separated on a 10% SDS-Laemmli gel, blotted onto nitrocellulose membranes, and...
Probe with specific antibodies. Antigens were detected with the enhanced-chemoluminescence system (Amersham-Pharmacia).

**Electrophoretic Mobility Shift Assay**

Nuclear binding of NF-κB was analyzed by electrophoretic mobility shift assays, with VSMC nuclear extracts used as described elsewhere.17

**Detection of Apoptosis**

Chromatin condensation was assessed by staining VSMCs with Hoechst Dye 333420 (DAPI, 10 μg/mL, Sigma) after treatment with 50 μmol/L MG132 or DMSO for 24 hours and fixation in 4% paraformaldehyde, pH 7.4.

For corresponding in vitro TUNEL staining, we used the In-Situ Cell Death Detection Kit, FITC (Roche Molecular Biochemicals). Apoptosis was analogously detected in vivo by the use of acetone-fixed cryosections of carotid arteries and by counterstaining with DAPI.

For FACS detection of TUNEL-positive cells, we stimulated the ApoDirect-Kit (PharMingen, Becton Dickinson), washed twice and harvested after 24 hours. We performed TUNEL staining with the ApoDirect-Kit (PharMingen, Becton Dickinson) and conducted FACS analysis with a Becton Dickinson FACS Calibur, with application of Cell Quest software.

DNA fragmentation was quantified in a sandwich ELISA by detection of histone-associated DNA. VSMCs were treated with one of the following: 1, 10, and 50 μmol/L MG132 or DMSO; staurosporine (1 μmol/L) for 24 hours; or with 1 μmol/L MG132 for 5 minutes. Both nonadherent as well as adherent cells were analyzed by means of the Cell-Death Detection ELISA™ assay (Roche Molecular Biochemicals).

**Immunofluorescence Analysis**

Cryosections of carotid arteries were stained for Ki67, PCNA, and ED-1: 8-mm-thick sections were fixed in ice-cold acetone for 10 minutes, blocked in TBST containing 10% FCS, and incubated with the antibodies overnight at 4°C. After washing, sections were incubated with Cy3-coupled secondary antibodies (Dianova), counterstained with DAPI, and embedded in mowiol (Calbiochem).

**Morphometric Analysis of Balloon-Injured Rat Carotid Arteries**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neointimal Area</th>
<th>Medial Area</th>
<th>Neointima/Media Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=14)</td>
<td>0.031±0.029</td>
<td>0.129±0.039</td>
<td>0.243±0.023</td>
</tr>
<tr>
<td>MG132 (n=15)</td>
<td>0.008±0.014</td>
<td>0.108±0.012</td>
<td>0.084±0.142</td>
</tr>
<tr>
<td>P</td>
<td>0.008</td>
<td>0.126</td>
<td>0.027</td>
</tr>
<tr>
<td>Decrease</td>
<td>−74%</td>
<td>−16%</td>
<td>−65%</td>
</tr>
</tbody>
</table>

**Figure 3.** Western blot analysis of accumulated multibiquitinylated proteins and specific proteasomal substrates on short-time stimulation of VSMCs with proteasome inhibitors. A, VSMCs were treated for 5 minutes with 1, 10, or 100 μmol/L MG132 or DMSO, washed, harvested after 12 hours, and analyzed for ubiquitin expression. B, VSMCs were treated for 5 minutes with 100 μmol/L MG132 (lanes 2, 5, 8), 100 μmol/L ALLM (lanes 3, 6, 9), or DMSO (lanes 1, 4, 7), harvested after 6, 12, or 24 hours, and analyzed for ubiquitin expression. C, VSMCs were treated for 5 minutes with 10 μmol/L MG262 (lanes 2, 4, 6) or DMSO (lanes 1, 3, 5), harvested after 6, 12, or 24 hours, and analyzed for ubiquitin expression. D, VSMCs were treated for 5 minutes with 100 μmol/L MG132 (lanes 2, 5, 8), 100 μmol/L ALLM (lanes 3, 6, 9), or DMSO (lanes 1, 4, 7), harvested after 6, 12, or 24 hours, and analyzed for expression of p53, c-jun, and p21.

**Rat Carotid Balloon Injury Model**

Animals were obtained and housed in conformity with international guidelines for the care and use of laboratory animals. Male Wistar rats (weight, 350 to 400 g; Charles River) were anesthetized with sodium pentobarbital, 60 mg/kg intraperitoneal. The left common carotid artery was denuded of endothelium as described elsewhere.18 For local drug delivery, 150 μL of 1 mmol/L MG132 (n=15) or DMSO (n=14) was administered for 5 minutes. At the indicated time points, animals were given an overdose of sodium pentobarbital. For morphometric analysis, arteries were perfusion-fixed with 4% paraformaldehyde and dissected. Sections of carotid artery were embedded in paraffin and stained with Verhoeff–van Gieson stain. Three representative sections per animal were chosen for blinded computerized morphometric analysis. We then calculated the areas covering the residual lumen, the media, and the neointima.

**Statistics**

Data are expressed as mean±SD unless otherwise indicated. For cell proliferation assay, significance was tested by comparison of regression coefficients of MG132 versus control groups. For DNA/Histone ELISA, dose dependency was calculated with Pearson’s test for parametric linear correlation. Neointima formation was compared by use of the Mann-Whitney U test. An error probability of P<0.05 was regarded as significant. We used SPSS 9.0 software for all statistical calculations.

**Results**

We assessed the role of the proteasome as a therapeutic target in the prevention of restenosis by using a hierarchical experimental setup. Initially, we functionally characterized proteasome inhibition with regard to proliferation, inflammation, and apoptosis in primary VSMCs of the rat carotid artery. We subsequently tested the effects of proteasome inhibition in a balloon injury model of the rat carotid artery and then analyzed the in vivo effects on proliferation, inflammation, and apoptosis. Specificity of proteasome inhibition was investigated by Western blot analysis.
MG132 and clasto-lactacystin (Figure 1C). 0.23, 10 \( \text{P/H11005} \) the first 2 days \( \text{P/H11005} 0.003 \). resulted in loss of adherent VSMCs by no less than 77% during 

Effects of proteasome inhibition on NF\( \kappa \)B activation were studied by analyzing the TNF\( \alpha \)-mediated degradation of the cytosolic inhibitor proteins IkB\( \alpha \) and \( \beta \) by Western blotting and by electrophoretic mobility shift assay analysis of NF\( \kappa \)B (Figure 1, B and C). Figure 1B shows the dose-dependent stabilization of IkB\( \alpha \) and \( \beta \) by MG132. TNF\( \alpha \)-induced activation of NF\( \kappa \)B was blocked dose-dependently by MG132 and clasto-lactacystin (Figure 1C).

Induction of Apoptosis
Proapoptotic effects of proteasome inhibition were tested by several independent methods. DNA staining of MG132-treated VSMCs, for example, revealed nuclear condensation and TUNEL-positive staining (Figure 2A). MG132-induced apoptosis, furthermore, was quantified by DNA/Histone-ELISA (Figure 2B), which disclosed a dose-dependent increase of apoptotic cell death 12 to 16 times greater than control. Even short-time incubation with high concentrations of MG132 (1 mmol/L) over 5 minutes, as used in the rat injury in vivo model, increased apoptotic cell death by a factor of 22. Quantification by TUNEL-FACS analysis accordingly revealed induction of DNA fragmentation in 83% of the cells for this MG132 regimen (Figure 2C). For the DNA/Histone-ELISA and TUNEL-FACS analysis, staurosporine treatment served as a positive control for quantitative induction of apoptosis.

Specificity of Proteasome Inhibition
To prove specificity of proteasome inhibition of short-time incubation with high doses of MG132, we analyzed accumulation of multiubiquitinylated proteins and specific substrates of the proteasome. Figure 3A shows accumulation of multiubiquitinylated proteins after 5-minute stimulation of VSMCs with 1 to 100 \( \mu \text{mol/L} \) MG132 (lanes 2 to 4). Evidently, no less than a dose of 100 \( \mu \text{mol/L} \) MG132 resulted in accumulation of multiubiquitinylated proteins (see Figure 3A, lane 4), which peaked at 12 hours (Figure 3B, compare lanes 2, 5, and 8). Similarly, 10 \( \mu \text{mol/L} \) of the specific proteasome inhibitor MG262, a boronic acid derivate of MG132,\textsuperscript{19,20} effectively induced accumulation of multiubiquitinylated proteins with the same time kinetics (Figure 3C, lanes 2, 4, and 6). In contrast, 100 \( \mu \text{mol/L} \) of the nonproteasomal, cathepsin-specific inhibitor ALLM\textsuperscript{21} did not lead to detectable accumulation of multiubiquitinylated proteins (Figure 3B, lanes 3, 6, and 9). Moreover, a 5-minute stimulation with 100 \( \mu \text{mol/L} \) MG132 resulted in accumulation of c-\textit{jun}, p21, and p53, which are known substrates of the proteasome (Figure 3D).

Prevention of Restenosis
We applied in vivo testing in a rat balloon injury model to determine the capability of proteasome inhibition to prevent restenosis. Local administration of 1 mmol/L MG132 for 5 minutes resulted in a 74% decrease \( (P=0.008) \) of neointima formation, as measured by neointimal area, and in a 65% decrease \( (P=0.027) \) of the neointima/media ratio (Table). Figure 4 depicts representative histological sections from control and MG132-treated animals and shows characteristic vessel wall structures with variable degrees of restenosis.

In Vivo Analysis
To understand the mechanism of proteasome inhibition in vivo, we investigated proliferation, inflammation, and apoptosis in rat carotid arteries (Figures 5 and 6).

To enable detection of proliferating cells, we stained cryosections of carotid arteries for the nuclear antigen Ki67, which is found in the outer parts of the nucleolus during late G1, G2, and M phases of the cell cycle. Figures 5A and B reveal distinct Ki67-positive staining after 14 days in control animals (Figure 5A), in contrast to animals treated with MG132 (Figure 5B).
Reduced proliferation in animals treated with proteasome inhibitor was confirmed by staining for a second proliferation marker, PCNA. For quantification, see Figure 5E.

Staining of the glycoprotein ED-1, which is expressed predominantly on the lysosomal membrane of tissue macrophages, disclosed pronounced macrophage infiltration in control animals after 14 days (Figure 5C), in contrast to the group treated with MG132 (Figure 5D). This result indicates that inflammation is reduced in animals treated with proteasome inhibitor (for quantification, see Figure 5E).

Apoptosis was analyzed by TUNEL staining of carotid arteries at different time points: Figure 6 (A and B) shows initial apoptosis in both groups after 30 minutes. Whereas TUNEL-positive cells in controls were no longer evident after 6 hours (Figure 6C), we observed distinct TUNEL staining after 6 hours after MG132 treatment (Figure 6D); that is, a result that indicates prolonged apoptosis. After 14 days we no longer found TUNEL-positive staining in either of the groups (Figure 6, E and F).

**Discussion**

This study shows for the first time the potential role of the ubiquitin-proteasome system as a therapeutic target in the prevention of restenosis. Our key finding may be summarized as follows: Inhibition of the proteasome system effectively prevents restenosis in the rat. In vitro and in vivo, proteasome inhibition exerts strong antiproliferative, antiinflammatory, and proapoptotic effects.

**Proliferation, Inflammation, and Apoptosis In Vitro and In Vivo**

Our proliferation assays clearly demonstrate dose-dependent inhibition of VSMC growth by MG132 (Figure 1A). These findings concur with those of Thyberg and Blomgren, who have shown...
that proteasome inhibitors not only block VSMC proliferation but suppress the transition of VSMCs from a contractile to a synthetic phenotype in vitro. Our in vitro data—that is, results with pronounced antiproliferative effects—were confirmed in vivo when rats treated with MG132 showed a markedly reduced proliferation rate in the injured carotid arteries.

Our second goal, inhibition of inflammation, was effectively achieved in vitro and in vivo. Activation of NFκB, a central mediator of inflammatory signals in restenosis, was efficiently blocked by MG132 in VSMCs. Moreover, we observed decreased macrophage infiltration into the vessel wall of animals treated with MG132, which indicates reduced inflammatory response.

The ubiquitin-proteasome system is involved in regulation of apoptosis: Several apoptotic accelerators such as p53 and c-myc—but also antiapoptotic mediators such as bcl-2 and inhibitors of apoptosis—are substrates of the proteasome. Moreover, suppression of the nuclear translocation of NFκB by proteasome inhibition eliminates its antiapoptotic properties. As an overall effect, these mechanisms apparently lead to conditions that favor apoptosis in VSMCs, as we were able to demonstrate in vitro and in vivo. In vivo, we observed initial apoptosis as described by Perlman et al in control and MG132-treated groups. However, MG132 treatment resulted in prolonged apoptosis that was still evident after 6 hours. Since apoptosis has been reported to suppress neointima formation (reviewed in Reference 30), the prolonged proapoptotic activity in the injured vessel wall observed with MG132 adds another favorable aspect to the therapeutic principle of proteasome inhibition.

Specificity of Proteasome Inhibition

The MG132 doses used in vivo and in a number of vitro experiments were greater by factors of 10 to 100 than doses for which specific proteasome inhibition was demonstrated in cell systems for incubation periods of several hours. Our rationale for this selection of high doses was the intention to imitate the animal experiments by means of short-time incubation of MG132 by stimulation of VSMCs for 5 minutes. To compensate for this short exposure time, we increased MG132 doses by the factors stated above. In this context, one may well question the specificity of these high doses. We provided proof of specificity, however, through several approaches. First, with incubation time of 5 minutes, accumulation of multiubiquitinylated proteins was achieved only with 100 μmol/L MG132 and with 10 μmol/L of the more specific boronate derivate MG262 and not with similar doses of the cathepsin-specific inhibitor ALLM. Moreover, only MG132 and not ALLM induced accumulation of specific substrates of the proteasomes that play important roles in proliferation (c-jun and p21) and apoptosis (p53). Although we cannot completely preclude the possibility that high doses of MG132 have unspecific targets, our MG132 data are confirmed by MG262, a peptide boronate that is not known to affect other types of proteases.

Prevention of Restenosis

In the well-established restenosis model of the rat carotid artery, local administration of the proteasome inhibitor MG132 for only 5 minutes leads to marked reduction of neointima formation. With 74% inhibition of neointimal hyperplasia, our approach, directed to the ubiquitin-proteasome system, is evidently highly effective. Notably, we did not observe systemic side effects in the rats after local application of 1 mmol/L MG132. Moreover, histological analysis of treated vessels revealed an intact vessel wall architecture and no alteration in overall cellular morphology (Figure 4). These promising results in the rat, however, require further confirmation in other animal models.

In conclusion, our data identify the ubiquitin-proteasome system as a novel therapeutic target in the prevention of vascular restenosis. Specific inhibitors of the proteasome
complex can be locally delivered in high concentrations in catheter-based therapy. They indeed appear to be promising tools, owing to their antiproliferative, anti-inflammatory, and proapoptotic properties.

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