PROTEIN KINASE C PATHWAYS AND THE DEVELOPMENT OF VASCULAR DISEASES

EGF Receptor Activity Modulates Apoptosis Induced by Inhibition of the Proteasome of Vascular Smooth Muscle Cells

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The observation that intracellular protein turnover rates participate directly in cell viability led to the development and clinical use of potent proteasome inhibitors. This study determined that the mechanism of apoptosis that is induced by inhibition of the proteasome of vascular smooth muscle cells (VSMC) was related to the intracellular accumulation of Bad, a BH3-only member of the Bcl-2 family of apoptosis regulators. Experiments confirmed that the apoptotic process was mitochondria- and caspase-dependent. Ubiquitination and accumulation of Bad in VSMC followed inhibition of the proteasome, and depletion of Bad using RNA interference prevented apoptosis that was induced by proteasome inhibition with PS-341. EGF receptor (EGFR) activation produced posttranslational modifications of Bad, providing the pro-survival signals that prevented apoptosis of smooth muscle cells during proteasome inhibition. Antagonists of the EGFR potentiated the apoptotic rate. In summary, the activities of the EGFR and the proteasome focused on Bad and the intrinsic apoptotic pathway and were used integrally in determining viability of VSMC. These findings might prove useful in the management of diseases in which proliferation of vascular smooth muscle cells plays a central role.


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Materials and Methods

Cell Culture and Experimental Conditions

The Institutional Animal Care and Use Committee at the University of Alabama at Birmingham approved this project. Primary cultures of...
VSMC were established by pooling thoracic aortas from male Sprague-Dawley rats, using standard enzymatic digestion techniques and culture conditions (29–31). The cells were grown in DMEM (Invitrogen Life Technology, Carlsbad, CA), supplemented with 10% FBS, in a mixture of humidified 5% CO₂/95% air. VSMC were used between the fourth and 12th subpassages. Exponentially growing cells were seeded at a density of 2 × 10⁵ cells/ml into new flasks 1 d before initiation of each experiment. All experiments were performed in serum-containing medium.

VSMC were incubated for 16 h in medium that contained PS-341 (Bortezomib) in concentrations between 0 and 1000 nM, with most experiments using 0.8 μM. In some experiments, various inhibitors were added at the time of addition of PS-341. 1,4-Diamino-2,3-dicyano-1, 4-bis[2-aminoethylthio]butadiene (U0126; Cell Signaling Technology, Beverly, MA), 10 μM, is a highly selective, noncompetitive inhibitor of MAPK kinase (MEK1/2) family members (32). 2-(4-Morpholino)-8-phenyl-4H-1-benzopyran-4-1 (LY294002; Cell Signaling Technology), 10 μM, is a highly specific inhibitor of PI3K (33). 4-(3-Chloroanilino)-6,7-dimethoxyquinazoline (Tyrophostin AG-1478; Calbiochem EMD Biosciences, La Jolla, CA), 20 μM, is a reversible inhibitor of EGFR (34,35), and 4-(3-bromophenylamino)-6-acrylamidoquinazoline (PD-168393; Calbiochem EMD Biosciences), 10 μM, is an irreversible inhibitor of EGFR (36). DEVD-CHO (Calbiochem EMD Biosciences), 1 to 50 μM, and Q-VD-OPh (R&D Systems, Minneapolis, MN), 1 to 50 μM, served as cell-permeable, irreversible, selective caspase-3 and nonspecific caspase inhibitors, respectively (37,38). Cyclosporin A (CsA; Calbiochem EMD Biosciences), 1 μM, inhibits the mitochondrial permeability transition pore (39). EGF (Cell Signaling Technology) was added in a final concentration of 100 ng/ml at the initiation of the experiment and again 1 h before termination of the experiment. As control groups, VSMC were incubated for the same times in medium that contained the same volumes of dimers or in medium that contained only U0126, LY294002, AG-1478, PD-168393, DEVD-CHO, Q-VD-OPh, or CsA.

**Evaluation of Apoptosis**

The percentage of apoptotic cells in each population of VSMC was determined by flow cytometry (Model BD LSR II; BD Biosciences, San Jose, CA) and vital staining with the use of a kit (Vybrant Apoptosis Assay Kit #11; Molecular Probes Eugene, OR). The kit contained recombination annexin V, conjugated to Alexa Fluor 488 dye, and 1H,5H,11H,15H-xanthene[2,3,4-ij:5,6-ij’]diquinoliniz-18-ium,9-[4 (chloromethyl)phenyl]-2,3,6,7,12,13,16,17-octahydro-chloride (MitoTracker Red, Molecular Probes, Eugene, OR). At the end of the incubation period, VSMC, approximately 5 × 10⁶ cells/ml, were stained according to the manufacturer’s instructions, with incubation in culture medium that contained 4 μl of 10 μM MitoTracker Red for 30 min at 37°C in a mixture of 5% CO₂/95% air. After washing in PBS, the cells were resuspended in 100 μl of Annexin binding buffer with 5 μl of Alexa Fluor 488 annexin V. The cells were incubated for 15 min at room temperature in the dark and then diluted and immediately analyzed by flow cytometry. For fluorescence microscopy, VSMC were grown on chamber slides and then incubated overnight in the experimental conditions described above. After staining with MitoTracker Red and Alexa Fluor 488 annexin V, the cells were fixed in 3.7% paraformaldehyde/PBS for 20 min. Cells were washed and coated with 100 μl of AntiFade reagent (Molecular Probes) and visualized using a fluorescence microscope (Leica, Heidelberg, Germany) equipped with a digital camera (Model CS3810; Hamamatsu Photonics K.K., Hamamatsu, Japan) at magnifications up to ×100.

**Protein Extraction and Immunoblotting**

Cells were lysed in chilled lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.0% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 2.5 mM sodium pyrophosphate, 1 mM NaVO₄ and 1 mM PMSF), and the insoluble material was cleared by centrifugation. The samples were normalized for total protein content using a kit (Micro BCA Protein Assay Reagent Kit; Pierce, Rockford, IL). Proteins were separated by SDS-PAGE under reducing conditions and were blotted onto nitrocellulose membranes for Western analysis as described (40–42), using lysates that contained 60 μg of total protein. Antibodies that were used in these studies were obtained from commercial sources and included antibodies that were directed against EGFR (Cell Signaling Technology), phospho-Y1068-EGFR (Cell Signaling Technology), cleaved caspase-3 (Cell Signaling Technology), cleaved caspase-9 (Cell Signaling Technology), phospho-S112-Bad (Cell Signaling Technology), cytochrome c (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-S136-Bad (Santa Cruz Biotechnology), Bad (Santa Cruz Biotechnology), and β-actin (Sigma Chemical Co., St. Louis, MO).

**Caspase Activity Assay**

VSMC were collected after each treatment protocol by scraping and lysed in cell lysis buffer on ice. Caspase activity was determined by an enzymatic assay, using fluorogenic peptides that served as substrates for caspase-3 (Ac-DEVAD-AMC) and caspase-9 (Ac-LEHD-AMC; both from Alexis Biochemicals, San Diego, CA) (37). The lysates were centrifuged at 12,000 × g for 5 min at 4°C. The supernatant fractions, which contained approximately 100 μg of protein in 100 μl) were added into individual wells of a 96-well plate, along with 100 μl of 2× caspase reaction buffer (40 mM HEPES [pH 7.5], 20% glycerol, and 4 mM dithiothreitol [DTT]) that contained the substrate in a final concentration of 50 μM. After incubation for 2 h at 37°C, production of AMC was quantified in relative fluorescence units at an excitation wavelength of 380 nm and emission wavelength of 460 nm, using a microplate fluorescence reader (Packard Fusion Universal Microplate Analyzer; Packard Biosciences, Meriden, CT). Background fluorescence was determined by using 100 μl of lysates and 100 μl of reaction buffer that contained 50 μM of substrate.

**Detection of Cytochrome c Release by Western Blotting**

Release of cytochrome c from mitochondria into cytosol was determined using a previously published technique (43). Cells (5 × 10⁶) were washed with PBS and permeabilized by incubation for 30 min at 4°C in 150 μl of PBS that contained 1 unit/μl streptolysin O (Sigma Chemical Co.), 1 mM PMSF, and 0.01% BSA. Cytochrome c levels in supernatant were determined by Western blotting using an anti-cytochrome c antibody (Santa Cruz Biotechnology).

**Ubiquitination of Bad in VSMC**

Because Bad is a cytosolic protein and member of the Bcl-2 family, whose levels are known to be regulated by the proteasome in other cells (18,44–47), ubiquitination of Bad was predicted and was determined in VSMC that were exposed to PS-341 or medium alone. The protocol was similar to that used by this laboratory previously (31). VSMC were exposed to PS-341, 0.8 μM, or medium alone for 6 h. Lysates were prepared by resuspending the cells in ice-cold hypotonic buffer (5 mM MgCl₂, 8 mM KCl, and 2 mM DTT in 20 mM Tris/HCl [pH 7.4]). Cytoplasmic protein extracts, 1 mg/ml, then were added to a reaction mix that contained 40 mM Tris-HCl (pH 7.6), 1 mM DTT, 10% glycerol, 1.0 pmol of [125I]ubiquitin (approximately 2 μCi), 1 μM ubiquitin aldehyde (100 ng/μl), 1 mg/ml methyl ubiquitin, 1 μM okadaic acid, and an ATP-regenerating system (2 mM ATP, 5 mM MgCl₂, 10 mM creatine phosphate, and 100 units/ml creatine phosphokinase). After
120 min of incubation, the reaction was halted by the addition of 200 μl of RIP buffer. Bad was immunoprecipitated from each sample using identical conditions with anti-Bad (Santa Cruz Biotechnology). A nonspecific IgG (SouthernBiotech, Birmingham, AL) served as a control for the immunoprecipitation reaction. The proteins were separated using 6% SDS-PAGE, and the gel was dried and exposed to X-ray film (Hawkins X-Ray Supply, Oneonta, AL).

**RNA Interference**

RNA interference (RNAi) was accomplished using small interfering RNA (siRNA) that targeted rat Bad. RNA duplexes that consisted of ratBad-specific sense and antisense RNA oligomers were synthesized commercially (M-093327-00; Dharmacon RNA Technologies, Lafayette, CO). VSMC at 70 to 80% confluence were transfected using a Dharmacon siRNA transfection reagent (DharmaFECT4; Dharmacon RNA Technologies).  

**Figure 1.** Representative photomicrographs of vascular smooth muscle cells (VSMC) exposed to the conditions of the study. Overnight incubation of cells with EGF (100 ng/ml) or PD-168393 (10 μM) did not alter cellular morphology, compared with cells incubated in medium alone (no treatment). Incubation with PS-341 (10 to 1000 μM) produced dosage-dependent increases in detached cells (arrows). In contrast, incubation of VSMC in medium that contained both PS-341 (800 nM) and EGF (100 ng/ml) prevented cell death, unless either U0126 (10 μM) or LY294002 (10 μM) was added simultaneously with EGF. PS-341 (800 nM) and PD-168393 (10 μM) together increased cell death. The last three photomicrographs are representative intravital immunofluorescence stains of adherent VSMC exposed to PS-341 (800 nM), using MitoTracker Red and annexin V conjugated to Alexa Fluor 488. Annexin V labeling, indicative of translocation of phosphatidylserine, was prominent in several cells after overnight incubation in medium that contained PS-341 and confirmed a role for apoptosis in this process. Bar = 20 μm.
chemiluminescence was captured using the VersaDoc imaging system

Phosphatase–conjugated anti-DIG antibody and CDP-Star reagent; 

in 0.1

bridged in DIG Easy Hybridization Buffer with DIG-labeled probes at

sequence of the 399-bp product was confirmed. Membranes were hy-

GAGGCTTTGTCGCATCTGTGTTGC-3

Western analysis proceeded in a standard manner as described in

with 2

fection efficiency and minimized toxicity. Bad siRNA was complexed

siTOX transfection control (Dharmacon RNA Technologies) were used

siRNA duplex (siCONTROL Non-Targeting siRNA; Dharmacon RNA

As an additional control, VSMC were transfected with an irrelevant

siRNA from each sample underwent electrophoresis in 1.2% agarose gels

thiocyanate-phenol-chloroform extraction. Twenty micrograms of total

EGF (100 ng/ml) promoted autophosphorylation of EGFR both

conditions listed below each lane of the gel. Incubation with

EGF (100 ng/ml) increased EGFR protein content in

PS-341 alone; *P < 0.05 versus PS-341 alone; *P < 0.05 versus
groups of VSMC incubated in PS-341 in dosages ≤100 nM.

Statistical Analyses

Data were expressed as mean ± SE. Significant differences among
data sets were determined by ANOVA with standard post hoc testing
(Statview, version 5.0; SAS Institute, Cary, NC) or unpaired t test,
where appropriate. P < 0.05 assigned statistical significance.

Results

Proteosome Inhibition Induced Apoptosis Modulated by
EGFR Activity

Primary cultures of subconfluent rat VSMC were incubated
overnight (16 h) in medium that contained PS-341 in concen-
trations that ranged between 10 and 1000 nM. A dosage-depen-
dent increase in detached cells was observed, with concentra-
tions as low as 10 nM demonstrating an effect (Figure 1); sub-
sequent experiments used 0.8 μM. Overnight (16 h) incuba-
tion in PS-341 (0.8 μM) increased EGFR protein content in
VSMC (Figure 2). Co-incubation of VSMC with EGF increased
autophosphorylation of EGFR and reversed PS-341–induced
cell detachment. PD-168393 (10 μM), a cell-permeable irrever-
sible inhibitor of EGFR (36), effectively prevented autophos-
phorylation of EGFR even in the presence of additional EGF
(Figure 2) and further increased cell detachment that was in-
duced by PS-341 (0.8 μM; Figure 1). PS-341 induced a dosage-
dependent increase in caspase-3 enzyme activity, and addition
of PD-168393 (10 μM) produced further increases in caspase-3
enzyme activity, as the dosage of PS-341 increased to ≥100 nM
(Figure 3). Overnight (16 h) incubation of VSMC with PD-
168393 (10 μM) alone did not increase cell detachment (Figure 1) or activate caspase-3 (Figure 3). Flow cytometry using (MitoTracker Red) and Annexin V quantified the apoptotic process (Figure 4). Apoptosis, determined by flow cytometry as the percentage of total number of cells that were Annexin V positive (indicating phosphatidylserine translocation) and MitoTracker Red negative (indicative of loss of mitochondrial respiratory activity), increased with addition of PS-341 and was inhibited by co-administration of EGF (Figure 4, Table 1). Furthermore, the antiapoptotic effect of EGF was lost with addition of the MEK1/2 inhibitor U0126 (10 μM) or the PI3K-specific inhibitor LY294002 (10 μM) simultaneously with EGF (Figures 1 and 4). The antiapoptotic effect of EGF also was lost with addition of the EGFR inhibitors PD-168393 and AG-1478 (Table 1). PD-168393 is an irreversible inhibitor of EGFR (36), whereas AG-1478 is a competitive inhibitor (34). In the concentration that was used in our study, AG-1478 did not completely prevent activation of EGFR (Figure 2). PS-341 also increased the numbers of dead VSMC (i.e., cells that lost mitochondrial respiratory activity and were Annexin V negative [quadrant 3]). Co-administration of EGF with PS-341 also decreased the numbers of VSMC in this quadrant. Presumably, the mechanism was related to inhibition of PS-341-mediated apoptosis by EGF.

Mechanism of Apoptosis Related to Proteasome Inhibition Was Mitochondria- and Caspase-Dependent

Overnight incubation of VSMC with PS-341 (0.8 μM) induced increases in the cytoplasmic concentration of cytochrome c (Figure 5). Addition of EGF decreased levels of cytoplasmic cytochrome c (Figure 5) and activated caspase-9 and caspase-3 (Figure 6); these antiapoptotic effects were lost with addition of U0126, LY294002, and the EGFR inhibitors AG-1478 and PD-168393 (Figures 5 and 6). Addition of U0126, LY294002, and the EGFR inhibitors AG-1478 and PD-168393 alone did not increase the activities of caspase-9 and caspase-3, compared with control conditions (data not shown). Caspase-9 enzyme activity correlated well with caspase-3 activity in every condition tested (Figure 6). PS-341 therefore induced apoptosis of VSMC by cytochrome c release from mitochondria and activation of caspase-9 and caspase-3. These findings were confirmed by co-incubation of VSMC with PS-341 (0.8 μM) and CsA (1 μM), which has

![Figure 4. Flow cytometry of VSMC that were incubated in the conditions shown, using MitoTracker Red and annexin V conjugated to Alexa Fluor 488. Overnight incubation in PS-341 (0.8 μM) increased intensity of Annexin V fluorescence and decreased fluorescence intensity of MitoTracker Red in a subpopulation of VSMC, indicating apoptosis. Addition of PS-341 (0.8 μM) with EGF (100 ng/ml) prevented apoptosis, whereas addition with either PD-168393 (10 μM) or AG-1478 (20 μM) increased apoptosis. The antiapoptotic effect of EGF was lost with the simultaneous addition of either U0126 (10 μM) or LY294002 (10 μM).](image-url)
been shown to prevent opening of the mitochondrial permeability transition pore (39) and inhibited apoptosis that was induced by PS-341 (Figure 7). In separate experiments (n = 5 in each group), CsA (1 μM) reduced PS-341–induced caspase-3 activity (118 ± 5 versus 50 ± 5 relative fluorescence units, PS-341 with and without CsA, respectively; P < 0.05) to levels that did not differ from activity levels in untreated cells (38 ± 4; P > 0.05) and in cells that were treated only with CsA (42 ± 5; P > 0.05). A pan-caspase inhibitor (Q-VD-OPh) and a caspase-3 inhibitor (DEVD-CHO) also produced dosage-dependent reductions in PS-341–induced apoptosis in VSMC (Figure 7). In additional experiments (n = 4 to 9 in each group), co-incubation of PS-341–treated cells with Q-VD-OPh produced a dosage-dependent reduction in caspase-3 activity, with 0.1, 10, and 20 μM Q-VD-OPh lowering caspase-3 activity from 121 ± 12 (PS-341 alone) to 68 ± 4, 26 ± 0.4, and 25 ± 0.2, respectively (P < 0.05). Immunofluorescence analysis of PS-341–treated VSMC did not reveal evidence of nuclear accumulation of apoptosis-inducing factor in the apoptotic cells, suggesting no significant role for apoptosis-inducing factor in this process. Thus, inhibition of the proteasome promoted apoptosis by a mitochondria-dependent and caspase-dependent process.

Proteasome Inhibition Increased Bad Levels and EGFR Activity Enhanced the Phosphorylation State of Bad at S112 and S136

After 6 h of pretreatment of VSMC with the proteasome inhibitor (PS-341), cell lysates were incubated in buffer that contained [125I]ubiquitin and an ATP-generating system, and then Bad was immunoprecipitated from the cytoplasmic extract. Autoradiography of the electrophoresed immunoprecipitates demonstrated multiple labeled bands consistent with polyubiquitination of Bad from the treated but not untreated cells (Figure 8). By 6 h of pretreatment of VSMC, Bad protein levels increased (Figure 9). Co-incubation with both PS-341 and EGF accentuated the phosphorylation of Bad at S112 and S136. As predicted from other studies (48,49), U0126 decreased phosphorylation of Bad at S112, whereas LY294002 decreased phosphorylation of Bad at S136. Addition of the EGFR inhibitors AG-1478 and PD-168393 decreased phosphorylation of Bad at both serine residues (Figure 9). An antiapoptotic effect of EGF was observed by addition of EGF to the culture medium within 1 h before termination of experiments in which VSMC had been incubated with PS-341 overnight (data not shown). These findings provided additional support for a nongenomic ef-

### Table 1. Comparison of the percentage of VSMC segregated by flow cytometry into four quadrants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>88.1 ± 2.9</td>
<td>5.0 ± 2.3</td>
<td>3.8 ± 0.8</td>
<td>3.1 ± 1.0</td>
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<tr>
<td>EGF</td>
<td>93.8 ± 0.6</td>
<td>3.3 ± 0.4</td>
<td>2.3 ± 0.7</td>
<td>0.7 ± 0.1</td>
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<tr>
<td>U0126</td>
<td>88.8 ± 2.8</td>
<td>1.8 ± 0.2</td>
<td>7.3 ± 2.7</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>LY294002</td>
<td>87.8 ± 0.4</td>
<td>1.6 ± 0.6</td>
<td>8.8 ± 0.8</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>AG-1478</td>
<td>86.9 ± 0.5</td>
<td>1.8 ± 0.4</td>
<td>9.6 ± 0.1</td>
<td>1.6 ± 0.2</td>
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<tr>
<td>PD-168393</td>
<td>85.9 ± 1.3</td>
<td>1.8 ± 0.2</td>
<td>10.0 ± 0.6</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td><strong>P value</strong></td>
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<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>PS-341</td>
<td>41.6 ± 6.4</td>
<td>21.7 ± 2.7</td>
<td>19.9 ± 1.6</td>
<td>16.9 ± 4.4</td>
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<tr>
<td>PS-341 + EGF</td>
<td>86.1 ± 1.7</td>
<td>7.2 ± 1.0</td>
<td>1.9 ± 0.2</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td><strong>P value</strong></td>
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<td>&lt;0.05</td>
<td>&lt;0.05</td>
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<tr>
<td>PS-341 + U0126</td>
<td>58.9 ± 4.7</td>
<td>16.8 ± 3.5</td>
<td>15.1 ± 2.0</td>
<td>8.2 ± 1.6</td>
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<tr>
<td>PS-341 + EGF + U0126</td>
<td>54.4 ± 5.5</td>
<td>18.1 ± 4.6</td>
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<td>10.5 ± 1.8</td>
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<td><strong>P value</strong></td>
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<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
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<tr>
<td>PS-341 + LY294002</td>
<td>51.2 ± 2.7</td>
<td>18.1 ± 1.9</td>
<td>21.4 ± 5.0</td>
<td>13.1 ± 1.9</td>
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<tr>
<td>PS-341 + EGF + LY294002</td>
<td>58.3 ± 1.6</td>
<td>11.7 ± 2.6</td>
<td>21.7 ± 3.6</td>
<td>8.4 ± 0.6</td>
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<td><strong>P value</strong></td>
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<tr>
<td>PS-341 + AG-1478</td>
<td>42.4 ± 4.8</td>
<td>24.2 ± 3.3</td>
<td>20.6 ± 1.6</td>
<td>12.8 ± 1.9</td>
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<tr>
<td>PS-341 + EGF + AG-1478</td>
<td>42.9 ± 4.5</td>
<td>25.5 ± 4.5</td>
<td>18.9 ± 2.4</td>
<td>12.6 ± 2.2</td>
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<tr>
<td><strong>P value</strong></td>
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<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>PS-341 + PD-168393</td>
<td>29.1 ± 7.6</td>
<td>33.1 ± 4.6</td>
<td>15.5 ± 1.1</td>
<td>22.3 ± 4.3</td>
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<tr>
<td>PS-341 + EGF + PD-168393</td>
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<td>25.3 ± 4.9</td>
</tr>
<tr>
<td><strong>P value</strong></td>
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<td>&gt;0.05</td>
<td>&gt;0.05</td>
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</tbody>
</table>

*Quadrant 1 contained metabolically active, annexin V–negative cells; quadrant 2 contained metabolically active, annexin V–positive cells; quadrant 3 contained metabolically inactive, annexin V–negative cells; quadrant 4 contained metabolically inactive, annexin V–positive cells, consistent with apoptosis.

**p > 0.05 versus no treatment.

*P < 0.05 versus no treatment and EGF treatment.
The effect of EGFR activation in modulating apoptosis in the setting of proteasome inhibition.

Using RNAi, production of Bad protein initially was inhibited in VSMC and then followed by incubation in PS-341 for up to 72 h. By Northern analysis, addition of siRNA produced a dosage-dependent decrease in Bad mRNA by 48 h after initiation of transfection (Figure 10) and a corresponding dosage-dependent decrease in Bad, which persisted over the subsequent 72 h of incubation in PS-341 (Figure 11). Coinciding with the decrease in Bad expression was a dosage-dependent decrease in apoptosis that was induced by proteasome inhibition (Figure 11).

Figure 5. Cytochrome c release as determined by Western analysis and ELISA in VSMC in the various experimental conditions of the study. Incubation of VSMC in medium that contained PS-341 (0.8 μM) induced cytochrome c release, whereas the concomitant introduction of EGF (100 ng/ml) decreased \( P < 0.05 \) versus PS-341) cytochrome c release. This effect was inhibited by addition of U0126, LY294002, and the EGFR inhibitors (AG-1478 and PD-168393), indicating EGF-induced activation of both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways in prevention of cytochrome c release.

Figure 6. Effect of PS-341 on caspase-3 and caspase-9. Western analysis using antibodies that recognize the cleaved forms of the caspases (top) demonstrated activation of both caspases by PS-341 and potential improvement with EGF. Activities of caspase-9 and caspase-3 were quantified by ELISA (bottom) at the end of the incubation periods (\( n = 7 \) to 14 experiments in each group). Incubation of VSMC in medium that contained PS-341 (0.8 μM) increased activities of caspase-9 and caspase-3, whereas the simultaneous addition of EGF (100 ng/ml) decreased caspase activities to baseline. The antiapoptotic effect of EGF was inhibited by addition of U0126, LY294002, and the EGFR inhibitors (AG-1478 and PD-168393). None of these compounds alone increased the activities of caspase-9 and caspase-3, compared with control conditions (data not shown).

In each experimental condition, caspase-9 activity correlated well with caspase-3 activity.
Discussion

Our study determined the mechanism of apoptosis that was caused by inhibition of the proteasome in VSMC and an important role for EGF in modulation of this process. Findings of this study included the following: (1) PS-341 induced a dosage-dependent increase in apoptosis of VSMC in culture; (2) pro-

Figure 7. Flow cytometry of VSMC incubated in the conditions shown, using MitoTracker Red and annexin V conjugated to Alexa Fluor 488. The addition of cyclosporin A (CsA; 1 μM) inhibited PS-341–induced apoptosis. The pan-caspase inhibitor (Q-VD-OPh) and the selective caspase-3 inhibitor (DEVD-CHO) produced a dosage-dependent decrease in PS-341–induced apoptosis.

Figure 8. Autoradiographic image of the effect of proteasome inhibition on ubiquitination of Bad. Six hours after exposure of VSMC to PS-341 (0.8 μM), polyubiquitination of immunoprecipitated Bad, denoted by the multiple bands noted in the first lane, was observed. The third lane represents immunoprecipitated Bad from untreated VSMC; a few faint bands were observed, indicating that once Bad is ubiquitinated, it is degraded rapidly by the proteasome. No bands were apparent in the second and fourth lanes, which were immunoprecipitation controls that used a nonspecific IgG.

Figure 9. Bad, phospho-Bad (S112), and phospho-Bad (S136) levels were determined by Western analysis 6 h after addition of PS-341 and the various inhibitors. Bad and phospho-Bad levels were detected faintly in control experiments (lanes 1 and 2). Incubation of VSMC with PS-341 (0.8 μM) produced large increases in Bad levels (lanes 3 to 12). Bad levels fell with addition of EGF (lane 4), whereas phospho-Bad (S112) and phospho-Bad (S136) levels increased. U0126 (10 μM) and LY294002 (10 μM) inhibited phosphorylation at S112 and S136, respectively. AG-1476 (20 μM) and PD-168393 (10 μM) inhibited EGF-induced phosphorylation of Bad at S112 and S136.
Bad, a BH3-only member of the Bcl-2 family (50), activates the multidomain Bcl-2 family members, such as Bcl-X<sub>L</sub>, and triggers cytochrome c release by mitochondria and caspase-3 activation (51). Increased expression of Bad sensitizes cells to apoptosis that is related to withdrawal of growth factor stimulation (52) and produces apoptosis in VSMC (53). In this study, the proapoptotic effect of proteasome inhibition in VSMC depended on expression of Bad, because siRNA that targeted Bad inhibited the apoptosis that was associated with PS-341. The polyubiquitination of Bad and increase in Bad levels after proteasome inhibition demonstrated an important role for the proteasome in regulation of Bad levels in VSMC and was consistent with published studies that demonstrated ubiquitination of Bad in other cell types (45). The findings also were consistent with studies that showed that overexpression of Bad but not Bax sensitized mammalian epithelial cell lines to EGFR inhibition (54). The experimental results complement the report by Meiners et al. (2), who showed that the proteasome inhibitor (MG132) prevented activation of the NF-κB pathway in VSMC.

Cell survival signals from growth factor and cytokine receptors focus on posttranslational modifications of Bad. The proteasome inhibition increased total EGFR and Bad levels in VSMC; (3) the mechanism of apoptosis that was induced by PS-341 depended on Bad expression and was mitochondria- and caspase-dependent; and (4) the apoptotic effect of proteasome inhibition was modulated by EGF-induced signaling mechanisms that included both MEK1/2 and PI3K, which respectively promoted serine phosphorylation of Bad at positions 112 and 136. Despite increased expression of EGFR that was induced by proteasome inhibition, the net effect was development of a proapoptotic state under these conditions, unless the medium was supplemented with EGF. The results demonstrated an interaction between the proteasome and signaling through the EGFR in determining the fate of VSMC through Bad and the intrinsic apoptotic pathway.

One of the consequences of hypertension is vascular hypertrophy and arterial remodeling. Although once thought to be a physiologic response, more recent studies have challenged this concept. For example, the Dahl/Rapp salt-sensitive rat develops hypertension rapidly when placed on a diet that is high in salt content. Within 2 wk after development of hypertension, wall thicknesses of small arteries and arterioles of the kidney increase, coinciding with vascular smooth muscle proliferation, and luminal area progressively decreases. These changes are associated with overexpression of EGFR in VSMC (4). Progressive tissue hypoxia in the kidney results in loss of renal function in these rats, which die from kidney failure (3,60). Whether proteasome inhibition will permit control of this process and prevent progressive kidney failure has not been tested, but administration of PS-341 improved hypertension and inhibited development of vascular hypertrophy in rats with DOCA salt-induced hypertension (61,62). Our studies have identified an important interaction between EGFR and the proteasome in mediating apoptosis of VSMC and further suggest a novel therapeutic strategy for proteasome inhibitors, alone or in combination with EGFR inhibition, in the
management of hypertension and prevention of target-organ damage or in other disease processes, such as vascular restenosis, in which abnormal proliferation of vascular smooth muscle is involved integrally.

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Disclosures
None.

References

Figure 11. Results of RNA interference (RNAi) experiments designed to reduce Bad expression in VSMC. Cells were transfected with siRNA 48 h before addition of PS-341 (0.8 μM). During the 3-d incubation period, VSMC that did not receive siRNA showed progressive decreases in cell numbers from apoptosis. In contrast, transfection of VSMC with increasing dosages of siRNA produced a dosage-dependent decrease in apoptotic rates and increase in cell numbers despite continued incubation in PS-341. A compilation of three immunoblot assays (bottom left) verified a persistent dosage-dependent reduction in Bad protein levels that was induced by siRNA for the 3 d of incubation in PS-341. (C) siRNA (100 mM), directed against an irrelevant mRNA sequence, had no effect on Bad protein expression and cell death that was induced by PS-341 (data not shown). Flow cytometry (bottom right) using VSMC that were obtained 48 h after addition of PS-341 confirmed the reduction in apoptosis. The numbers in the right corner of each graph represent the percentage of the total VSMC that were localized to the right lower quadrant of the graph. VSMC that are labeled “No treatment” were exposed to transfection medium but not to PS-341.


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