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 involved 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.


Proliferation of vascular smooth muscle cells (VSMC) (1) is involved integrally in vascular stenosis after endothelial disruption (1,2) and in the development of hypertension-associated progressive renal injury (3,4). EGF is prominent in the list of potential growth factors and cytokines that might participate in this process. EGF receptor (EGFR; also known as HER1) is a receptor tyrosine kinase family member that is involved integrally in fundamental processes of growth and regulation of normal cell populations (5). VSMC express functional EGFR, and EGF induces a dosage-dependent increase in proliferation (6). EGFR has gained increasing attention and were involved 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.

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 \times 10^5 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 \mu M. In some experiments, various inhibitors were added at the time of addition of PS-341. 1,4-Diamino-2,3-di-cyan-1, 4-bis[2-aminophenylthio]butadiene (U0126; Cell Signaling Technology, Beverly, MA), 10 \mu M, is a highly selective, noncompetitive inhibitor of MAPK kinase (MEKI/1) family members (32). 2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-1 (LY294002; Cell Signaling Technology), 10 \mu M, is a highly specific inhibitor of PI3K (33). 4-(3-Chloroanilino)-6,7-dimethoxyquinazoline (Tyrophostin AG-1478; Calbiochem EMD Biosciences, La Jolla, CA), 20 \mu M, is a reversible inhibitor of EGFR (34,35), and 4-[3-bromophenylamino]-6-acrylamidoquinazoline (PD-168393; Calbiochem EMD Biosciences), 10 \mu M, is an irreversible inhibitor of EGFR (36). DEVD-CHO (Calbiochem EMD Biosciences), 1 to 10 \mu M, and Q-VD-Oph (R&D Systems, Minneapolis, MN), 1 to 50 \mu M, served as cell-permeable, irreversible, selective caspase-3 and nonselective caspase inhibitors, respectively (37,38). Cyclosporin A (CsA; Calbiochem EMD Biosciences), 1 \mu M, 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 dilituents 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 LSRII; BD Biosciences, San Jose, CA) and vital staining with the use of a kit (Vybrant Apoptosis Assay Kit #1; Molecular Probes Eugene, OR). The kit contained re-combinant annexin V, conjugated to Alexa Fluor 488 dye, and 1H,5H,11H,15H-xanthone[2,3,4-ij;5,6,7-i’j’]diquinolizin-18-ium,9-[4 (chloromethyl)phenyl]-2,3,6,7,12,13,16,17-octahydro-chloride (Mito-Tracker Red, Molecular Probes, Eugene, OR). At the end of the incubation period, VSMC, approximately 5 \times 10^6 cells/ml, were stained according to the manufacturer’s instructions, with incubation in culture medium that contained 4 \mu l of 10 \mu M 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 \mu l of Annexin binding buffer with 5 \mu l 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 \mu l of Antifade reagent (Molecular Probes) and visualized using a fluorescence microscope (Leica, Heidelberg, Germany) equipped with a digital camera (Model C5810; Hamamatsu Photonics K.K., Hamamatsu City, Japan) at magnifications up to \times 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 Na2VO4 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 \mu g 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 \beta-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-DEVDD-AMC) and caspase-9 (Ac-LEHD-AMC); both from Alexis Biochemicals, San Diego, CA (37). The lysates were centrifuged at 12,000 \times g for 5 min at 4°C. The supernatant fractions, which contained approximately 100 \mu g of protein in 100 \mu l were added into individual wells of a 96-well plate, along with 100 \mu l of 2 \times 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 \mu M. 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 \mu l of lysates and 100 \mu l of reaction buffer that contained 50 \mu M 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 \times 10^5) were washed with PBS and permeabilized by incubation for 30 min at 4°C in 150 \mu l of PBS that contained 1 unit/\mu l 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 \mu M, 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 [32p]ubiquitin (approximately 2 \mu Ci), 1 \mu M ubiquitin aldehyde (100 ng/\mu l), 1 mg/ml methyl ubiquitin, 1 \mu M okaaic 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 RIPA buffer. Bad was immunoprecipitated from each sample using identical conditions with anti-Bad (Santa Cruz Biotechnology). A non-specific 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 HXR 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 rat Bad-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](image)

*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 nM) 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 and phosphatase-conjugated anti-DIG antibody and CDP-Star reagent; in 0.1 M HEPES (pH 7.3) overnight. After hybridization, membranes finally were washed with 2× SSC/0.1% SDS. Bound probes were detected using alkaline phosphatase–conjugated anti-DIG antibody and CDP-Star reagent; chemiluminescence was captured using the VersaDoc imaging system.

Technologies) that contained varying amounts (0 to 100 nM) of siRNA. As an additional control, VSMC were transfected with an irrelevant siRNA duplex (siCONTROL Non-Targeting siRNA; Dharmacon RNA Technologies), using the same protocol. Preliminary experiments using siTOX transfection control (Dharmacon RNA Technologies) were used to determine the optimum exposure conditions that maximized transfection efficiency and minimized toxicity. Bad siRNA was complexed with 2 μl of DharmaFECT4 in 200-μl total volume and then added to complete medium in a final volume of 1 ml for each well in a 12-well plate. After incubation in the transfection solution for 12 h, the medium was replaced and incubation was continued up to 36 h. The cells then were treated with 0.8 μM PS-341 and incubated for an additional 24 to 72 h. Western analysis proceeded in a standard manner as described in the previous section, using samples that contained 40 μg of total protein. The membranes were probed using antibody directed against Bad (Santa Cruz Biotechnology). As controls, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in each sample was detected using antibody directed against GAPDH (Bio-Rad, Hercules, CA). The membranes then were stripped and rehybridized with digoxigenin-labeled GAPDH that was obtained through the American Type Culture Collection (Rockville, MD). The density of the GAPDH band in the same lane was used to control for potential differences in RNA loading.

Statistical Analyses

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

Results

Proteasome 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 concentrations that ranged between 10 and 1000 nM. A dosage-dependent increase in detached cells was observed, with concentrations as low as 10 nM demonstrating an effect (Figure 1); subsequent experiments used 0.8 μM. Overnight (16 h) incubation 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 irreversible inhibitor of EGFR (36), effectively prevented autophosphorylation of EGFR even in the presence of additional EGF (Figure 2) and further increased cell detachment that was induced 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 the 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 antia apoptotic 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

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**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).
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.

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% in Each Quadrant</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>No treatment</td>
<td>88.1 ± 2.9</td>
</tr>
<tr>
<td>EGF</td>
<td>93.8 ± 0.6</td>
</tr>
<tr>
<td>U0126</td>
<td>88.8 ± 2.8</td>
</tr>
<tr>
<td>LY294002</td>
<td>87.8 ± 0.4</td>
</tr>
<tr>
<td>AG-1478</td>
<td>86.9 ± 0.5</td>
</tr>
<tr>
<td>PD-168393</td>
<td>85.9 ± 1.3</td>
</tr>
<tr>
<td>P value</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>PS-341</td>
<td>41.6 ± 6.4</td>
</tr>
<tr>
<td>PS-341 + EGF</td>
<td>86.1 ± 1.7</td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>PS-341 + U0126</td>
<td>58.9 ± 4.7</td>
</tr>
<tr>
<td>PS-341 + EGF + U0126</td>
<td>54.4 ± 5.5</td>
</tr>
<tr>
<td>P value</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>PS-341 + LY294002</td>
<td>51.2 ± 2.7</td>
</tr>
<tr>
<td>PS-341 + EGF + LY294002</td>
<td>58.3 ± 1.6</td>
</tr>
<tr>
<td>P value</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>PS-341 + AG-1478</td>
<td>42.4 ± 4.8</td>
</tr>
<tr>
<td>PS-341 + EGF + AG-1478</td>
<td>42.9 ± 4.5</td>
</tr>
<tr>
<td>P value</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>PS-341 + PD-168393</td>
<td>29.1 ± 7.6</td>
</tr>
<tr>
<td>PS-341 + EGF + PD-168393</td>
<td>27.7 ± 7.0</td>
</tr>
<tr>
<td>P value</td>
<td>&gt;0.05</td>
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<sup>a</sup>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.

<sup>b</sup>P > 0.05 versus no treatment.

<sup>c</sup>P < 0.05 versus no treatment and EGF treatment.

**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 [125]lubiquitin 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 incubation in PS-341 (0.8 µM), 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-
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-

![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.](image)

![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.](image)

![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.](image)
Role of Bad in Apoptosis of Vascular Smooth Muscle

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. Progressive tissue hypoxia in the kidney results in loss of renal function in these rats, which die from kidney failure. 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. 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 treatment of vascular remodeling.

Figure 10. Northern analysis of VSMC 48 h after transfection using different amounts of small interfering RNA (siRNA) designed to reduce Bad expression. The first lane showed that transfection with nontargeting control siRNA did not alter Bad mRNA expression. Compared with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a dosage-dependent decrease in mRNA for Bad was observed using siRNA targeted against Bad, indicating a direct effect of the siRNA on steady-state mRNA levels of Bad.

Control (100 nM) | siRNA dose (nM)
--- | ---
100 | 10 | 1.0 | 0.1 | 0

Survival signal that is provided particularly by EGF depends on expression of Bad. The signal transduction mechanisms involve both PI3K and MEK1/2, with Akt activation promoting phosphorylation of Bad at the serine residue at position 136 (48) and the Ras-MAPK pathway phosphorylating the serine residue at position 112 (49). EGFR stimulation activates both pathways in VSMC. Serine-phosphorylated Bad binds to 14-3-3 in the cytoplasm, preventing interaction with Bcl-XL and inhibition of Bcl-XL function (55). Because Bad has tandem repeats that contain phosphoserine residues, binding of the doubly serine-phosphorylated Bad (at S112 and S136) to 14-3-3 is extremely tight (56). Only nonphosphorylated Bad induces apoptosis by forming a heterodimer with Bcl-XL through the BH3 domain, thereby promoting the mitochondrial release of cytochrome c (55,57). Thus, activation of both signaling pathways results in posttranslational modifications of Bad and permits the integration of antiapoptotic signals from growth factor receptors into the intrinsic apoptotic signal cascade. In these studies, activation of EGFR signaling cascades resulted in increases in the phosphorylation state of both S112 and S136 of Bad. Inhibition of either PI3K or MEK1/2 prevented the antiapoptotic effect of EGF. These findings agree with Hayakawa et al. (58), who demonstrated that inhibition of phosphorylation of Bad at either S112 or S136 sensitized an ovarian cancer cell line to cisplatin-mediated death. Our study also agrees with Jung et al. (59), who demonstrated that hydrogen peroxide–induced apoptosis of VSMC was prevented by addition of FCS, which activated both PI3K and MEK1/2. Bai et al. (53) also demonstrated that IGF-I–mediated inhibition of apoptosis of VSMC involved Akt-mediated phosphorylation of Bad. Recently, Gilmore et al. (54) demonstrated that the IGF-I receptor transactivated EGFR to permit activation of the Ras-MAPK pathway, which, along with the PI3K-activated pathway, participated in limiting apoptosis in mammary epithelial cell lines. Both pathways therefore seem to participate in conveying the antiapoptotic signal after growth factor receptor stimulation, particularly EGF and IGF-I.

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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.

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