

# PR39 Inhibits Apoptosis in Hypoxic Endothelial Cells

## Role of Inhibitor Apoptosis Protein-2

Jiaping Wu, MD; Cherie Parungo, MD\*; Guifu Wu, MD, PhD\*; Peter M. Kang, MD; Roger J. Laham, MD; Frank W. Sellke, MD; Michael Simons, MD; Jian Li, MD, PhD

**Background**—PR39 is a proline- and arginine-rich peptide implicated in wound healing and myocardial ischemia protection. To determine the potential mechanisms of PR39 in ischemia, we examined the role of PR39 in hypoxia-induced apoptosis in vascular endothelial cells.

**Methods and Results**—Hypoxia results in an increase of apoptosis in bovine aortic endothelial cells (BAECs), as determined by terminal deoxynucleotidyl transferase-mediated dUTP biotin nick-end labeling (TUNEL) analysis and caspase-3 activity. Hypoxia induced  $66.2 \pm 2.7\%$  TUNEL-positive cells, whereas in the presence of synthesized PR39 peptide, TUNEL-positive cells were reduced to  $29.6 \pm 1.9\%$  ( $P < 0.05$ ). After 24 hours of hypoxia, the addition of PR39 reduced caspase-3 activity to  $3.17 \pm 0.47$  pMol/min from  $10.52 \pm 0.55$  pMol/min in hypoxic BAECs. Moreover, PR39 increased inhibitor of apoptosis protein-2 (IAP-2) gene and protein expression by 3-fold in a time- and dose-dependent manner. The induction of IAP-2 by PR39 conferred an increase in IAP-2 gene transcription and IAP-2 mRNA stability. Furthermore, inhibiting IAP-2 with second mitochondria-derived activator of caspase (Smac) and with small interfering RNA targeting IAP-2 abrogated the ability of PR39 to reduce caspase-3 activity.

**Conclusions**—We provide the first direct evidence for PR39 as an antiapoptotic factor in endothelial cells during hypoxia. These data suggest that PR39 inhibits hypoxia-induced apoptosis and decreases caspase-3 activity in endothelial cells through an increase of IAP-2 expression. (*Circulation*. 2004;109:1660-1667.)

**Key Words:** peptides ■ hypoxia ■ apoptosis ■ cells, endothelial

Apoptosis is critical for various physiological and pathological processes. Mounting evidence strongly suggests that vascular endothelial cells are impaired through apoptosis, contributing to the overall endothelial dysfunction in a range of clinic settings, including ischemic heart diseases.<sup>1</sup> Therefore, preventing endothelial cell apoptosis could result in improved angiogenesis and endothelial function in patients with myocardial ischemia.<sup>2</sup> Consequently, understanding the pathways involved in vascular endothelial cell apoptosis and identifying strategies to inhibit this process would have important clinical implications.

Antiapoptosis is a complex process involving several factors. Two major protein families have been delineated. Proteins of the Bcl-2 family are major regulators of the mitochondria-initiated caspase activation pathway,<sup>3</sup> whereas inhibitor of apoptosis protein (IAP) regulates the cytochrome c/Apaf-1 caspase-activating pathway. Endothelial cells stimulated by severe hypoxia/anoxia increase IAP-2 and become resistant to apoptosis.<sup>4</sup> Although some cytokines, such as

vascular endothelial growth factor (VEGF)<sup>5</sup> and fibroblast growth factor-2 (FGF-2),<sup>6</sup> have been reported to have antiapoptotic properties in vascular endothelial cells, little is known about hypoxic regulation of genes that are directly involved in cell death or death resistance.

We report here that a novel peptide, PR39, has an ability to inhibit apoptosis in endothelial cells. PR39, a proline (P)- and arginine (R)-rich peptide with 39 amino acids, was originally isolated from pig intestine and identified in neutrophil azurophilic granules and macrophages.<sup>7,8</sup> Recently, the peptide has been found to induce syndecan expression in mesenchymal cells and to influence cell motility and metastatic potential in wound repair.<sup>9</sup> The peptide binds to the cytosolic component of NADPH oxidase complex protein p47<sup>phox</sup><sup>7</sup> and a signaling adaptor protein p130<sup>Cas</sup>. Previously we reported that PR39 plays a critical role in limiting cardiac injury through induction of angiogenesis.<sup>10</sup> However, that could not entirely explain the mechanism of the cardiovascular-protective effect of PR39. Accordingly, we explore the role of PR39 in

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From the Angiogenesis Research Center, Divisions of Cardiology (J.W., C.P., G.W., P.M.K., R.J.L., J.L.) and Cardiothoracic Surgery (F.W.S.), Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Mass, and Section of Cardiology, Dartmouth-Hitchcock Medical Center, Dartmouth Medical School, Hanover, NH (M.S.).

\*Drs C. Parungo and G. Wu contributed equally to this work.

Correspondence to Jian Li, MD, PhD, Division of Cardiology, Beth Israel Deaconess Medical Center, Harvard Medical School, 330 Brookline Ave, Boston, MA 02215. E-mail jli@bidmc.harvard.edu

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apoptosis in vascular endothelial cells. We examined the effect of administering PR39 in hypoxia-provoked terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL)-positive cells and caspase-3 activity and analyzed the apoptotic-related gene and proteins induced by PR39. This study provides the first evidence indicating that PR39, as an antiapoptotic factor, plays an important role in hypoxia-induced apoptosis underlying the mechanisms of PR39 in limiting infarct size and ischemia/reperfusion injury in the heart.

## Methods

### Cell Culture and Hypoxia

Bovine aortic endothelial cells (BAECs) were harvested from bovine aorta by enzymatic dissociation. The cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. BAECs were passed every 4 to 5 days, and experiments were performed on cells in 3 to 4 passages. After cells had grown to confluence, they were placed in a quiescent medium (0.5% fetal bovine serum) for 16 hours. Hypoxia was induced with the use of a modular incubator chamber (Billups-Rothenberg). The oxygen level in the chamber was monitored with an oxygen analyzer (Vascular Technology Inc), and it remained at 1% to 3% O<sub>2</sub> for up to 72 hours.

### Peptide Synthesis

PR39 peptide was synthesized and purified by high-performance liquid chromatography (Genemed Synthesis Inc), dissolved in PBS, and stored at -20°C until use. The control peptide, PR39 scrambled sequence order (PR39-SO), was synthesized with the same amino acids as PR39 but in a randomly scrambled order, keeping the same positive charge as PR39. The compared sequences are as follows: PR39: RRRPRPPYLPRRPPPPFFPRLPRIPPGFPPRFPFRFP; PR39-SO: RRRPRPFPGPIPPFPPLRPPFPFPRPRPYRPLR.

### Immunofluorescent Study

BAECs were cultured in chamber slides and fixed in 4% paraformaldehyde. The slides were incubated with an antibody against PR39 (1:500, Mabtech) and biotinylated antibody against mouse IgG (1:400, Vector Laboratory), followed by streptavidin (1:200, Amersham). For localization of synthesized PR39 in endothelial cells, PR39 was synthesized with hemagglutinin protein (HA) (YPYDVP-DYA) tag on the c-terminus. After incubation of PR39-HA for 6 hours, slides were stained with anti-PR39 or anti-HA antibody (Roche) and photographed with a confocal microscope (BioRad).

### Apoptotic Analysis

The TUNEL method was performed with the use of the TUNEL detection kit (Roche) according to the manufacturer's instructions. BAECs were cultured in chamber slides and double labeled with ToPro-3 (nuclei label, Molecular Probe) and TUNEL kits. Sequential images were obtained from 5 random fields per slides. Cells stained with both TUNEL and ToPro-3 were considered positive for apoptosis. Apoptotic BAECs were also quantified by flow cytometry after the TUNEL staining. A total of 10 000 cells were analyzed from each sample. Data analysis was performed with Multicycle software for Coulter flow cytometry. Caspase-3 activity in cell extracts was measured with a caspase-3 cellular activity assay kit (Calbiochem) according to the manufacturer's directions. Caspase-3 activity was expressed as picomoles  $\rho$ -nitroaniline released per minute per microgram cellular protein.

### RNA Blot Hybridization

Total RNA was extracted from cells by TriReagent (Sigma). The RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose membrane, then hybridized at 68°C for 3 hours with a random-primed, <sup>32</sup>P-labeled IAP-2 cDNA probe in QuikHyb solution (Stratagene). The IAP-2 probe was prepared by

reverse transcription-polymerase chain reaction (PCR) with 5'-AGT CTT GCT CGT GCT GGT TT-3' and 5'-ATT CGA GCT GCA TGT GTC TG-3', corresponding to 433 to 1055 in human IAP-2 cDNA sequence.

### Western Blot Analysis

Whole-cell lysates were obtained from cultured cells with RIPA solution (Boston Bioproducts Inc). Sixty micrograms of total proteins was fractionated by 10% or 15% SDS-polyacrylamide gel (for PR39) and transferred to polyvinylidene fluoride membranes (Millipore). PR39 was detected by monoclonal anti-PR39 antibody (a gift from Dr Ross, Kansas State University). Anti-IAP-2, anti-IAP-1, and anti-BAX (Santa Cruz); anti-Bcl-2 and anti-XIAP (Transduction Laboratories); and anti-second mitochondria-derived activator of caspase (Smac)/DIABLO (Imgenex) antibodies were used. After incubation of anti-IgG for 1 hour, the proteins were visualized with an ECL detection system (Amersham).

### RNA Stability Assay

Actinomycin D (ACD) (5  $\mu$ g/mL) was added to BAECs. Ten micrograms of total RNA extracted for each time point was subjected to Northern blot with an IAP-2 cDNA probe. Signal was quantified with the use of Alpha Imager 2200 and adjusted by GAPDH levels. The corrected density was then plotted as a percentage of the 0-hour value against time, with the decay rate constant derived from the slope of the decay curve.

### IAP-2 Transcription Studies

A nucleotide fragment (-1066 to +38 nucleotide of human sequence at GeneBank AF233684) encompassing the basal elements of human IAP-2 promoter was cloned into a PGL-3 vector (Promega) containing the luciferase reporter. BAECs were transfected with the IAP-2 promoter constructor with the use of LipofectAMINE (Invitrogen). PR39 (3  $\mu$ mol/L) was added, cells were lysed 6 hours later, and luciferase activity was determined with the use of the luciferase assay system (Promega).

### Smac Constructor and Transfection

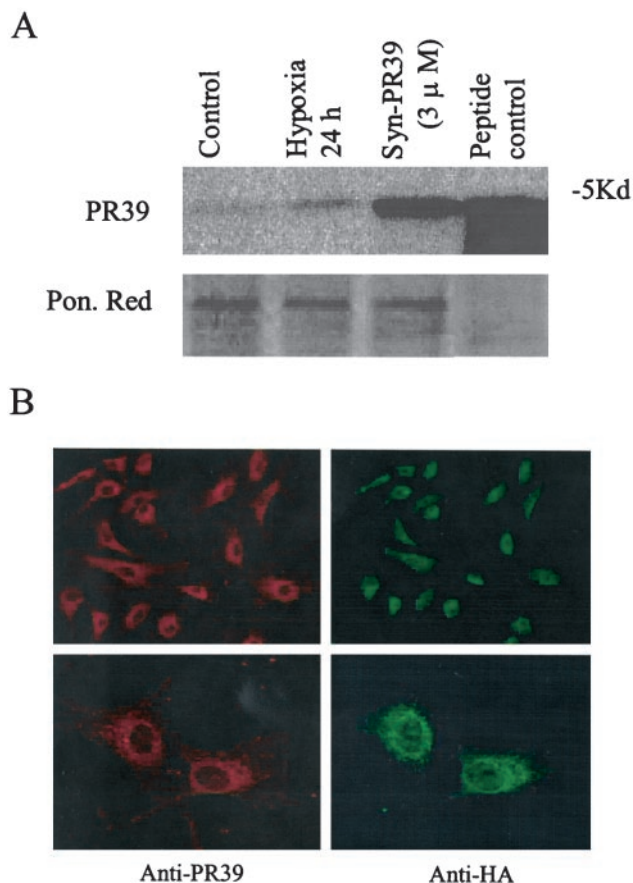
Full-length Smac cDNA (750 bp) was generated by PCR with the use of forward primer 5'-AGCTTGGTACCCGCTGCACAATGGCGGCTCT-3' and reverse primer 5'-CTAGACTCGAGACAGGGCAGTGTGCTCAGGC-3', corresponding to human Smac sequence. The PCR product was cloned into the expression vector pcDNA4 (Invitrogen). Constructor of Smac was transfected into cultured BAECs with LipofectAMINE (Invitrogen). Transfection rate reached 60% to 70% of total cells on the basis of green fluorescent protein cotransfection. Expression of Smac was confirmed by a Western blot with a polyclonal antibody against Smac (Imgenex).

### Preparation and Transfection of Short Interfering RNA Targeting to IAP-2

Short interfering RNA targeting human IAP-2 (IAP-2-siRNA) was designed online and synthesized by QIAGEN. The sequences of IAP-2-siRNA corresponded to the coding regions 191 to 211 relative to the first nucleotide of the start codon, as follows: 5'-AGG AGU CUU GCU CGU GCU GTT-3' and 5'-CAG CAC GAG CAA GAC UCC UTT-3'. Transfection of siRNA was performed according to the manufacturer's direction (Targeting Systems). siRNA was transfected into BAECs at the final concentration of 100 nmol/L. The efficacy of IAP-2 knockdown was assessed by Western blotting.

### Statistical Methods

Data are expressed as mean  $\pm$  SD. Continuous variables were compared by paired Student *t* test (baseline and follow-up). TUNEL analyses were compared by ANOVA. All reported probability values were 2 tailed, and a probability value <0.05 was considered statistically significant.



**Figure 1.** PR39 is induced by hypoxia; exogenous PR39 is expressed in the cells. A, Western blotting analysis demonstrates the expression of endogenous PR39 in BAECs incubated for 24 hours in normoxia (lane 1) or hypoxia (lane 2) and the expression of exogenous PR39 peptide in the cells (lane 3). Lane 4 was loaded with the PR39 peptide only and was used to determine the molecular weight of PR39 and specificity of anti-PR39 antibody. Ponceau S Red (Pon. Red) staining shows as protein loading controls. Note that PR39 expression was induced in hypoxia. Incubating exogenous synthesized PR39 (Syn-PR39) in BAECs increased PR39 expression. B, Representative immunofluorescent image of localization of exogenous PR39 expression in BAECs. After incubation with synthetic PR39-HA peptide for 6 hours, expression of exogenous PR39 in the cells was detected by staining with anti-PR39 and anti-HA antibodies (top, low magnification). With high magnification, note the synthetic PR39 localized in cytoplasm of BAECs.

## Results

### PR39 Is Induced by Hypoxia, and Exogenous PR39 Is Expressed in Cells

We initially examined PR39 expression in hypoxic endothelial cells. After 24 hours of hypoxia, PR39 protein expression increased, as illustrated by Western blot analysis (Figure 1A, lane 2 versus lane 1). This finding is consistent with our previous study, which demonstrates that PR39 increases in the heart after myocardial infarction.<sup>8</sup> Because endogenous levels of PR39 may limit our ability to determine its biological activity, PR39 peptide was synthesized and incubated with BAECs. As shown in Figure 1A, lane 3, synthesized PR39 is highly expressed in the cell lysate. Both endogenous and exogenous PR39, expressed as matured peptides with

molecular weight 4.7 kD, are the same size as the positive control (Figure 1A lane 4, load peptide only). To determine the localization of the exogenous PR39 in BAECs, HA-labeled synthesized PR39 was incubated and stained by immunofluorescent analysis. Figure 1B demonstrates that exogenous PR39 penetrated the cell membrane, expressed in the cytoplasm of BAECs.

### PR39 Inhibits Hypoxia-Induced Apoptosis

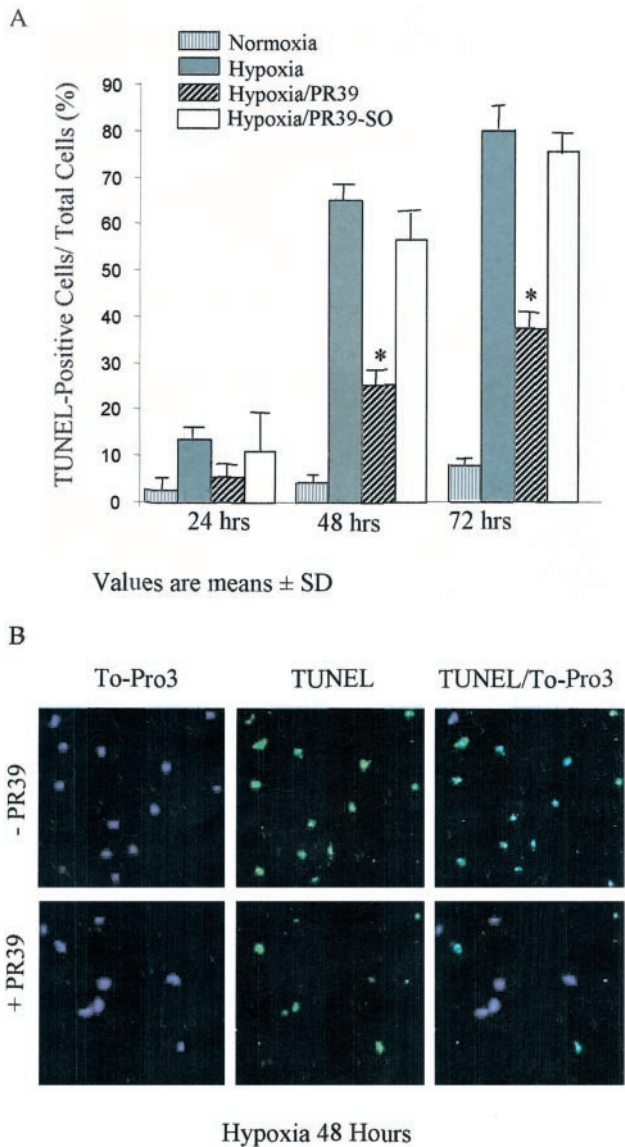
BAECs exposed to hypoxia showed a time-dependent induction of apoptosis, as detected by TUNEL assay. TUNEL-positive cells were counted with flow cytometry (Figure 2A) and immunofluorescent staining (Figure 2B). There was no significant difference in TUNEL-positive cells between hypoxia and normoxia after 24 hours of incubation. However, after 48 hours of hypoxia,  $66.2 \pm 2.7\%$  of BAECs developed an apoptotic phenotype. The percentage of apoptotic cells was significantly reduced to  $29.6 \pm 1.9\%$  ( $P < 0.05$ ) in BAECs pretreated with PR39 ( $3 \mu\text{mol/L}$  for 3 hours). Similarly, the percentage of apoptotic cells after 72 hours of hypoxia was  $79.9 \pm 5\%$  in untreated cells and  $38.1 \pm 3.2\%$  in PR39-treated BAECs (Figure 2A). These results indicate that PR39 has protective effects during hypoxia in endothelial cells. To eliminate the nonspecific effect of charged molecules such as proline and arginine, which are abundant in PR39, a control peptide was synthesized with the same amino acids in a scrambled order (PR39-SO). The scrambled-order peptide did not reduce TUNEL-positive cells during hypoxia (Figure 2A).

### PR39 Reduces Activation of Caspase-3 Activity

Caspase-3 is one of the key proteins in the apoptosis pathway. Because the TUNEL assay is used only to detect the late stage of apoptosis, caspase-3 activity was measured in hypoxic BAECs in the presence or absence of PR39 treatment. Caspase-3 activation was significantly increased within 4 hours and peaked at 16 hours of hypoxia (Figure 3A), suggesting that apoptosis occurred as early as 4 hours after hypoxia, although the TUNEL assay did not indicate significant change even in 24-hour hypoxic cells. In the presence of PR39 ( $3 \mu\text{mol/L}$ ), there was a significant attenuation of hypoxia-induced caspase-3 activation (Figure 3B). Unlike PR39, the scrambled-order peptide failed to reduce caspase-3 activity (Figure 3B). These data suggest that PR39 inhibits apoptosis in hypoxic endothelial cells by diminishing caspase-3 activity.

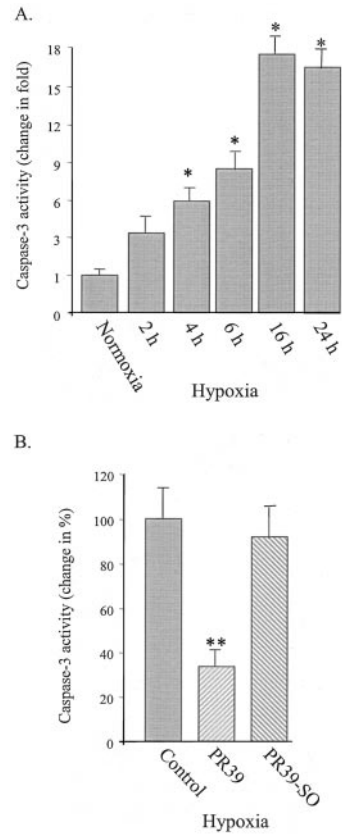
### PR39 Increases IAP-2 Expression

To delineate the mechanism of PR39 in inhibition of apoptosis, we screened the genes that were regulated by PR39 with DNA array analysis. Of  $>200$  genes regulated by PR39, IAP-2 mRNA exhibited a 3-fold increase, whereas mRNA levels of other preapoptosis or antiapoptosis genes, such as those of the Bcl-2 gene family and tumor necrosis factor- $\alpha$ , did not change in response to PR39 (data not shown). To further confirm this result, RNA and protein levels of IAP-2 were examined in BAECs treated with exogenous PR39. RNA blots of BAECs treated with PR39 ( $3 \mu\text{mol/L}$ ) demonstrated a profound increase in IAP-2 expression over the time course of the study. The expression of IAP-2 increased



**Figure 2.** PR39 reduces apoptosis induced by hypoxia in endothelial cells. A, Quantitative analysis of apoptotic cells with flow cytometry, shown as the ratio between TUNEL-positive cells and total cells in absence (gray bars) or presence (striped bars) of PR39. Note a significant reduction of apoptotic cells in the presence of PR39 after 48 and 72 hours of hypoxia. (\* $P < 0.05$ ). PR39-SO did not change apoptotic cells induced by hypoxia. (Each data point represents mean  $\pm$  SD of 3 separate experiments). B, Confocal image shows TUNEL-positive cells and total cells in the presence or absence of PR39 after 48 hours of hypoxia in BAECs. There were fewer TUNEL-positive cells with PR39 treatment.

dramatically as early as 6 hours, reaching a peak of 3.2-fold increase over baseline at 24 hours (Figure 4A, top), whereas a control peptide, PR39-SO, did not affect IAP-2 expression (Figure 4A, bottom). Western blot analysis also showed that PR39 increased IAP-2 protein level in a dose-dependent fashion (Figure 4B). To further determine the specificity of PR39-induced IAP-2 expression, the time course of PR39 treatment up to 72 hours in BAECs was analyzed by immunoblotting. As Figure 4C shows, changes in IAP-2 protein expression by PR39 closely paralleled those of IAP-2

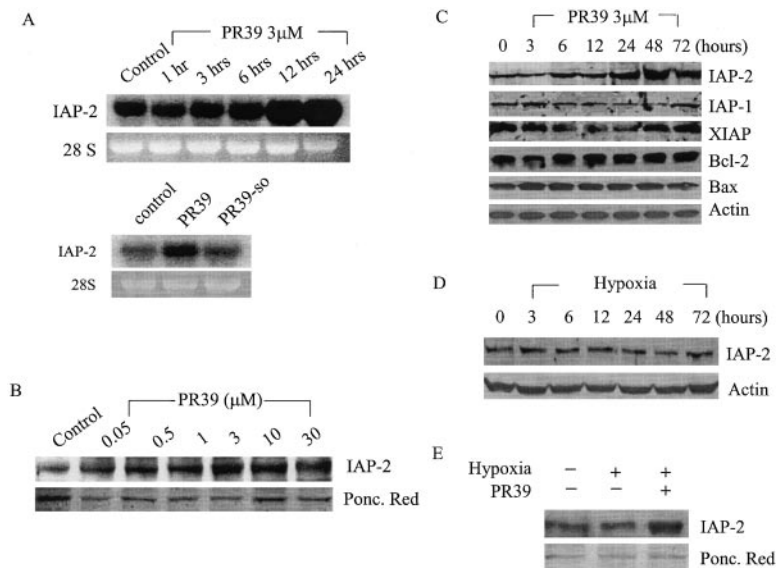


**Figure 3.** PR39 reduces caspase-3 activity induced by hypoxia in endothelial cells. A, Time course of caspase-3 activation was analyzed in BAECs in hypoxia. Caspase-3 activity was then examined with a colorimetric assay. The significant induction of caspase-3 activity was as early as 4 hours and reached a maximum in 16 hours of hypoxia. B, Caspase-3 activity induced by 16 hours of hypoxia was abolished when BAECs were pre-treated with PR39. Reduction of caspase activity did not occur in the cells with PR39-SO. Each data point represents mean  $\pm$  SD of 3 separate experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ .

mRNA, showing an early increase in expression at 6 hours and remaining significantly elevated above baseline values up to 72 hours, whereas changes in levels of IAP-1 and XIAP were not observed. In addition, Bcl-2 and Bax, the molecules belonging to another antiapoptotic protein family, did not show significant changes in response to PR39, except that Bcl-2 protein upmigrated after incubation with PR39 for 6 hours. This migration may be due to Bcl-2 phosphorylation. However, further investigation is necessary to make this determination. Overall, these results indicated that PR39-induced IAP-2 expression is relatively specific, which led us to hypothesize that PR39 inhibits hypoxia-induced apoptosis by means of an increase of IAP-2 expression. Furthermore, regulation of IAP-2 per se in hypoxia was examined. As Figure 4D shows, IAP-2 was not induced by hypoxia up to 72 hours. However, in PR39-treated cells, expression of IAP-2 increased by 2-fold after 24 hours of hypoxia (Figure 4E).

### Mechanism of PR39-Mediated Increase of IAP-2 Expression

To determine whether PR39 regulates IAP-2 gene expression by transcriptional or posttranscriptional mechanisms, we



**Figure 4.** PR39 induces expression of IAP-2 in endothelial cells. A, BAECs were treated with PR39 at indicated time periods. IAP-2 mRNA was significantly stimulated by PR39 at 12 and 24 hours (top). PR39-SO failed to induce IAP-2 expression after 24 hours of treatment (bottom). B, Western blot analysis of expression of IAP-2 protein induced by PR39 showing dose dependence. C, Western blot analysis of expression of apoptotic-related proteins in BAECs treated with PR39. PR39 increased IAP-2 protein level as early as 6 hours and peaked at 48 hours, whereas levels of IAP-1, XIAP, and Bax were not affected by PR39. The expression of Bcl-2 protein upregulated after incubation with PR39 for 6 hours. D, Western blot analysis of expression of IAP-2 by hypoxia in BAECs. The protein level of IAP-2 did not change in either short- or long-term hypoxia. E, Western blot analysis of BAECs with or without PR39 treatment under hypoxic conditions. Expression of IAP-2 was constant after 24 hours of hypoxia without PR39 and increased when the cells were preincubated with PR39. Ponceau S Red (Ponc. Red) staining shows as protein loading controls.

measured the activity of a luciferase construct under the control of the human IAP-2 promoter. When BAECs transfected with a construct of the IAP-2 promoter region were exposed to PR39, there was a  $1.75 \pm 0.5$ -fold increase in luciferase activity (Figure 5A). In addition, to assess the effect of PR39 on IAP-2 mRNA stability, we measured the steady state level of IAP-2 mRNA affected by PR39 via IAP-2 half-life assay in the presence of ACD ( $5 \mu\text{g/mL}$ ). IAP-2 mRNA half-life was 2.3 hours in the absence of PR39 and 3.5 hours in the presence of  $3 \mu\text{mol/L}$  PR39 (Figure 5B). Thus, PR39-induced increase in the level of IAP-2 mRNA in BAECs is due to an increase in transcription rate and mRNA stability.

### Inhibition of Apoptosis by PR39 Is Mediated by Increase in IAP-2 Expression

It is known that IAP-2 is a pivotal antiapoptotic factor, acting through inhibition of caspases.<sup>4,11</sup> We hypothesized that PR39 acts in hypoxia-related apoptosis by increasing IAP-2. To test this hypothesis, function of IAP-2 was blocked to test whether PR39 imparts its antiapoptotic effects on hypoxic cells. The inhibition of IAP-2 was achieved by overexpressing Smac, a known IAP-2 inhibitor,<sup>11</sup> in BAECs. Overexpression of the full length of human Smac cDNA was detected by Western blotting (Figure 6A). PR39 decreased caspase-3 activity induced by hypoxia, as shown in Figure 3B, but this was not observed in Smac-overexpressing cells (Figure 6B), suggesting that Smac blocks IAP-2 sensitivity to inhibit caspase-3, thereby blocking PR39 from decreasing caspase-3 activity.

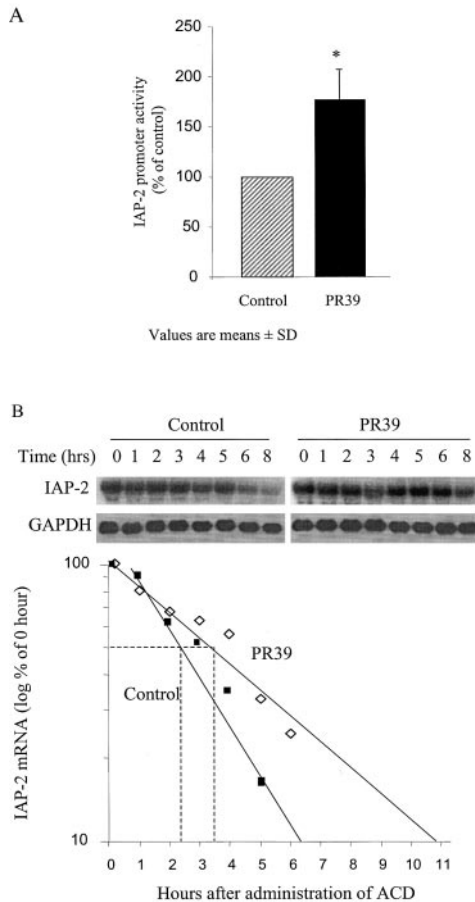
To further confirm that IAP-2 is a key molecule in inhibition of apoptosis by PR39, IAP-2-targeting siRNA was applied to BAECs. As shown in Figure 6C, a significant reduction of IAP-2 was seen in BAECs. Either IAP-1 or XIAP was not affected by IAP-2 siRNA. The efficacy of IAP-2 knockdown by IAP-2 siRNA was analyzed by hypoxia-induced caspase-3 activity. Interestingly, a 2-fold increase of caspase-3 activity was obtained when IAP-2 expression was blocked compared with the nontransfected cells. Mean-

while, the reduction of caspase-3 activity by PR39 was attenuated in IAP-2siRNA-transfected BAECs. Taken together, these results indicate that PR39 inhibits apoptosis mediated by IAP-2.

### Discussion

The principal finding of this study is that PR39 inhibits hypoxia-induced apoptosis in cultured endothelial cells. We report that the mechanism of this effect is via PR39-dependent induction of IAP-2 expression. Several lines of evidence support this finding. First, PR39 expression was induced by hypoxia. Second, treatment of cultured endothelial cells with PR39 significantly reduced caspase-3 activity and induced expression of IAP-2. Finally, the ability of PR39 to inhibit caspase-3 activity could be blocked by inhibition of IAP-2. Our studies are complementary to prior reports demonstrating the important role of PR39 in angiogenesis in both in vivo and in vitro models of ischemia.<sup>10</sup> In addition to the angiogenesis effect, the inhibition of apoptosis by PR39 may play a role in its ability to reduce infarct size during myocardial infarction,<sup>12</sup> attenuate myocardial ischemia/reperfusion injury, and prevent postischemic microvascular dysfunction.<sup>13</sup>

PR39 is secreted in a prepro-peptide form that includes a canonical leader sequence. It rapidly undergoes cleavage of the N-terminal portion to generate a mature form composed of 39 amino acids, which is rich in proline and arginine and thus carries a highly positive charge.<sup>14</sup> In the present study, the antiapoptotic effect of PR39 is not caused by its highly positive charge, because scramble-ordered sequence of the same 39 amino acids in this study did not abolish hypoxia-induced caspase-3 activity or induce IAP-2 expression. Despite the general consideration that molecules with highly positive charges do not penetrate cell membrane efficiently, recent studies have demonstrated that arginine is an important amino acid that leads proteins to enter the nuclei. Arginine-containing peptides are able to penetrate cellular membrane in an energy-independent pathway.<sup>15</sup> This was supported by the report showing that the reduction of myeloperoxidase



**Figure 5.** Mechanism of PR39-induced increase in IAP-2 expression in endothelial cells. A, PR39 stimulated IAP-2 promoter activity. Shown is analysis of luciferase reporter activity in BAECs transiently transfected with a construct containing human sequence of IAP-2 promoter fragment linked to a luciferase gene. There was a  $1.75 \pm 0.5$ -fold increase in luciferase activity in the BAECs with PR39 treatment (mean  $\pm$  SD;  $P < 0.05$ ). B, PR39 affected the half-life of mRNA of IAP-2. BAECs were exposed to PR39 ( $3 \mu\text{mol/L}$ ) for 24 hours before the addition of ACD ( $5 \mu\text{g/mL}$ ). Total RNA extracted at indicated times after ACD administration was subjected to Northern blots with IAP-2 probe. The density of each band was corrected by GAPDH, then plotted as a percentage of the 0-hour value against time. Note the substantially prolonged IAP-2 mRNA half-life in PR39-treated cells.

activity by PR39 in ischemia/reperfusion injury was completely abrogated by mutating 3 arginines in the N-terminal by 3 alanines.<sup>12</sup> In addition, we also proved in this report that synthesized PR39 penetrated the cell membrane and localized to the cytoplasm in endothelial cells.

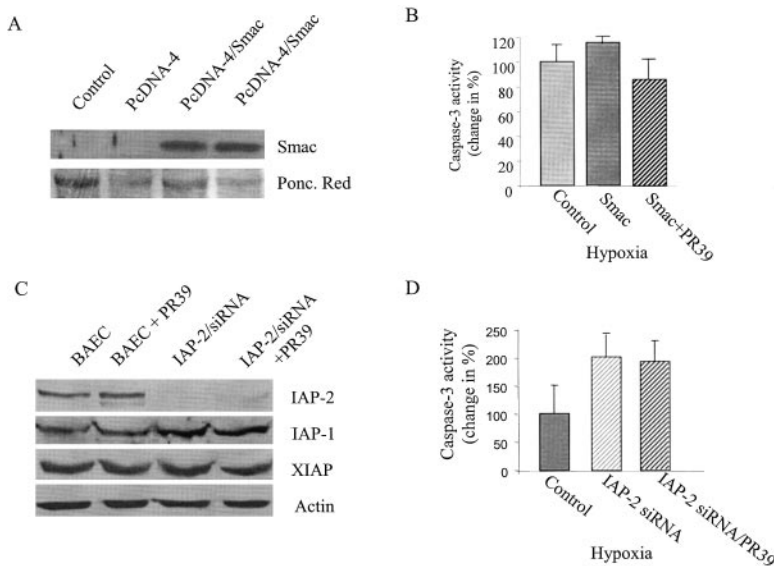
Our data provide evidence that PR39 inhibition of apoptosis is mediated by an increase of IAP-2 in gene and protein levels. The identification of PR39 as an inducer of IAP-2 is especially significant because IAP-2 is the major pathway of hypoxia-induced apoptosis. The effect of hypoxia on apoptosis is still controversial. Dong et al<sup>4</sup> demonstrated that severe hypoxia (anoxia) protects against apoptosis by inducing IAP-2 in proximal tubule cells and showed that the induction of IAP-2 by anoxia is not cell type specific. In the present study we demonstrate that moderate hypoxia induces apoptosis without changing the IAP-2 expression in BAECs. How-

ever, when neonatal cardiac myocytes were subjected to severe hypoxia, apoptosis was also induced.<sup>16</sup> Taken together, the degree of hypoxia and cell type specificity may not be the critical factors that explain these opposite responses. Further investigation is needed to determine whether other factors are involved in the regulation of apoptosis by hypoxia.

Our observation is consistent with other studies demonstrating that other antiapoptotic factors, including Bcl-2/Bax or IAP-1/XIAP, were not significantly induced in hypoxic cells.<sup>4</sup> However, PR39 may cause Bcl-2 phosphorylation after 6 hours of incubation, as shown by the Bcl-2 upmigration. Recent evidence suggests that inhibition of apoptosis by Bcl-2 is regulated by serine and threonine phosphorylation.<sup>17</sup> Whether phosphorylation of Bcl-2 enhances or inhibits its antiapoptotic function is still debated. Certain phosphorylations induced by cytokines, such as interleukin-3, are antiapoptotic,<sup>18</sup> whereas other phosphorylations triggered by chemotherapeutic drugs, such as Paclitaxel, are apoptotic.<sup>19</sup> The widely accepted hypothesis is that single-site phosphorylation activates and multiple-site phosphorylation inactivates Bcl-2. Thus, phosphorylation of Bcl-2 by PR39 might be another important process involved in the regulation of apoptosis. Ongoing studies in our laboratory include the analysis of PR39-induced Bcl-2 phosphorylation to identify the critical site of Bcl-2 to be phosphorylated.

We have previously shown that PR39 inhibited the degradation of hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ), suggesting that HIF-1 $\alpha$  plays an important role in the effects of PR39 under hypoxic conditions.<sup>10</sup> Whether HIF-1 $\alpha$  mediates the inhibition of apoptosis by PR39 in hypoxia was not examined in this study. A previous study has shown that the maximal IAP-2 expression detected in severe hypoxia/anoxia was HIF-1 $\alpha$  independent.<sup>4</sup> However, HIF-1 $\alpha$  was upregulated by hypoxia in a hypoxia model similar to that used in this study. We have shown that the expression of IAP-2 was not changed after 24 hours of hypoxia, whereas with pretreatment with PR39, IAP-2 was increased during hypoxia, indicating that PR39 is involved in regulation of IAP-2 in hypoxia-induced apoptosis. Another possible explanation for the effect of PR39 in hypoxia is that PR39 has been shown to be an inhibitor of NADPH oxidase activity by interacting with the p47<sup>phox</sup> subunit.<sup>7</sup> There are 2 contradictory hypotheses regarding the significance of cellular redox status in gene expression during hypoxia. One model proposes that cellular levels of reactive oxygen species production are increased under hypoxia in stabilization of HIF-1 $\alpha$ .<sup>20</sup> Another model suggests that a lower level of reactive oxygen species is generated in hypoxic conditions and that gene regulation is involved in inhibition of reactive oxygen species generation.<sup>21</sup> However, neither the antioxidants nor the pro-oxidants could induce IAP-2 expression.<sup>4</sup> The induction of IAP-2 by PR39 is unlikely to be mediated by its effect on NADPH oxidase activity.

When it is considered that the regulation of IAP-2 is the major pathway protecting against hypoxia-induced apoptosis, our results have demonstrated a critical role of PR39 in the induction of IAP-2 mRNA and protein levels. The construction of an IAP-2 promoter fragment with a luciferase reporter gene demonstrated that PR39 stimulates IAP-2 promoter



**Figure 6.** Effect of PR39 on hypoxia-induced caspase-3 activation mediated by IAP-2. A, BAECs with pcDNA4-Smac transfection successfully expressed Smac in Western blotting with anti-Smac antibody. Ponceau S Red (Ponc. Red) staining shows as protein loading controls. B, Caspase-3 activity was examined with a colorimetric assay. The caspase-3 activity induced by hypoxia is shown as control. Smac overexpression cells did not change caspase-3 activity in hypoxia; however, caspase-3 activity reduced by PR39, shown in Figure 3B, did not occur in Smac expression cells. C, Western blotting analysis indicated that transfection of siRNA targeting IAP-2 leads to silencing the corresponding IAP-2 but not blocking IAP-1 and XIAP. D, PR39 failed to reduce hypoxia-induced caspase-3 activity in IAP-2-siRNA-transfected BAECs. The experiments were repeated 3 times and are presented as mean $\pm$ SD.

activity. In the IAP-2 promoter regulatory region, other investigators have identified several critical cis-acting elements, including cAMP response element binding protein (CREB), nuclear factor-1, HIF-1 $\alpha$ , nuclear factor- $\kappa$ B (NF- $\kappa$ B), c-Myc, and TATA box.<sup>22</sup> Among these factors, CREB binding sites are identified as enhancer sequencers that regulate IAP-2 gene expression.<sup>22</sup> CREB and HIF-1 $\alpha$  binding sites are present in promoters of genes that enhance endothelial cell function.<sup>23</sup> Induction of promoter activity of genes in endothelial cells by PR39 has not been studied fully. However, in particular, the presence of ACTCAT, a novel cis element for proline,<sup>24</sup> in the IAP-2 promoter region may be identified by PR39 via its proline- and arginine-rich sequences that enhance IAP-2 expression. Certainly, identification of interaction of PR39 with such elements would be the focus of further investigation.

Besides PR39-induced gene expression of IAP-2 via stimulating IAP-2 promoter activity, our results have demonstrated that PR39 increased IAP-2 mRNA half-life. In anti-apoptotic processes, a conserved adenylate/uridylylated (AU)-rich element (AREs) in the 3' untranslated region of Bcl-2 mRNA is endowed with a destabilizing function that is involved in Bcl-2 downregulation during apoptosis.<sup>25</sup> Furthermore, RNA-stabilizing proteins such as HuR could bind to AREs located in the 3' UTR of several RNAs to stabilize mRNA transcripts, to enhance translation, or to perform a combination of these posttranscriptional regulatory steps. Hypoxia specifically increases the binding of HuR to AREs.<sup>26</sup> The role of HuR binding to AREs in IAP-2 gene and its involvement in IAP-2 posttranscriptional regulation by PR39 requires further study. Posttranscriptional regulation of IAP-2 by PR39 is also suggested by protein changes up to 72 hours in our experiments. However, the prolongation of IAP-2 mRNA half-life does not exclude potential IAP-2 regulation by PR39 at the posttranscriptional level because ACD could reduce but not completely abolish IAP-2 expression in the presence of PR39. Yang et al<sup>27</sup> has shown that auto-ubiquitination and degradation of IAPs may be the key event in the apoptotic program. When it is considered that PR39 has

the ability to selectively inhibit proteasomes,<sup>28</sup> the increases of IAP-2 protein expression by PR39 may be possible through its selective inhibition of IAP-2 degradation in the proteasome-ubiquitin pathway, which is suggestive of another pathway that regulates IAP-2 expression.

IAPs suppress cell death by inhibiting the activity of caspases in the mitochondrial pathway of apoptosis.<sup>29</sup> The mitochondrial protein Smac promotes apoptosis by eliminating the inhibitory effect of IAPs through physical interaction. Thus, an apparent role of IAP-2 in apoptosis could be reduced while the cells express Smac. We have compared the apoptotic sensitivity of endothelial cells in hypoxia between Smac cDNA transfected cells and control cells. The results show that PR39 reduced caspase-3 activity in control cells, which was not observed in cells that overexpressed Smac. Furthermore, transfection of siRNA targeting IAP-2 enabled knockdown of the ability of IAP-2 to inhibit hypoxia-induced caspase-3; it also abolished PR39 in inhibition of apoptosis. Thus, this study indicates that either by silencing IAP-2 expression with siRNA or blocking IAP-2 sensitivity with Smac overexpression, PR39 lost its ability to inhibit caspase-3 activity, suggesting that IAP-2 is a key mediator in PR39 inhibition of apoptosis.

We have previously shown that PR39 inhibits I $\kappa$ B $\alpha$  degradation as proteasome inhibitor in cultured cells and in animal models.<sup>28</sup> The functional effects of PR39 treatment are mediated by inhibition of NF- $\kappa$ B-dependent gene expression. NF- $\kappa$ B has been reported to be involved in antiapoptotic activity related to proteasome inhibitors. The ability to inhibit I $\kappa$ B $\alpha$  degradation by PR39 will decrease NF- $\kappa$ B activation, an inhibition that may conflict with the ability of PR39 to inhibit apoptosis. The dual reaction of PR39 could be one of the reasons that explain why PR39 prevents only 50% of cells from undergoing apoptosis, although most of exogenous PR39 could penetrate into the cells.

In summary, we have shown that PR39 was induced by hypoxia. Exogenous PR39 inhibits apoptosis in endothelial cells subjected to hypoxic injury. The antiapoptotic effect of PR39 is mediated by increased IAP-2 expression via tran-

scriptional and posttranscriptional regulation. This characterization will enhance our understanding of PR39-dependent antiapoptosis and may lead to the development of novel therapeutic approaches in the treatment of ischemic heart disease.

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