Role of calpain in hypoxic inhibition of nitric oxide synthase activity in pulmonary endothelial cells

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Su, Yunchao, and Edward R. Block. Role of calpain in hypoxic inhibition of nitric oxide synthase activity in pulmonary endothelial cells. Am J Physiol Lung Cell Mol Physiol 278: L1204–L1212, 2000.—Pulmonary artery endothelial cells (PAEC) were exposed to normoxia or hypoxia (0% O₂-95% N₂-5% CO₂) in the presence and absence of calpain inhibitor I or calpeptin, after which endothelial nitric oxide synthase (eNOS) activity and protein content were assayed. Exposure to hypoxia decreased eNOS activity but not eNOS protein content. Both calpain inhibitor I and calpeptin prevented the hypoxic decrease of eNOS activity. Incubation of calpain with total membrane preparations of PAEC caused dose-dependent decreases in eNOS activity independent of changes in eNOS protein content. Exposure of PAEC to hypoxia also caused time-dependent decreases of heat shock protein 90 (HSP90) that were prevented by calpain inhibitor I and calpeptin. Moreover, the HSP90 content in anti-eNOS antibody-immunoprecipitates from hypoxic PAEC lysates was reduced, and repletion of HSP90 reversed the decrease of eNOS activity in these immunoprecipitates. Incubation of PAEC with a specific inhibitor of HSP90 (geldanamycin) mimicked the hypoxic decrease of eNOS activity. These results indicate that the hypoxia-induced reduction in eNOS activity in PAEC is due to a decrease in HSP90 caused by calpain activation.

hypoxia; heat shock protein 90; pulmonary artery

Calpain is the name used to describe a family of calcium-activated, nonlysosomal neutral cysteine peptidases that are ubiquitously distributed in all mammalian cells (15, 17, 21). For the most part, calpain isoforms consist of a distinct large catalytic subunit (about 80 kDa) and a common smaller subunit (about 30 kDa) that help regulate their activity (10, 21). The cDNA of the subunits has thus far been cloned in a variety of tissues, including endothelial cells (6, 10, 29). Calpain exists in the cytosol as procalpain, an inactive proenzyme that translocates from the cytosol to the cell membrane in the presence of calcium. Autocatalytic activation of procalpain to active calpain occurs at the membrane in the presence of physiological levels of calcium and phosphatidylinositol (4). Substrates for calpain include the cytoskeletal proteins fodrin, talin, and filamin; microtubule-associated proteins; and a number of membrane proteins, including growth factor receptors (e.g., epidermal growth factor receptor), adhesion molecules (e.g., integrin, cadherin), and ion transporters (e.g., Ca²⁺-ATPase), as well as enzymes such as protein kinase C, myosin light chain kinase, calmodulin-dependent kinase, phosphatases, and phospholipase C (21). Calpain has been found to have an important role in transmembrane signaling, cell differentiation, transcriptional regulation, cytokine processing, and apoptosis (4, 19, 22). Calpain is also implicated in a variety of pathological states, including ischemia, hypoxia, vasospasm, Alzheimer’s disease, sepsis, inflammation, and muscular dystrophy (4, 19, 24, 29).

Exposure of endothelial cells to hypoxia alters many aspects of endothelial cell function and metabolism, including membrane function, cellular enzyme activity, cytokine secretion, protein synthesis, and mRNA expression (1, 11, 14, 20, 28, 30). The effect of hypoxia on the activity of the endothelial isoform of nitric oxide synthase (eNOS) is controversial. McQuillan et al. (14) and Liao et al. (11) reported that incubation of cultured endothelial cells in 0–3% O₂ for 24–48 h resulted in the reduction of eNOS gene expression, eNOS protein content, and eNOS activity. Xue et al. (28) demonstrated that exposure of rats to 10% O₂ for 2–4 wk induced upregulation of eNOS expression in the pulmonary endothelium. However, in this study, NOS activity in the particulate fraction of hypoxic lung tissue homogenates (where the eNOS is located) remained unchanged. Finally, Arn et al. (1) reported that exposure to hypoxia (1% O₂) for 6–24 h increased eNOS mRNA and protein content in cultured bovine aortic endothelial cells. However, the relative NOS specific activity, i.e., NOS activity per NOS protein, decreased in hypoxic endothelial cells.

The experiments of Xue et al. (28) and Arn et al. (1) hint that hypoxia modifies eNOS activity by a posttranslational mechanism. Recently, we reported that hypoxia increases calpain activity and expression in cultured porcine pulmonary artery endothelial cells (PAEC) (24, 29) and that the increased calpain activity has a critical role in the mobilization of L-arginine from proteins in hypoxic cells (24). In the present study, we found that exposure of PAEC to hypoxia for 24 h reduces eNOS specific activity but not eNOS protein content, and we tested the hypothesis that calpain activation is responsible for the hypoxia-induced decrease of eNOS activity observed in porcine PAEC. Our results indicate that a calpain-mediated mechanism is

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Fig. 1. Effect of hypoxia on endothelial nitric oxide synthase (eNOS) activity in pulmonary artery endothelial cells (PAEC). Cells were exposed to normoxia (control) or hypoxia for 4–24 h, after which eNOS activity was determined as described in METHODS. Results are expressed as means ± SE; n = 5 experiments. *P < 0.01 vs. control.

Fig. 2. Effect of hypoxia on eNOS protein content in PAEC. Cells were incubated in presence of normoxia (control) for 24 h or in presence of hypoxia for 2–24 h, after which eNOS protein was measured by Western blot analysis as described in METHODS. A: representative Western blot of eNOS protein content. B: bar graph depicting eNOS protein contents quantified by scanning densitometry. Comparable results were obtained in 4 separate experiments.
were exposed at 37°C to 0% O2-5% CO2-95% N2 (hypoxia) or

**METHODS**

reduction in heat shock protein 90 (HSP90) content. This activity in PAEC and that this mechanism involves a responsible for the hypoxia-induced decrease in eNOS activity in PAEC and that this mechanism involves a reduction in heat shock protein 90 (HSP90) content.

**METHODS**

Cell culture. Endothelial cells were obtained from the main pulmonary artery of 6- to 7-mo-old pigs and were cultured as previously reported (24, 29). Third- to sixth-passage cells in monolayer culture were maintained in RPMI 1640 medium containing 4% fetal bovine serum and antibiotics (10 U/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamicin, and 2 µg/ml Fungizone) and were used 2 or 3 days after confluence.

All monolayers were initially identified as endothelial cells by phase-contrast microscopy. Selected dishes of cells were further characterized by electron microscopy or by indirect immunofluorescent staining for factor VIII antigen or both. By use of these techniques, monolayer cultures were estimated to be pure endothelial cells.

Exposure to hypoxia. The confluent monolayers of PAEC were exposed at 37°C to 0% O2-5% CO2-95% N2 (hypoxia) or air-5% CO2 (normoxia) at 1 atmosphere absolute for 4–24 h as previously reported (24, 29) in the presence and absence of the following agents: calpain inhibitor I (10 µM); calpeptin (50 µM); tyrosine kinase inhibitor genistein (10 µM); tyrosine phosphatase inhibitor vanadate (20 µM); protein kinase C inhibitors staurosporine (5 nM), calphostin C (200 nM), and chelerythrine (1 µM); or geldanamycin (1 µg/ml), a specific inhibitor of HSP90. The percentage of oxygen in the gas phase was 19–21% during exposure.

**Determination of NOS activity**

After exposure to normoxic or hypoxic environments, the PAEC were washed with phosphate-buffered solution and then lysed in boiling sample buffer (0.06 M Tris·HCl, pH 7.4, 1% sodium dodecyl sulfate, and 5% glycerol, pH 6.8). The lysates were boiled in a water bath for 5 min to remove insoluble materials. The lysate proteins (20 µg) were fractionated on 7.5% SDS-PAGE gels and blotted onto polyvinylidene difluoride membranes (Bio-Rad; Hercules, CA). The blots were incubated in blocking solution (10% nonfat milk in 0.2% Tris·HCl, pH 7.5) and then hybridized with 1:5,000 diluted monoclonal antibodies against eNOS or HSP90 (Transduction Laboratories; Lexington, KY) at room temperature for 1 h. The bands were detected using an immunochromoluminescence method, and the density of the bands was quantitated using a Fluor-S-MultiImager (Bio-Rad).

**Effect of calpain on eNOS activity and eNOS protein content**

To study possible posttranslational modification of eNOS activity by calpain, PAEC total membrane fractions isolated in the absence of protease inhibitors were incubated with calpain in vitro. The 200-µl reaction mixture (300–400 µg of protein) contained 1 mM CaCl₂, 5 mM cysteine, and 0.05–1.0 U/200 µl calpain I (Calbiochem; San Diego, CA). After incubation at 30°C for 30 min, the reactions were terminated by adding calpain inhibitor I to a final concentration of 100 µM. A 10-µl aliquot was used for Western blot for 60 min at 4°C, and the total membrane pellet was resuspended in buffer B (buffer A + 2.5 mM CaCl₂). The resulting suspension was used for determination of eNOS activity by monitoring the formation of L-[³H]citrulline from L-[³H]arginine (25). The total membrane fractions (100 µg of protein) were incubated (total volume 0.4 ml) in buffer B containing 1 mM NADPH, 100 nM calmodulin, 10 µM tetrahydrobiopterin, and 5 µM combined L-arginine and purified L-[³H]arginine (0.6 µCi; specific activity 69 Ci/mmol; NEN; Boston, MA) for 30 min at 37°C. Purification of L-[³H]arginine and measurement of L-[³H]citrulline formation were carried out as described previously (25). The specific activity of NOS is expressed as pmol L-citrulline·min⁻¹·mg protein⁻¹ and reflects eNOS activity because our cells do not exhibit basal or hypoxia-induced NOS activity. Total membrane protein content was determined by the method of Lowry et al. (13).

Western blot analysis of NOS protein and HSP90. After exposure to normoxic or hypoxic environments, the PAEC were washed with phosphate-buffered solution and then lysed in boiling sample buffer (0.06 M Tris·HCl, 2% SDS, and 5% glycerol, pH 6.8). The lysates were boiled in a water bath for 5 min to remove insoluble materials. The lysate proteins (20 µg) were fractionated on 7.5% SDS-PAGE gels and blotted onto polyvinylidene difluoride membranes (Bio-Rad; Hercules, CA). The blots were incubated in blocking solution (0.06 M Tris·HCl, 0.2% nonfat milk, 100 mM NaCl, and 0.1% Tween 20, pH 7.5) and then hybridized with 1:5,000 diluted monoclonal antibodies against eNOS or HSP90 (Transduction Laboratories; Lexington, KY) at room temperature for 1 h. The bands were detected using an immunochromoluminescence method, and the density of the bands was quantitated using a Fluor-S-MultiImager (Bio-Rad).

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analysis of eNOS protein content, and the rest of the reaction mixture was used for determination of eNOS activity by the method described previously.

Coprecipitation of HSP90 and caveolin-1 by anti-eNOS antibody. PAEC exposed to normoxic or hypoxic conditions for 24 h were lysed in ice-cold buffer containing 20 mM Tris·HCl, pH 7.4, 2.5 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 100 mM NaCl, 10 mM NaF, 1 mM Na3VO4, 1 mM Pefabloc, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µM calpain inhibitor I, and 10 µg/ml pepstatin A. The cell lysates were centrifuged at 10,000 g for 20 min to remove insoluble material. Then 500 µl of the lysates (500 µg protein) from normoxic or hypoxic cells were incubated with 2.5 µg of anti-eNOS antibody at 4°C for 4–5 h. The cell lysates were centrifuged at 10,000 g for 20 min to remove insoluble material. Then 500 µl of the lysates (500 µg protein) from normoxic or hypoxic cells were incubated with 2.5 µg of anti-eNOS antibody at 4°C for 4–5 h. Protein A Sepharose (30 µl) was added, and the samples were further incubated for 2 h at 4°C. The immunoprecipitates were recovered by centrifugation and washed three times in buffer containing 50 mM Tris·HCl, pH 8.0, 150 mM NaCl, and 0.1% Triton X-100. Immunoprecipitated proteins were eluted from the Sepharose beads by boiling the samples for 5 min in 30 µl of SDS immune-blotting sample buffer. The Sepharose beads were then pelleted by centrifugation at 14,000 g, and eNOS, caveolin-1, and HSP90 protein contents in the supernatant were analyzed by Western blot as described previously. Caveolin-1 was detected using a commercially available monoclonal antibody (Transduction Laboratories).

Determination of NOS activity in anti-eNOS antibody immunoprecipitates (immuno-NOS assay). The immuno-NOS assay was done according to the method described by García-Cardeña et al. (8). Briefly, eNOS antibody-induced immunoprecipitates were obtained from PAEC exposed to normoxic and hypoxic conditions as described in Coprecipitation of HSP90 and caveolin-1 by anti-eNOS antibody, except that the detergent was 10 mM 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate instead of 1% Triton X-100 and 0.1% SDS. The protein A-Sepharose beads were resuspended in 0.6 ml of NOS assay buffer (buffer B). eNOS activities in the presence or absence of HSP90 (30 µg/ml) or N⁵-monomethyl-
L-arginine (1 mM) were determined as described in Determination of NOS activity.

Data analysis. In each experiment, experimental and control endothelial cells were matched for cell line, age, seeding density, number of passages, and number of days postconfluence to avoid variation in tissue culture factors that can influence the measurement of NOS activity and NOS protein content. Results are shown as means ± SE for n experiments. Student’s t-test was used to determine the significance of differences between means of hypoxic and normoxic groups, and P < 0.05 was considered significant.

RESULTS

Exposure of PAEC to hypoxia for 4–24 h resulted in a time-dependent decrease of eNOS activity (Fig. 1), whereas eNOS protein content was unchanged in hypoxic PAEC (Fig. 2). To determine whether the hypoxia-induced decrease of eNOS activity was associated with calpain, PAEC were exposed to hypoxia for 24 h in the presence and absence of calpain inhibitor I (10 µM). As indicated in Fig. 3, calpain inhibitor I prevents the loss of eNOS activity in PAEC exposed to hypoxia for 24 h. Similar results were observed with calpeptin, another specific inhibitor of calpain.

To confirm that calpain can decrease eNOS activity in PAEC and to ascertain whether this is a direct effect

density (0–24 h) after which HSP90 content was measured as described in METHODS. A: representative Western blot of HSP90. B: bar graph depicting HSP90 content. Results are expressed as means ± SE; n = 5 experiments. *P < 0.05 vs. control (0-h exposure).

Caveolin and HSP90 have been reported to bind to eNOS and to regulate its catalytic activity (7, 8, 16). To determine whether the calpain-mediated inhibition of eNOS activity that we observed in hypoxic PAEC is associated with a change in the content of one of these regulatory proteins, we measured caveolin-1 and HSP90 protein contents in normoxic and hypoxic PAEC. Exposure to hypoxia did not affect caveolin-1 content in PAEC (data not shown). In contrast, exposure to hypoxia for 2–24 h resulted in a time-dependent decrease of HSP90 protein content that parallels the time course

Fig. 5. Effect of hypoxia on heat shock protein 90 (HSP90) content. PAEC were exposed to hypoxia for 2–24 h after which HSP90 content was measured as described in METHODS. A: representative Western blot of HSP90. B: bar graph depicting HSP90 content. Results are expressed as means ± SE; n = 5 experiments. *P < 0.05 vs. control (0-h exposure).

of calpain on eNOS, membrane fractions of PAEC were incubated with calpain I (0.05–1.0 U/200 µl) in vitro for 30 min at 30°C. Incubation of PAEC membranes with calpain I resulted in a dose-dependent decrease of eNOS activity (Fig. 4). Reduction in eNOS activity occurred with as little as 0.1 U calpain I/200 µl. In contrast, eNOS protein content was not affected by calpain I concentrations of up to 0.5 U/200 µl. eNOS protein content was decreased in the presence of 1.0 U/200 µl of calpain I, a concentration that is 10-fold higher than that which we have observed in hypoxic PAEC (10). Incubation with calpain inhibitor I prevented the decreases in eNOS activity and content (Fig. 4).

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Fig. 6. Effect of calpain inhibitor I on hypoxia-induced decrease of HSP90 content. PAEC were exposed to normoxia or hypoxia for 24 h in presence and absence (control) of calpain inhibitor I (10 µM) after which HSP90 content was measured as described in METHODS. A: representative Western blot of HSP90. B: bar graph depicting HSP90 content. Results are expressed as means ± SE; n = 4 experiments. *P < 0.05 vs. normoxic control.
of the decrease of eNOS protein content in hypoxic cells (Fig. 5). Calpain inhibitor I prevented the hypoxia-induced decrease of HSP90 protein content (Fig. 6). Similar results were obtained with calpeptin.

To study whether the protein-protein interactions between eNOS and the two regulatory proteins, caveolin-1 and HSP90, were affected by hypoxia, the lysates from PAEC exposed to normoxia or hypoxia were immunoprecipitated with antibody directed against eNOS. Immunoprecipitation of eNOS from PAEC lysates resulted in the coprecipitation of caveolin-1 and HSP90. Exposure to hypoxia did not alter the caveolin-1 content coprecipitated with eNOS (not shown), but exposure to hypoxia did significantly reduce the amount of HSP90 coprecipitated with eNOS (Fig. 7). Purified HSP90 expressed in Escherichia coli added back to the eNOS-HSP90 complex immunoprecipitated by anti-eNOS antibody reversed the decrease of eNOS activity in the immunoprecipitates from hypoxic PAEC (Fig. 8).

To evaluate further the relationship between HSP90 and eNOS activity, we used geldanamycin, a specific inhibitor of HSP90. Geldanamycin binds to the nucleotide-binding site of HSP90 and specifically blocks HSP90 function (9). Incubation of PAEC with geldanamycin not only resulted in a dose-dependent decrease of eNOS activity, but it also prevented the hypoxia-induced decrease of eNOS activity (Fig. 9). Geldanamycin did not inhibit eNOS activity when added directly to the total membrane fraction of PAEC.

Several groups have reported that tyrosine phosphorylation and serine phosphorylation of eNOS alter its activity (2, 8). To investigate whether tyrosine phosphorylation or serine phosphorylation is responsible for the hypoxia-induced loss of eNOS activity observed in the present study, PAEC were exposed to normoxia or hypoxia for 24 h in the presence and absence of the tyrosine kinase inhibitor genistein, the tyrosine phosphatase inhibitor vanadate, and the protein kinase C inhibitors staurosporine, calphostin C, and chelery-
Chelerythrine, did not have any effect on the hypoxia-induced loss of eNOS activity in our cells. Second, tyrosine phosphorylation of eNOS may affect its activity (8, 26). However, neither genistein nor vanadate affected the hypoxia-induced decrease of eNOS activity. Moreover, hypoxia did not alter tyrosine kinase and tyrosine phosphatase activities in PAEC (unpublished data). Third, eNOS can be myristoylated or palmitoylated (3, 12). However, neither of these modifications has been reported to change the in vitro catalytic activity of eNOS (3, 12).

Our results indicate that calpain is responsible for the hypoxia-mediated decrease in eNOS activity in porcine PAEC. The specific calpain inhibitors calpain inhibitor I and calpeptin prevented the hypoxia-induced loss of eNOS activity. Moreover, incubation of PAEC total membrane fractions with concentrations of calpain I observed in hypoxic PAEC resulted in decreases in eNOS activity in the absence of changes in eNOS protein content that were prevented by calpain inhibitor I.

To explore further the mechanism by which calpain mediates the decrease in eNOS activity observed in hypoxic PAEC, we considered the possibility that calpain activation was either resulting in the inhibition of an activator of eNOS or causing the activation of an inhibitor of eNOS. There are several reports that eNOS activity can be regulated by the interaction between the eNOS protein and other proteins such as caveolin-1 (8, 16), calmodulin (16), and HSP90 (7). Interaction of eNOS with caveolin leads to inhibition of eNOS activity. The binding of Ca\(^{2+}\)/calmodulin to eNOS disrupts this inhibitory eNOS-caveolin complex, leading to enzyme activation. However, in the present study, hypoxia did not change the caveolin-1 content that was coimmunoprecipitated with eNOS protein. An alteration of calmodulin content is unlikely to be responsible for the decreased eNOS activity in hypoxic PAEC because 100 nM calmodulin was included in the reaction.

**Table 1.** Effects of staurosporine, calphostin C, chelerythrine, genistein, and vanadate on hypoxia-induced reduction of eNOS activity in PAEC

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Normoxia</th>
<th>Hypoxia</th>
<th>Normoxia + Inhibitor</th>
<th>Hypoxia + Inhibitor</th>
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</thead>
<tbody>
<tr>
<td>Staurosporine (5 nM)</td>
<td>9.01 ± 0.81</td>
<td>6.02 ± 0.75*</td>
<td>11.5 ± 0.94</td>
<td>6.54 ± 0.72†</td>
</tr>
<tr>
<td>Calphostin C (200 nM)</td>
<td>8.26 ± 0.92</td>
<td>4.46 ± 0.34*</td>
<td>6.05 ± 0.72</td>
<td>3.62 ± 0.22†</td>
</tr>
<tr>
<td>Chelerythrine (1 µM)</td>
<td>8.64 ± 0.85</td>
<td>5.03 ± 0.41*</td>
<td>11.6 ± 0.92</td>
<td>6.78 ± 0.66†</td>
</tr>
<tr>
<td>Genistein (10 µM)</td>
<td>8.02 ± 0.82</td>
<td>4.52 ± 0.21*</td>
<td>7.68 ± 0.65</td>
<td>4.35 ± 0.33†</td>
</tr>
<tr>
<td>Vanadate (20 µM)</td>
<td>9.12 ± 0.95</td>
<td>5.34 ± 0.43*</td>
<td>8.67 ± 0.78</td>
<td>4.42 ± 0.51†</td>
</tr>
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Values are means ± SE; n = 4 experiments. Pulmonary artery endothelial cells (PAEC) were exposed to normoxia or hypoxia for 24 h in presence and absence of staurosporine, calphostin C, chelerythrine, genistein, and vanadate after which eNOS activity was assayed. Doses of inhibitors represent maximal concentrations that can be used to inhibit in porcine PAEC without causing cytotoxicity. Unit of eNOS activity is pmol L-citrulline·min\(^{-1}\)·mg protein\(^{-1}\).*P < 0.01 vs. normoxia. †P < 0.01 vs. normoxia + inhibitor group.
tion mixture used to measure eNOS activity in our in vitro assay. Recently, Garcia-Cardena et al. (7) found that binding of HSP90 to eNOS enhances the activation of eNOS. Our results indicate that hypoxia caused a time-dependent decrease of HSP90 that corresponded to the time course of change of eNOS activity in the hypoxic PAEC. Calpain inhibitor I and calpeptin prevented this hypoxia-induced decrease of HSP90 protein content. Moreover, the HSP90 content that was coprecipitated by the anti-eNOS antibody was reduced in hypoxic PAEC compared with normoxic cells. Repletion of HSP90 in the anti-eNOS antibody-induced-immunoprecipitates restored eNOS activity in the pellets from hypoxic PAEC. Finally, the specific inhibitor of HSP90 ( geldanamycin) mimicked the effect of hypoxia, i.e., it decreased eNOS activity to the same degree as exposure to hypoxia for 24 h and prevented further decrease of eNOS activity by subsequent exposure to hypoxia. Taken together, these results indicate that a reduction in HSP90 is responsible for the calpain-mediated decrease of eNOS activity observed in hypoxic PAEC.

Pulmonary endothelial cells are an important source of NO, and NO is an important endogenous vasodilator that contributes to the low vascular resistance in the pulmonary circulation (23). Several reports indicate that hypoxia causes a decrease in the synthesis and/or release of NO from pulmonary endothelial cells (18, 27). Moreover, mice with targeted disruption of the eNOS gene become hyperresponsive to mild hypoxia (5). Our study suggests calpain is involved in hypoxia-induced reduction of eNOS activity. Ruetten and Thiemermann (19) have reported that calpain has an important role in the release of NO in the condition of sepsis. Thus calpain, serving as a signal transduction molecule, may have an important role in the regulation of vascular function, especially under hypoxic conditions.

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