Proteasome Inhibition Down-regulates Endothelial Nitric-oxide Synthase Phosphorylation and Function*

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Endothelial nitric-oxide synthase (eNOS) function is fundamentally modulated by protein phosphorylation. In particular, phosphorylation of serine 1179 (bovine)/1177 (human) by Akt has been shown to be the central mechanism of eNOS regulation. Here we revealed a novel role of proteasome in controlling eNOS serine 1179 phosphorylation and function. Rather than affecting eNOS turnover, proteasomal inhibition specifically dephosphorylated eNOS serine 1179, leading to decreased enzymatic activity. Blocking protein phosphatase 2A (PP2A) by okadaic acid or PP2A knockdown restored eNOS serine 1179 phosphorylation and activity in proteasome-inhibited cells. Although total PP2A expression and activity in cells were not affected by proteasome inhibitors, proteasomal inhibition induced PP2A ubiquitination and ubiquitinated PP2A translocated from cytosol to membrane. Further biochemical analyses demonstrated that eNOS associated with PP2A on cell membranes. Proteasomal inhibition markedly enhanced PP2A association to eNOS, and this increase of PP2A dephosphorylated eNOS and its upstream kinase Akt. Taken together, these studies identified a novel pathway in which proteasome modulates eNOS phosphorylation by inducing intracellular PP2A translocation.

Nitric oxide (NO) is a ubiquitous signaling molecule in a variety of biological processes (1, 2). Biological NO synthesis is catalyzed by a family of NO synthase (NOS),2 which converts L-arginine and oxygen to NO and L-citrulline (3). Unlike the conventional signaling molecules, NO is a free radical gas. Because NO is highly diffusible and cannot be stored in intracellular compartments, modulation of NO signaling intensity is primarily achieved by tuning NO activity. So far, three NOS isoforms have been identified in mammalian cells as neuronal NOS (type I), inducible NOS (type II), and endothelial NOS (eNOS, type III) (4). Among them, regulation of eNOS function appears to be most multifaceted (5). Previously thought to be solely activated by elevated cytosolic Ca2+ concentrations, eNOS function is now known to be critically modulated by protein phosphorylation, protein-protein interactions, and subcellular localization (6).

Several kinases have been identified to phosphorylate eNOS at a number of consensus motifs (5). These include protein kinases A, B (PKB/Akt), and C, calmodulin kinase II, AMP-activated kinase, etc. The predominant one is the phosphatidylinositol 3-kinase-Akt pathway. It was first reported that vascular endothelial growth factor or shear stress activated Akt through phosphatidylinositol 3-kinase and subsequently Akt phosphorylated the serine 1179/1177 (bovine/human) residue of eNOS (7, 8). Phosphorylation of serine 1179 enhances eNOS function, and remarkably, the catalytic activity of phospho-eNOS does not require the rise of intracellular Ca2+ (9). Since the original reports that vascular endothelial growth factor and shear stress activated eNOS through the phosphatidylinositol 3-kinase-Akt pathway, there are increasing number of hormones or bioactive substances found to modulate eNOS function via Akt. These include insulin, insulin-like growth factor, angiopoietin-1, estrogen, leptin, sphingosine 1-phosphate, reactive oxygen species, corticosteroids, etc. (10). Thus, phosphorylation of eNOS serine 1179/1177 by Akt has emerged as a central mechanism of eNOS regulation under both physiological and pathological conditions.

The phosphorylation status of a protein is determined by the balanced actions of protein kinases and phosphatases that, respectively, add and remove phosphate groups from their target proteins (11, 12). In contrast to the extensive characterization of the kinases that phosphorylate eNOS, the process of eNOS dephosphorylation is understood in much less detail. Nevertheless, there were reports that serine 1179 of eNOS was distinctively dephosphorylated by protein phosphatase 2A (PP2A) (13). PP2A is a highly conserved and ubiquitous serine/threonine phosphatase that is often thought to dephosphorylate proteins with poor substrate specificity (14). It remains unknown how PP2A is directed to specifically control eNOS serine 1179 phosphorylation.

The ubiquitin-proteasome system is responsible for the majority of protein degradation in eukaryotic cells and, hence, plays important roles in many fundamental cellular processes including signal transduction (15, 16). The core component of this system is the 26 S proteasome in which proteolysis takes place. In an effort to define the roles of the ubiquitin-proteasome system in regulating eNOS turnover, we unexpectedly found that proteasome profoundly affected eNOS serine 1179

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2 The abbreviations used are: NOS, NO synthase; eNOS, endothelial NOS; PP2A, protein phosphatase 2A; JNK, c-Jun NH2-terminal kinase; BAEC, bovine aortic endothelial cell; HEK, human embryonic kidney; siRNA, small interfering RNA.

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phosphorylation and function. Inhibition of proteasome function led to eNOS serine 1179 dephosphorylation and consequent decrease of enzymatic activity. Further studies demonstrated that the effects of proteasome on eNOS were mediated by the membrane translocation of PP2A. We also provided evidence suggesting that ubiquitination of PP2A regulatory and structural subunits may be required for its membrane translocation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture materials were obtained from Invitrogen. MG132 and lacticystin were purchased from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA). Okadaic acid was the product of Calbiochem. 2′,5′-ADP-Sepharose 4B was the product of Amersham Biosciences. Antibodies against eNOS and 3-phosphoinositide-dependent kinase 1 were purchased from BD Transduction Laboratories (San Diego, CA). Antibodies against phospho-eNOS (serine 1179), Akt, phospho-Akt (serine 473), phospho-Akt (threonine 308), and phospho-p38 were products of Cell Signaling Technology (Beverly, MA). The antibody against phospho-eNOS (threonine 497) was purchased from Upstate Biotechnology (Lake Placid, NY). Antibodies against PP2A-Aα/β, PP2A-B56-α, PP2A-C, cyclin B1, ubiquitin, phospho-JNK, β-tubulin, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). l-[14C]Arginine was purchased from PerkinElmer Life Sciences. The protease inhibitor tablet was the product of Roche Applied Science. Calmodulin, NADPH, l-arginine, tetrahydrobiopterin, N-nitro-l-arginine methyl ester, and other reagents were purchased from Sigma unless otherwise indicated.

**Cell Culture and Transfection**—Bovine aortic endothelial cells were purchased from Cell Systems (Kirkland, WA) and grown in the medium provided by the manufacturer. Human embryonic kidney 293 (HEK 293, ATCC) cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum (Invitrogen). HEK 293 cells neither contain eNOS mRNA nor express eNOS protein. Wild-type bovine eNOS cDNA in mammalian expression vector pcDNA3 was transfected to HEK 293 cells using Lipofectamine and PLUS reagents (Invitrogen) according to the manufacturer’s instruction. Transfected cells were cultured in selective media (complete Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Invitrogen)). HEK 293 cells neither contain eNOS mRNA nor express eNOS protein. Wild-type bovine eNOS cDNA in mammalian expression vector pcDNA3 was transfected to HEK 293 cells using Lipofectamine and PLUS reagents (Invitrogen) according to the manufacturer’s instruction. Transfected cells were cultured in selective media (complete Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Invitrogen)).

**Small Interfering RNA (siRNA)**—The siRNA SMARTpools for human PP2A-C and non-targeting control were purchased from (Dharmacon). In 12-well plates, cells were plated the day before transfection and grown to 30–50% confluence. siRNA oligonucleotides (100 nM) were transfected into cells with Oligofectamine reagent (Invitrogen) (32). After 48 h of transfection, Western blotting was carried out to examine the knockdown of targeted proteins.

**Cellular Fractionation**—Cells were harvested and suspended in homogenization buffer (20 mM Hapes-NaOH, pH 7.4, 10 mM NaCl, 1 mM dithiothreitol, 50 mM NaF, 1 mM Na3VO4, and protease inhibitor tablet). After a 30-min incubation on ice, cells were homogenized with a Dounce homogenizer and then centrifuged at 1000 × g for 10 min to remove the unbroken cells and nuclei. The supernatants were transferred to a fresh tube and designated as the cytosolic fraction. The pellets were resuspended and washed once with homogenization buffer. After another centrifugation (100,000 × g for 1 h, 4 °C), the pellets were recovered as the membrane fraction. In some experiments the cytosolic and membrane fractions were directly boiled in SDS/PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 40 mM dithiothreitol, and 10% glycerol) and analyzed by Western blotting. In other experiments, target proteins in cytosolic and membrane fractions were resuspended in radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 50 mM NaF, 1 mM Na3VO4, 5 mM sodium pyrophosphate, 1 mM EDTA, and protease inhibitor tablet), and immunoprecipitations were carried out with appropriate antibodies.

**Co-immunoprecipitation and Pulldown Assay**—Cells were harvested and lysed on ice for 30 min in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF, 1 mM Na3VO4, 5 mM sodium pyrophosphate, and protease inhibitor tablet). The cell lysates were centrifuged at 14,000 × g for 15 min, and the supernatants were recovered. Supernatants containing equal amounts of proteins were incubated with 2.5 mg of primary antibodies overnight at 4 °C. The immunoprecipitates were harvested by protein G PLUS-agarose beads (Santa Cruz Biotechnology). The beads were washed once with regular washing buffer (50 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA and 0.5% Nonidet P-40), twice with high salt washing buffer (50 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), and another time with regular washing buffer. Immunoprecipitates were then eluted by 5 min boiling of the beads in SDS/PAGE sample buffer and characterized by Western blotting with appropriate antibodies.

For the eNOS pulldown assay, 2′,5′-ADP-Sepharose 4B resins (100 ml of 50% slurry) were added to the cell lysates and incubated with a rotator for 2 h at 4 °C. The resins were washed similarly as described in the co-immunoprecipitation assay, and pulled down proteins were eluted by boiling the beads in SDS/PAGE sample buffer and analyzed by Western blotting.

**Western Blotting**—Cells were lysed on ice for 30 min in modified radioimmune precipitation assay buffer containing 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 50 mM NaF, 1 mM Na3VO4, 5 mM sodium pyrophosphate, and protease inhibitor tablet. Cell lysates were centrifuged at 14,000 × g for 15 min, and the supernatants were recovered. The total protein concentrations were determined by using the DC-protein assay reagent (Bio-Rad). The lysates were boiled in SDS sample buffer, and the proteins were separated by SDS/PAGE on 4–20% gradient gels (Invitrogen) and then transferred to nitrocellulose membranes by using semidry transfer cell (Bio-Rad). After blocking, the membranes were probed with the appropriate primary antibodies. Membrane-bound primary antibodies were detected using secondary antibodies conjugated with horseradish peroxidase. Immunoblots
were developed on films using the enhanced chemiluminescence technique (SuperSignal West Pico, Pierce).

**NOS Activity Assay**—eNOS activity was measured by the L-[14C]arginine to L-[14C]citrulline conversion assay (30, 31). To measure the activity of phospho-eNOS, the assay was performed in the presence of 10 mM Ca2+ as previously reported (8, 32). Briefly, cells were harvested in homogenate buffer (50 mM Tris-Cl, pH 7.4, 2 mM dithiothreitol, 50 mM NaF, 1 mM Na3VO4, and protease inhibitor mixture) and made homogeneous by pulse sonication. After centrifugation (14,000 × g for 15 min at 4 °C), the pellets were recovered, washed, and resuspended in homogenate buffer. Cell lysates (45 mg of protein) was added to the reaction mixture containing 50 mM Tris-HCl, pH 7.4, 0.5 mM calmodulin, 10 μM tetrahydrobiopterin, 5 μM L-[14C]arginine, and 45 μM L-arginine. After 45 min of incubation at 37 °C, the reactions were terminated by ice-cold stop buffer. L-[14C]Citrulline was separated by passing the reaction mixture through Dowex AG 50W-X8 (Na+ form, Sigma) cation exchange columns and quantitated by liquid scintillation counting.

**In Vitro Phosphatase Activity Assay**—Cells were lysed on ice for 30 min with phosphatase lysis buffer containing 50 mM Tris-HCl, pH 7.4, 0.1% Nonidet P-40, 1 mM EDTA, 2 mM dithiothreitol, and a protease inhibitor tablet. PP2A immunoprecipitates or 2.5'-ADP-Sepharose 4B pulldown complexes were prepared as described above. These beads were extensively washed with regular immunoprecipitation washing buffer and high salt (500 mM NaCl) washing buffer. The beads were further washed once with phosphatase buffer (50 mM Tris-HCl, pH 7.0, 100 mM CaCl2). Activities of PP2A in the beads were determined by using a malachite green phosphatase assay protocol with a phosphopeptide (KRpTIR; pT is phosphorylated Thr) as the substrate (Upstate Biotechnology) followed by the measurement of absorbance at 650 nm with a SPECTRAMax 190 microplate spectrophotometer.

**Statistics**—Data were expressed as the mean ± S.E. Comparisons were made using a two-tailed Student’s paired or unpaired t test. Differences were considered to be statistically significant at p < 0.05.

**RESULTS**

**Proteasomal Inhibition Induced eNOS Serine 1179 Dephosphorylation**—To determine the roles of proteasome in regulating eNOS turnover and phosphorylation, we examined the effects of a proteasome inhibitor MG132 on the levels of total eNOS and phospho-eNOS (serine 1179) in an eNOS stably transfected cell line (eNOS-HEK 293). As shown in Fig. 1A, MG132 treatment time-dependently decreased serum 1179-phosphorylated eNOS, whereas the levels of total eNOS were not significantly affected. MG132-induced serum 1179 dephosphorylation was a highly specific event because the phosphorylation status of other residues in eNOS such as threonine 497 or serine 635 was not significantly affected. MG132-induced serum 1179 dephosphorylation was observed in the stably eNOS-transfected HEK 293 cells (Fig. 1A). MG132 (10 μM) caused time-dependent eNOS serine 1179 dephosphorylation (p). The phosphorylation status of eNOS threonine 497, serine 635, and total eNOS content remained unchanged. MG132 treatment time-dependently decreased serum 1179 dephosphorylation, eNOS activity was reduced in MG132-treated cells (Fig. 1A). The classic NOS inhibitor L-NAME, another proteasome inhibitor that is structurally different from MG132, also selectively dephosphorylated eNOS serine 1179. D, proteasome inhibition in MG132 and lactacystin-treated cells was evidenced by the accumulation of cyclin B1. Representative blots were shown from six independent experiments.
ically dephosphorylated eNOS serine 1179, leading to decreased enzymatic activity.

To ascertain that our findings from eNOS-HEK 293 cells occurred in native endothelial cells, we also determined the effects of proteasome inhibition on eNOS phosphorylation in bovine aortic endothelial cells (BAECs). In consistent with the results from eNOS-HEK 293 cells, MG132 selectively reduced eNOS serine 1179 phosphorylation in a time-dependent manner (Fig. 2). These data demonstrated that the eNOS-HEK 293 cell was a valid model to investigate the regulation of eNOS phosphorylation by proteasome in endothelial cells.

PP2A Blockade or Knockdown Reversed the eNOS Dephosphorylation Induced by Proteasomal Inhibition—Because eNOS serine 1179 was reported to be dephosphorylated by PP2A (13), we sought to determine the roles of PP2A in proteasome inhibition-induced eNOS dephosphorylation. Okadaic acid at the range of 1–1000 nM has been demonstrated to only block PP2A (18). As shown in Fig. 3A, adding okadaic acid (50 nM) reversed MG132-induced eNOS serine 1179 dephosphorylation. As a result, phospho-eNOS activity in MG132-treated cells was also restored by okadaic acid treatment (Fig. 3B). To corroborate the results obtained with okadaic acid, we used siRNA to selectively knock down PP2A. As shown in Fig. 4, PP2A knockdown prevented MG132-induced eNOS serine 1179 dephosphorylation and preserved phospho-eNOS activity. These PP2A siRNA silence data were in accordance with the results from okadaic acid and further confirmed that that proteasome inhibition-induced eNOS serine 1179 dephosphorylation was mediated by PP2A.

To determine whether eNOS serine 1179 dephosphorylation by proteasomal inhibition was due to PP2A up-regulation, we investigated the effects of MG132 on PP2A expression and activity. PP2A is a heterotrimeric enzyme consisting of a catalytic subunit C, a structural subunit A, and a regulatory subunit B (19). Interestingly, the intracellular levels of the three PP2A subunits were not significantly affected by MG132 treatment (Fig. 3C). Although PP2A expression was unchanged by proteasomal inhibition, it was possible that PP2A activity might be enhanced. To rule out this possibility, we immunoprecipitated PP2A from cells and measured phosphatase activity. As shown in Fig. 3D, PP2A immunoprecipitates from control and MG132-treated cells displayed similar activities. Thus, dephosphorylation of eNOS serine 1179 in proteasome-inhibited cells was not due to increased PP2A expression or enhanced catalytic activity.

Proteasomal Inhibition Enhanced PP2A Association to eNOS—Because total PP2A expression and activity were not affected by proteasomal inhibition, we then asked whether PP2A subcellular localization was changed and, if so, whether PP2A translocation resulted in eNOS serine 1179 dephosphorylation. Because eNOS is a membrane-associated enzyme, experiments were conducted to determine the effects of MG132 on the distribution of PP2A in cytosolic and membrane fractions of eNOS-HEK 293 cells. As shown in Fig. 5A, in the...
control untreated cells PP2A mainly resided in cytosol. Only small amounts of PP2A were detected in the membrane fractions. After MG132 treatment, a significant increase of PP2A content was seen in the membrane fractions. These results indicated that cytosolic PP2A translocated to membrane after proteasome inhibition.

To determine whether PP2A and eNOS can physically associate each other on membrane, PP2A was immunoprecipitated from the cells. As shown in Fig. 5B, immunoprecipitation (IP) of PP2A resulted in a co-precipitation of eNOS in cells. Western blot (WB), eNOS pulldown assay showed the increase of PP2A association with eNOS in MG132-treated cells. Elevated PP2A activity was measured in pulled down eNOS after MG132 treatment (**, p < 0.01, versus control, n = 4). This figure was a representative of four separate experiments.

Inhibition augmented PP2A association to eNOS, leading to serine 1179 dephosphorylation.

Deactivation of eNOS-associated Akt in Proteasome-inhibited Cells—In addition to increased PP2A association, reduction of eNOS serine 1179 phosphorylation may also result from loss or deactivation of its upstream kinase Akt. Because Akt was reported to associate with eNOS by the intermediation of heat shock protein 90 (Hsp90) (20), we pulled down eNOS and studied the effects of proteasome inhibition on eNOS-associated Akt. Activation of Akt requires phosphorylation of its threonine 308 and serine 473 residues by 3-phosphoinositide-dependent kinase 1 (21). As shown in Fig. 6A, MG132 treatment dramatically decreased threonine 308- and serine 473-phosphorylated Akt, indicating that Akt was deactivated by proteasomal inhibition. This Akt deactivation was not due to the loss of eNOS-Akt association because the total amounts of Akt bound to eNOS remained the same. MG132 treatments also did not change the sum levels of Akt or 3-phosphoinositide-dependent kinase 1 (PDK1) in cells (Fig. 6B). Because proteasome inhibition augmented PP2A association to eNOS and phospho-Akt is known to be a substrate of PP2A (18), we hypothesized that deactivation of Akt was mediated by PP2A. Indeed, the addition of okadaic acid completely reversed the dephosphorylation of eNOS-associated Akt (threonine 308/serine 473) in MG132-treated cells (Fig. 6C). To ascertain that PP2A-mediated kinase dephosphorylation specifically occurred to eNOS-associated phospho-Akt, we also monitored the effects of MG132 on the phosphorylation status of p38 kinase and JNK, which are also known to be the substrates of PP2A (18).
shown in Fig. 6D, neither phospho-p38 nor phospho-JNK was affected by MG132 treatment. These data strongly indicated that increased PP2A association to eNOS dephosphorylated phospho-Akt, leading to Akt deactivation in proteasome-inhibited cells.

**Roles of Ubiquitination in Proteasome Inhibition-induced PP2A Membrane Translocation**—Finally, we explored the possible mechanisms underlying the membrane translocation of PP2A after proteasome inhibition. Because the most predominant consequence of proteasome inhibition is the accumulation of ubiquitinated proteins, we reasoned that ubiquitination of PP2A might involve in the process of its membrane translocation. To test this hypothesis, we immunoprecipitated PP2A-A (PR65) and -B subunits (B56) from cellular fractions and determined their ubiquitination status. In the absence of MG132, no significant ubiquitination was found to occur to the PR65 and B56 in the cytosol (Fig. 7, A and B). However, PR65 and B56 in cellular membrane fractions were weakly ubiquitinated. These data suggested that ubiquitination of PR65 and B56 subunits might be required for its membrane association. If this hypothesis was correct, it would predict that the increased PP2A in the membranes of proteasome-inhibited cells were ubiquitinated. Indeed, dramatic increases of ubiquitinated PR65 and B56 were seen in cellular membrane fractions after MG132 treatment (Fig. 7, A and B). Neither PR65 nor B56 in the cytosol of MG132-treated cells was detected to be ubiquitinated despite the massive accumulation of other ubiquitinated proteins (Fig. 7C). These data further supported the notion that ubiquitination of PR65 and B56 might mediate the cytosol-to-membrane translocation of PP2A after proteasomal inhibition.

**DISCUSSION**

The results described above unveiled a previously uncharacterized role of proteasome in controlling eNOS phosphorylation and function. The cardiovascular system heavily relies on phosphorylation regulation of eNOS to maintain its homeostasis (5, 6). Aberrant eNOS regulation and function have been implicated in the pathogenesis of a number of cardiovascular diseases such as atherosclerosis, hypertension, and stroke (1). Numerous biological stimuli ranging from hormones and physical stretch to the cholesterol-lowering drug simvastatin activate eNOS through the Akt-eNOS serine 1179 phosphorylation axis (10). The present study identified the ubiquitin-proteasome system as a novel modulator of eNOS phosphorylation. As the chief intracellular protein degradation pathway, the ubiquitin-proteasome system has been reported to be involved in neuronal NOS and inducible NOS turnover (22). Interestingly, no significant eNOS accumulation was seen in cells after proteasomal inhibition in the present study. On the other hand, dephosphorylation of eNOS serine 1179 occurred. Proteasomal inhibition-induced eNOS dephosphorylation was unique to its serine 1179 residue because the phosphorylation status of threonine 497 or serine 635 was unchanged. The functional significance of this dephosphorylation was evidenced by the fact that eNOS activity was markedly reduced in proteasome-inhibited cells. There was a study reporting that MG132 caused a moderate increase of eNOS levels in pulmonary artery endothelial cells (23). With BAECs, we did not observe significant changes in eNOS levels after MG132 treatment. The varied results may be due to different types of endothelial cells used. Govers et al. (24) also reported that neither MG132 nor lactacystin affected eNOS expression in BAECs. Interestingly, they observed that NO productions from BAECs were reduced by MG132 treatment. The mechanism was unknown. Our findings that proteasome inhibition down-regulated eNOS serine 1179 phosphorylation and activity may provide a plausible explanation for their observations. Together, our findings demonstrated that the ubiquitin-proteasome system regulates eNOS function by
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modulating its serine 1179 phosphorylation rather than protein turnover.

Blockade of PP2A by okadaic acid or PP2A knockdown completely reversed MG132-induced eNOS serine 1179 dephosphorylation and recovered enzymatic activity. These data indicated that PP2A is involved in the dephosphorylating action of proteasome on eNOS. Interestingly, our studies demonstrated that proteasomal inhibition-induced eNOS serine 1179 dephosphorylation was due to PP2A subcellular redistribution rather than up-regulation of PP2A expression or activity. This is probably because PP2A is already highly abundant in cells. It was estimated that PP2A constitutes near 1% total cellular proteins (14). PP2A is constitutively active and accounts for the major portion of serine/threonine phosphatase activity in cells and tissues. Proteasomal inhibition induced a cytosol-to-membrane translocation of PP2A. This subcellular PP2A redistribution rendered enhanced phosphatase activity on membrane, which carried out dephosphorylations of membrane-bound proteins such as eNOS without the need to raise total PP2A expression or activity in cells.

In the traditional view, PP2A passively counterbalances the actions of protein kinases by removing phosphate groups from the substrates of these kinases. Recent studies have changed this view and shown that PP2A can actively participate in signal transduction (19). However, unlike the kinases which recognize specific motifs of their target proteins, PP2A dephosphorylates phosphoserine and phosphothreonine residues in substrate proteins with no particular sequence specificity. How PP2A engages in a particular signaling pathway remains not fully understood. One way to achieve specific actions is to couple PP2A with its targets through protein-protein interactions (12). For example, it was reported that PP2A specifically modulates the Wnt-β-catenin signaling pathway through the interaction between its B56 subunit and adenomatous polyposis coli protein (25). In the present study we found that PP2A coupled with eNOS. Proteasomal inhibition induced more PP2A recruited to eNOS, leading to serine 1179 dephosphorylation. The specific recruitment of PP2A to eNOS also provided a plausible explanation why dephosphorylation distinctively occurred at serine 1179 but not threonine 497, because the later was shown to be selectively dephosphorylated by PP1 (26). The details of eNOS-PP2A association remain to be determined. Future studies are needed to elucidate whether eNOS directly couples with PP2A or they interact with each other via intermediate proteins.

Akt has been reported to associate eNOS through the scaffolding of heat shock protein 90 (20). Our current study revealed PP2A as another component of eNOS subproteome. This close assembling of PP2A and Akt to eNOS established a local Yin-Yan mechanism for the precise control of eNOS serine 1179 phosphorylation and function. Although most prior studies focused on the action of Akt, the present study highlighted that PP2A may play a dominant role in the overall control of eNOS serine 1179 phosphorylation. Upon proteasomal inhibition, recruitment of PP2A to eNOS dephosphorylated both phospho-eNOS and phospho-Akt. Dephosphorylation of the threonine 308 and serine 473 deactivated Akt. Thus, recruitment of PP2A to eNOS is an extremely effective way to down-regulate eNOS phosphorylation because it not only eliminated existing phospho-eNOS but also prevented de novo phospho-eNOS formation. Furthermore, the recruitment of PP2A to eNOS also ensured that dephosphorylation discriminately occurred to eNOS. Indeed, we found that other PP2A kinase substrates such as phospho-p38 and phospho-JNK were not affected in proteasome-inhibited cells.

Although proteasome-mediated proteolysis still dominates the function of ubiquitin, it has become to appreciate the important roles of ubiquitination in non-proteolysis processes such as protein trafficking, regulation of protein function, and protein-protein interactions (27). For example, monoubiquitination has been shown to be a necessary and sufficient signal for internalization of many cell surface proteins into the endocytic pathway (28). Ubiquitination was also found to critically modulate the activity of certain transcriptional factors. Ubiquitination of MEKK1 inhibited MKK1 and MKK4 as well as their downstream extracellular signal-regulated kinase 1/2 and JNK activation, and this was not due to proteasome-mediated protein degradation (29). A number of ubiquitin-binding proteins have been identified to play important roles in regulating a wide array of protein-protein interactions (27). The results in the present study suggested that ubiquitination of PP2A PR65 and B56 subunits may be essential for its membrane association and translocation. The diverse B subunits of PP2A have been long implicated in the process of PP2A subcellular localization (14, 19). But exactly how the B subunits position PP2A in cells remains not fully understood. Our findings that membrane-bound but not the cytosolic B56 was ubiquitinated suggested ubiquitination of B56 may be required for its membrane localization. The PP2A-A subunit PR65 in the cell membrane was also ubiquitinated. These data argued that ubiquitination of B56 and PR65 might involve in the process of PP2A membrane association. Indeed, proteasomal inhibition increased ubiquitinated PR65 and B56, and a simultaneous increase of PP2A presence in membrane was detected. Regardless whether ubiquitination is required for PP2A to associate with eNOS, future investigations are still needed. Nevertheless, these results suggested that ubiquitination was another important posttranslational modification mechanism to direct PP2A to a particular protein complex, such as Akt-eNOS, to modulate protein phosphorylation and signal transduction.

In summary, the findings of this study suggest the following mechanism for the interaction between proteasome function and eNOS phosphorylation. Normally, small amounts of PP2A were associated with eNOS to counterbalance the action of Akt. Under the condition of proteasomal inhibition, PP2A, likely via ubiquitination of PR65 and B56, translocated to the cell membrane and targeted on eNOS. PP2A subsequently dephosphorylated eNOS serine 1179 and phospho-Akt, leading to reduced enzymatic activity. The physiological and pathological significances of these findings remain to be explored. In light of the increasing evidence that proteasome dysfunction occurred in various diseases, it will be important to investigate if altered eNOS phosphorylation and function participate in the pathogenesis of these diseases.

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