Reactive Oxygen Species Sensitivity of Angiotensin II-dependent Translation Initiation in Vascular Smooth Muscle Cells*

Translated initiation, the rate-limiting step in protein synthesis, is a key event in vascular smooth muscle cell growth, a major component of vascular disease. Translation initiation is regulated by interaction between PHAS-I and the eukaryotic initiation factor 4E (eIF4E). Although angiotensin II (Ang II)-induced vascular smooth muscle cell hypertrophy requires the generation of reactive oxygen species (ROS), the ROS sensitivity of these events and their upstream activators remain unclear. Here, we investigated the role of ROS in the regulation of PHAS-I phosphorylation on Thr-70 and Ser-65, an event required for the release of eIF4E from PHAS-I. Ang II-induced Ser-65 phosphorylation was ROS-dependent as assessed by pretreatment with ebselen (3.6 ± 0.2 versus 1.1 ± 0.2), diphenylene iodonium (3.6 ± 0.2 versus 1.0 ± 0.1), and N-acetyl cysteine (3.6 ± 0.2 versus 1.2 ± 0.1), but Ang II-stimulated phosphorylation of Thr-70 was ROS-insensitive. Although phosphatidylinositol 3-kinase pathway inhibition by LY294002 blocked both Ser-65 and Thr-70 phosphorylation (3.8 ± 0.1 versus 0.8 ± 0.1 and 3.2 ± 0.2 versus 1.0 ± 0.01, respectively), protein phosphatase 2A inhibition by okadaic acid selectively increased (3.3 ± 0.1 versus 5.2 ± 0.1) and p38 mitogen-activated protein kinase inhibition by SB203580 selectively decreased (3.8 ± 0.1 versus 1.4 ± 0.3) Ser-65 phosphorylation. Dominant negative Akt adenovirus also inhibited only Ser-65 phosphorylation (3.7 ± 0.1 versus 1.0 ± 0.03). These results demonstrate a unique differential ROS sensitivity of two separate residues on PHAS-I, which seems to be explained by the selective involvement of distinct signaling pathways in the regulation of these phosphorylation events.

Oxidative stress has been shown to be a major contributing factor in the pathogenesis of vascular disease, including hypertension, atherosclerosis, and restenosis following angioplasty (1). A major source of vascular production of reactive oxygen species (ROS)

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1 The abbreviations used are: ROS, reactive oxygen species; VSMC, vascular smooth muscle cells; Ang II, angiotensin II; MAP, mitogen-activated protein; MAPK, MAP kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PPK2A, protein phosphatase 2A; NAC, N-acetyl cysteine; DN-Akt-Adv, dominant negative Akt adenovirus; PI, phosphatidylinositol.

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Oxidative stress has been shown to be a major contributing factor in the pathogenesis of vascular disease, including hypertension, atherosclerosis, and restenosis following angioplasty (1). A major source of vascular production of reactive oxygen species (ROS)

Dependent increase in O₂ production in the vasculature and in cultured vascular smooth muscle cells (VSMCs) (4). NAD(P)H oxidase-derived ROS are required for Ang II-induced VSMC hypertrophy, as shown by the ability of the flavin-containing oxidase inhibitor diphenylene iodonium (DPI), expression of p22phox antisense, or catalase overexpression to decrease VSMC hypertrophy (4, 5).

VSMC hypertrophy is characterized by an increase in Ang II-dependent protein synthesis in the absence of DNA synthesis (6). Translation initiation, the limiting step for protein synthesis, is critically dependent on phosphorylation of the PHAS-I-eIF4E complex. eIF4E is tightly regulated by interaction with PHAS-I, which acts as a translational repressor (7). When eIF4E is bound to PHAS-I, it binds to mRNA but cannot form an active initiation complex (8). However, upon stimulation with growth factors such as Ang II or IGF-1, eIF4E is released from PHAS-I and forms an active complex with eIF4G (9, 10). PHAS-I is phosphorylated on multiple serine and threonine residues including Thr-70, Thr-37, Thr-46, and Ser-65 (11). The temporal sequence of these phosphorylations remains debatable; however, there is good consensus that phosphorylation of Ser-65, which is necessary for the dissociation of PHAS-I from eIF4E (12), requires prior phosphorylation of Thr-37, Thr-46, Thr-70 (11).

Several Ang II-dependent signaling pathways have been implicated in the regulation of PHAS-I phosphorylation. Among these are ERK1/2 MAP kinases (7), p38 MAP kinase (14), and the PI 3-kinase signaling pathway, including Akt (15–17). Of these signaling intermediates, Akt (18) and p38 MAP kinase (19) have been shown to be redox-sensitive. However, it remains to be determined whether the sensitivity of these known regulators of PHAS-I to ROS mediates the Ang II response and confers ROS sensitivity to PHAS-I phosphorylation by Ang II.

Little attention has been given to the possible role of phosphatases in the regulation of translation initiation and PHAS-I phosphorylation, although recent results suggest the possibility that protein phosphatase 2A (PP2A) is involved in this event (20). In yeast, PP2A activation causes a dramatic decrease in translation initiation (20). Although the mechanism for this effect is unclear, it may involve dephosphorylation of either PHAS-I or eIF4E. Treatment with okadaic acid increases PHAS-I phosphorylation in 3T3-L1 adipocytes (21). Moreover, PP2A co-immunoprecipitates with p70S6 kinase, a rapamycin-sensitive, upstream regulator of Ang II-induced PHAS-I phosphorylation (22). This is interesting because PP2A has been shown to be ROS-sensitive: sublethal levels of oxidant stress have been shown to suppress PP2A in T cells (23), and PP2A activity has been found to be modulated by H₂O₂ and glutathione (24). Taken together, these data suggest that PP2A is a potential mediator of Ang II-regulated translation initiation.

The ROS sensitivity of Ang II-dependent PHAS-I phosphorylation and the signaling pathways that regulate this event

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remain unclear. In this study, we show differential, site-specific ROS sensitivity of Ang II-dependent PHAS-I phosphorylation on two sites, Thr-70 and Ser-65. In addition, we demonstrate the involvement of both ROS-sensitive and ROS-insensitive signaling pathways that are differentially and specifically involved in the regulation of these phosphorylation events. We show, for the first time, the ROS sensitivity of PP2A activity in response to Ang II and its involvement in the regulation of PHAS-I phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Materials**—DPI was purchased from Alexis Biochemicals. Ebselen and N-acetyl cysteine (NAC) were from Sigma. LY294002 was purchased from Calbiochem. U0126, okadaic acid, 4-amino-1-tet-butyl-3-(p-methylphenyl)pyrazol[3,4-d] pyrimidine (PP1), polyethylene glycol-superoxide dismutase, and SB203580 were from Sigma. Akt inhibitor (1L-6-hydroxymethyl-chiro-inositol 2-2(O)-O-methyl-3-O-sacetylcylcarbonate) was from Calbiochem. Anti-total PHAS-I antibodies were from Oncogene Research Products. Anti-phospho PHAS-I, anti-phospho-eIF4E, anti-Akt, anti-phospho Akt, anti-p38, anti-phospho p38, and anti-phospho ERK 1/2 MAP kinase antibodies were from Cell Signaling. Dominant negative Akt adenovirus (DN-Akt-Adv) was kindly provided by Dr. Hanjoong Jo (Emory University) (25).

**Cell Culture**—VSMCs were isolated from rat thoracic aorta by enzymatic digestion as described previously. Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 2 mm glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells between passage 6 and 20 were made quiescent in 0.1% calf serum for 24–48 h and used at confluence for experiments. For VSMCs stably transfected with a vector overexpressing catalase or with an empty vector control (26), Geneticin (400 μg/ml) in kinase reaction buffer for 10 min as described (27), and Ser-65 antibodies (Cell Signaling) were used for Western blotting. Bands were visualized by enhanced chemiluminescence (ECL, Amersham Biosciences) and quantified using NIH Image software.

**In Vitro Kinase Assay**—Growth-arrested VSMCs were treated with 10 μM SB203580 or 5 μM Akt inhibitor for 45 min prior to treatment with Ang II for 30 min. VSMC lysates were prepared as described (27) and immunoprecipitated using anti-Akt or anti-phospho MAP kinase antibodies. Samples were incubated with GST-PHAS-I (5 μg) and ATP (100 μM) in kinase reaction buffer for 10 min as described (27), and Ser-65 phosphorylation was determined using phospho-specific anti-Ser-65 antibodies.

**PP2A Activity Assay**—VSMC lysates were prepared in a phosphate-free lysis buffer (27) without sodium pyrophosphate, NaF, deoxycholic acid, SDS, and glycerol and then immunoprecipitated in the same buffer using anti-PP2A antibodies (Upstate Biotechnology). PP2A activity was measured according to the protocol supplied by Calbiochem. Briefly, PP2A immunoprecipitates were incubated with a PP2A-specific substrate for 5 min in PP2A reaction buffer (Calbiochem). The reaction was terminated by the addition of molybdate dye/additive mixture. Concentrations of free phosphate, released by PP2A, were measured in 96-well plates in a microplate reader at 540 nm.

**Data Analysis**—Blots shown are representative of at least three experiments. Data are expressed as mean ± S.E. Analysis of variance, followed by t test, was used for comparisons among multiple groups. Differences among means were considered significant at p < 0.05. Data were analyzed using Prism software.

**RESULTS**

**Differential ROS Sensitivity of Ang II-induced PHAS-I Phosphorylation on Thr-70 and Ser-65**—In this study, we focused on the phosphorylation of two critical residues of PHAS-I, Ser-65, a site required for the release of eIF4E and its subsequent function in translation initiation, and Thr-70, the phosphorylation of which must precede that of Ser-65. Fig. 1 shows the time course of PHAS-I phosphorylation by Ang II on these two sites as detected by site-specific anti-phospho PHAS-I antibodies. Maximal PHAS-I phosphorylation on Thr-70 occurred at 15 min (3.6 ± 0.1-fold versus control), whereas maximal phosphorylation of Ser-65 followed at 30 min (3.6 ± 0.2) (Fig. 1).

To determine the ROS sensitivity of the phosphorylation events that regulate translation initiation, we pretreated the cells with a panel of ROS inhibitors: the glutathione peroxidase mimetic ebselen, DPI to inhibit flavin-containing oxidases and NAC, and examined the effects of these antioxidants on site-specific PHAS-I phosphorylation. Preliminary experiments (data not shown) and previous studies (4, 28) verified that these compounds completely inhibit Ang II-induced ROS formation. All inhibitors completely blocked Ang II-dependent phosphorylation of Ser-65 (ebeslen (3.6 ± 0.2 versus 1.0 ± 0.2), DPI (3.6 ± 0.2 versus 1.0 ± 0.1), and NAC (3.6 ± 0.2 versus 1.2 ± 0.1), while having no significant effect on the phosphorylation of Thr-70 (Fig. 2A). Similar results for Ser-65 (3.5 ± 0.1 versus 1.2 ± 0.3) and Thr-70 (3.3 ± 0.1 versus 3.2 ± 0.1) were obtained in cells stably overexpressing catalase to prevent hydrogen peroxide formation (Fig. 2B). Incubation of VSMCs with 100 units/ml polyethylene glycol-superoxide dismutase for 24 h to scavenge superoxide (29) had no effect on Ser-65 or Thr-70 PHAS-I phosphorylation (Fig. 2C). Taken together, these data indicate that the phosphorylation of Ser-65 is mediated by H2O2, whereas the phosphorylation of Thr-70 is not.

Because eIF4E cannot be phosphorylated unless it is released from PHAS-I, and the release of eIF4E depends on PHAS-I phosphorylation, we hypothesized that eIF4E phosphorylation would also be ROS-sensitive. Both ebselen and DPI significantly attenuated Ang II-induced eIF4E phosphorylation at 30 min, confirming the role of ROS in translation initiation (data not shown).
The Roles of ERK1/2, p38, PI 3-kinase, and Akt in Site-specific PHAS-I Phosphorylation—Previous reports have established a possible role for the ERK1/2 (7, 30), p38 MAPK (31), and PI 3-kinase (15, 30, 32) signaling pathways in the regulation of PHAS-I phosphorylation in some cell types. Using site-specific anti-phospho Thr-70 and Ser-65 antibodies, we investigated the relative roles of these signaling pathways in the regulation of both Ser-65 and Thr-70 phosphorylation in VSMCs (Fig. 3).

Both the MEK inhibitor, U0126, and the specific PI 3-kinase inhibitor, LY294002, at concentrations that block activation of
ROS Sensitivity of PHAS-I Phosphorylation in VSMCs

**The Role of PP2A Phosphatase in Site-specific PHAS-I Phosphorylation**—To gain further insight into the differential ROS sensitivity of PHAS-I phosphorylation, we next focused our attention on the PP2A phosphatase, a protein that has been shown to be involved in protein synthesis in yeast. We determined the effect of okadaic acid, an inhibitor of the PP2A phosphatase, on Thr-70 and Ser-65 phosphorylation of PHAS-I (Fig. 4). Okadaic acid had no effect on Ang II-induced Thr-70 phosphorylation. However, it significantly increased Ang II-stimulated phosphorylation of Ser-65 (3.3 ± 0.1 versus 5.2 ± 0.1), indicating that PP2A phosphatase regulates Ser-65, but not Thr-70, phosphorylation.

If PP2A phosphatase contributes to the increase in Ser-65 phosphorylation during agonist stimulation, one would predict that phosphatase activity would be decreased by agonist. We measured PP2A activity as nanograms of free phosphate released from a PP2A-specific substrate. In this assay, okadaic acid completely inhibits PP2A activity, confirming specificity for PP2A. We found that PP2A activity was markedly inhibited (3.1 ± 0.1 versus 1.0 ± 0.1) after a 5-min stimulation of VSMCs by agonist.

The respective kinases (33), completely inhibited both Thr-70 and Ser-65 phosphorylation (Fig. 3A, lanes 3 and 4). In contrast, SB203580, the specific inhibitor of the redox-sensitive p38 MAPK, had no effect on the phosphorylation of Thr-70 but inhibited Ang II-dependent Ser-65 phosphorylation by 67 ± 2% and 74 ± 3%, respectively (Fig. 3A, lane 5, and 3B). To determine whether Akt or p38 MAP kinase is the actual kinase responsible for direct phosphorylation of Ser-65, we performed in vitro kinase assays in which we either immunoprecipitated p38 MAP kinase in the presence of 5 μM Akt inhibitor or immunoprecipitated Akt in the presence of 10 μM SB203580. Ser-65 phosphorylation, as detected by the phospho-specific antibody, was observed in both immunoprecipitates because Akt and p38 MAP kinase form a complex in VSMCs (data not shown). However, p38 MAP kinase inhibition had no effect on Ang II-induced Ser-65 phosphorylation, whereas Akt inhibition blocked it completely (Fig. 3C). The same concentration of Akt inhibitor completely blocked Ang II-dependent Akt phosphorylation but had no effect on p38 MAP kinase phosphorylation (data not shown). These data suggest the involvement of ERK1/2 and PI 3-kinase, but not of p38 MAPK or Akt, in the phosphorylation of Thr-70, whereas the subsequent phosphorylation of Ser-65 is dependent on all these signaling intermediates, with Akt as the final effector.

**Fig. 3.** ERK1/2, p38, Akt differentially regulate site-specific PHAS-I phosphorylation in response to Ang II. A. Left, growth-arrested VSMCs were pretreated with 5 μM U126, 10 μM LY294002, or 10 μM SB203580 for 45 min prior to treatment with 100 nM Ang II for 15 min. Western blot analysis was performed using anti-phospho-Thr-70-PHAS-I antibodies (top blot) or anti-PHAS-I antibodies (bottom blot). Lower panel, average data (mean ± S.E.) from three experiments, *p < 0.05 versus control; †p < 0.05 versus Ang II alone at the indicated time. Right, same as left panel, except that VSMCs were treated with Ang II for 30 min, and Western blot analysis was performed using anti-phospho-Thr-70-PHAS-I antibodies. B, VSMCs were infected with DN-Akt-Adv or Lac Z-Adv for 24 h, and then treated with 100 nM Ang II for 15 or 30 min. Left, Western blot analysis was performed using anti-phospho-Thr-70-PHAS-I antibodies (top blot) or anti-PHAS-I antibodies (bottom blot). Right, same as left panel, except that Western blot analysis was performed using anti-phospho-Ser-65-PHAS-I antibodies. Lower panels, average data (mean ± S.E.) from three experiments, *p < 0.05 versus control, †p < 0.05 versus Ang II alone at the indicated time. C, growth-arrested VSMCs were treated with 5 μM Akt inhibitor or with SB203580 for 45 min prior to treatment with 100 nM Ang II for 30 min. Cell lysates were immunoprecipitated (IP) with anti-p38 MAP kinase or anti-Akt antibodies as indicated. Western blot analysis was performed using anti-phospho-Ser-65-PHAS-I antibodies.
with Ang II (Fig. 5). Since antioxidants block the phosphorylation of Ser-65, it is possible that antioxidants act by preventing the Ang II-stimulated decrease in PP2A activity. As shown in Fig. 5A, ebselen (1.0 ± 0.1 versus 2.0 ± 0.1), DPI (1.0 ± 0.1 versus 2.1 ± 0.4), and NAC (1.0 ± 0.1 versus 1.9 ± 0.1) significantly attenuated the Ang II-induced inhibition of PP2A activity. Similar results were obtained in cells stably overexpressing catalase (1.0 ± 0.1 versus 1.8 ± 0.1) (Fig. 5A). These data support a partial ROS sensitivity of Ang II-induced PP2A inhibition.

The ROS sensitivity of PP2A could be the result of direct action of ROS on PP2A or the involvement of known ROS-sensitive signaling pathways in PP2A inhibition by Ang II. To determine the kinases that might regulate Ang II-induced PP2A inhibition, we used specific inhibitors of PI 3-kinase, p38 MAPK, Akt, and MEK. LY294002, SB203580, and U0126 prevented the Ang II-induced decrease in PP2A activity (Fig. 5B). On the other hand, DN-Akt-Adv was unable to reverse the effect of Ang II (Fig. 5B). These data implicate PI 3-kinase, p38 MAP kinase, and ERK1/2, but not Akt, as upstream regulators of PP2A activation.

**DISCUSSION**

Ang II-induced hypertrophy of VSMCs has been clearly shown to be dependent upon ROS (18, 26). We have previously shown that specific signal transduction mechanisms in VSMCs are ROS-dependent, including p38MAPK and Akt, whereas others, such as ERK1/2, are not (18, 19). It is possible that these proximal proteins are the major target of ROS and that the other enzymes display ROS sensitivity merely because they are downstream. It is likely to be more complicated than this simple scheme, however, because many pathways converge to activate a single kinase or gene, and ROS may modulate protein activity at many levels. Furthermore, all but the most proximal agonist-activated enzymes have multiple inputs that sometimes act in a compensatory manner, so it is important to understand the ROS sensitivity of each step in the signaling pathways leading to growth. PHAS-I is the immediate regulator of the eIF4E translation initiation factor (7, 12), the rate-limiting step in translation initiation, and is the target of multiple signaling pathways (14, 16, 17, 21). Because PHAS-I action requires phosphorylation on multiple sites (11), we postulated that it might serve as an integrator of ROS-dependent and -insensitive pathways. Our data clearly indicate that two critical residues of PHAS-I, Thr-70 and Ser-65, are differently regulated by ROS and are among the first to show that specific
phosphorylation sites of the same molecule can exhibit differential ROS sensitivity. These differences appear to be a direct consequence of the upstream Ang II-dependent signaling pathways that converge on each site.

Dissociation of the PHAS-I-eIF4E complex is the critical event for translation initiation. Stimulation of cells with hypertrophic factors leads to the sequential phosphorylation of PHAS-I in multiple residues, culminating in the phosphorylation of Ser-65 and resulting in the release of eIF4E (12). It has been previously demonstrated that the hypertrophic factor Ang II phosphorylates both PHAS-I and eIF4E (34), but neither the ROS sensitivity of this event nor the sites of PHAS-I targeted by Ang II have been investigated. In this study, we found that in Ang II-stimulated VSMCs, phosphorylation of Thr-70 (maximal at 15 min) precedes phosphorylation of Ser-65 (maximal at 30 min). These results are in agreement with previous reports indicating that phosphorylation of Thr-70 is required for the phosphorylation of Ser-65 (12).

Although Ang II-dependent protein synthesis has been shown to be ROS-dependent, the ROS sensitivity of PHAS-I and eIF4E phosphorylation in response to G-protein coupled agonists has not been investigated in detail. Others have shown that eIF4E can be phosphorylated by hydrogen peroxide and oxidized low density lipoprotein (35, 36), and we found that both PHAS-I and eIF4E phosphorylations are inhibited by a panel of antioxidants, but only at 30 min. Taken together with the time course shown in Fig. 1, these observations suggest that only the later events of PHAS-I phosphorylation would be expected to be ROS-sensitive. This is in fact what we found (Figs. 1 and 2). Phosphorylation of Thr-70 peaks at 15 min and is not affected by antioxidants, whereas phosphorylation of Ser-65 is not evident until 30 min and is completely blocked by antioxidants or catalase overexpression. Since the release of eIF4E critically depends on the ROS-dependent phosphorylation of Ser-65 of PHAS-I, and eIF4E cannot be phosphorylated until it is released, the ROS sensitivity of eIF4E phosphorylation is expected.

This observed ROS dependence of Ser-65 phosphorylation either may be due to direct effects of ROS on PHAS-I itself or may be a result of upstream ROS-sensitive signaling pathways that are involved in the regulation of PHAS-I phosphorylation. Thus, we investigated the possible contribution of known ROS-sensitive signaling pathways activated by Ang II in the site-specific phosphorylation of Thr-70 and Ser-65. Several signaling pathways have been implicated in the phosphorylation of PHAS-I in different cell types, including ERK1/2 (14), PI 3-kinase (15, 16, 37), p38 MAPK (14), and Akt (16, 17). These data are, however, controversial. Some investigators suggest that PHAS-I is phosphorylated in a PI 3-kinase-dependent manner and indicate that Akt is necessary and sufficient for the phosphorylation of PHAS-I (15, 16, 37). In support of this, growth factor-induced PHAS-I phosphorylation was shown to be mediated by a wortmannin- and rapamycin-sensitive pathway that was separate from ERK1/2 (38). In contrast, others report that PHAS-I is phosphorylated via a Ca^{2+}-sensitive and ERK1/2-dependent, but PI 3-kinase/Akt-independent, pathway (39). The discrepancies in these reports are likely due to the involvement of these signaling pathways in the phosphorylation of different sites on PHAS-I (11, 12, 40). Our data concerning the differential effect of kinase and phosphatase inhibitors on these sites support this interpretation and suggest a basis for the differential ROS sensitivity.

Of the ERK1/2, PI 3-kinase, p38 MAPK, and Akt signaling pathways, p38 MAPK and Akt have been shown to be ROS-dependent in VSMCs in response to Ang II (18, 19). Therefore we expected these latter pathways to be upstream of the ROS-sensitive Ser-65. Akt and p38 MAPK inhibition blocked Ser-65, but not Thr-70, phosphorylation by Ang II, suggesting that these signaling pathways are indeed responsible for conferring ROS sensitivity to Ser-65 phosphorylation. Since Akt, but not p38 MAP kinase, was able to phosphorylate Ser-65 in an in vitro kinase assay, we conclude that Akt is the actual kinase responsible for Ser-65 phosphorylation. The inhibitory effect of the p38 MAP kinase inhibitor, SB203580, is most likely due to the fact that p38 MAP kinase is an upstream regulator of Akt activation (41). However, Ser-65 phosphorylation was also attenuated by ERK1/2 and PI 3-kinase inhibition. This is most likely due to the temporal sequence of PHAS-I phosphorylation. Our data show that both ERK1/2 and PI 3-kinase are required for Thr-70 phosphorylation. Since Thr-70 phosphorylation precedes and is necessary for Ser-65 phosphorylation, blocking Thr-70 should also block Ser-65 phosphorylation, which is in fact what we found.

Another possible explanation for the differential ROS sensitivity that we observed in PHAS-I phosphorylation is phophatase activity targeted to a specific site. In yeast, activation of the serine/threonine phosphatase PP2A causes a dramatic decrease in translation initiation (20). Although the mechanism for this effect is unclear, it may involve dephosphorylation of either PHAS-I or eIF4E. Purified PP2A dephosphorylates eIF4E in vitro (42, 43), and conversely, treatment with okadaic acid increases PHAS-I phosphorylation in 3T3-L1 adipocytes (21). Thus, we examined PP2A involvement in the regulation of PHAS-I phosphorylation. When PP2A activity was blocked with a concentration of okadaic acid that specifically inhibits PP2A, Ser-65, but not Thr-70, phosphorylation was notably increased, suggesting that this phosphatase, as well as the mentioned kinases, plays a role in the regulation of Ser-65 phosphorylation by Ang II. This effect of PP2A may not be direct but may reflect regulation of other upstream pathways such as mammalian target of rapamycin (mTOR) (13). Of interest, PP2A inhibition by Ang II is partially ROS-sensitive, as assessed by antioxidant inhibition and catalase overexpression. These results are the first to show modulation of PP2A sensitivity by a wortmannin- and rapamycin-sensitive pathway that is in fact what we found.
activity by ROS in response to Ang II. This is consistent with studies showing that H$_2$O$_2$ modulates PP2A activation (24). ROS sensitivity could also be conferred by upstream signaling pathways since inhibitor experiments showed that ERK1/2, PI 3-kinase, and p38 MAPK are involved in Ang II inhibition of PP2A (Fig. 5).

It is evident that multiple ROS-sensitive and ROS-insensitive signaling pathways regulate the site-specific phosphorylation of PHAS-I. Fig. 6 schematically shows our proposed relationship between these pathways in Ang II-stimulated VSMCs. PHAS-I phosphorylation is regulated by both ROS-insensitive and ROS-sensitive signaling pathways so that the phosphorylation of the ROS-insensitive site, Thr-70, is regulated by ROS-insensitive ERK1/2 and PI 3-kinase. This phosphorylation of the ROS-insensitive site, Thr-70, is regulated by ROS-insensitive and ROS-sensitive signaling pathways so that the phosphorylation of PHAS-I that allows ROS-dependent phosphorylation of Ser-65, the site that is critical for PHAS-I function and the release of eIF4E. The phosphorylation of Ser-65 is regulated directly or indirectly by ROS-sensitive kinases and phosphatases, including p38 MAP kinase, Akt and the PP2A phosphatase. These observations provide novel insight into the complex ROS-sensitive molecular mechanisms regulating smooth muscle cell hypertrophy, an integral process in multiple vascular diseases.

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