Cross-talk between Arg methylation and Ser phosphorylation modulates apoptosis signal–regulating kinase 1 activation in endothelial cells

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ABSTRACT We describe a novel functional interaction between ASK1 and PRMT5. We show that PRMT5 interacts with and methylates ASK1 at arginine residue 89 and thereby negatively regulates its activity by promoting the interaction between ASK1 and Akt and thus phosphorylating ASK1 at serine residue 83. Furthermore, the association between ASK1 and Akt is enhanced by VEGF stimulation, and PRMT5 is required for this association. Moreover, PRMT5-mediated ASK1 methylation impaired the H2O2-induced activity of ASK1, and this inhibitory effect of PRMT5 was abolished by replacement of arginine 89 with Trp or depletion of PRMT5 expression by RNA interference. Together the results demonstrate cross-talk between arginine methylation and serine phosphorylation in ASK1.

INTRODUCTION

Apoptosis signal–regulating kinase 1 (ASK1), a 155-kDa protein, is a member of the mitogen-activated protein kinase (MAPK) kinase kinase family, which are activated in response to proinflammatory stimuli and cellular stress, leading to activation of MAPK c-Jun N-terminal kinase (JNK)/p38 signaling cascades (Ichijo et al., 1997; Hayakawa et al., 2006; Bunkoczi et al., 2007). For example, ASK1 is strongly activated in cells exposed to various oxidants, such as H2O2 and diamide (Matsuzawa et al., 2005; Soga et al., 2012). Oxidative stress–induced activation of ASK1 leads to apoptosis in various types of cells (Kadowaki et al., 2005; Hyun et al., 2010). Indeed, ASK1 is a central target of many cellular survival factors that bind to different domains of ASK1 to keep ASK1 in an inactive state (Du et al., 2004; Kim et al., 2012; Kosek et al., 2014). ASK1 can be phosphorylated at several sites, and these phosphorylation sites regulate ASK1 activity in both positive and negative manners (Kim et al., 2001; Tobiume et al., 2002; Fujii et al., 2004; Gu et al., 2009; Seong et al., 2010). In resting cells, ASK1 constantly forms an inactive complex with thioredoxin (Trx), but upon treatment of cells with tumor necrosis factor α or oxidants such as H2O2, ASK1 is dissociated from Trx and activated by subsequent modifications, including phosphorylation at the sites of Thr-845 (Fujino et al., 2002; Gu et al., 2009; Seong et al., 2010). Conversely, several serine/threonine protein kinases, such as Akt, directly phosphorylate ASK1 at Ser-83 to inhibit ASK1-induced apoptosis (Kim et al., 2001). Consistently, inhibition of Akt by phosphatidylinositol 3-kinase inhibitor significantly induces activation of p38 and JNK (Xie et al., 2009; Lu et al., 2010). The mechanism for ASK1 activation in response to apoptotic stimuli has not been fully elucidated.

Recent work shows that ASK1 is closely linked to cardiac diseases, such as cardiac hypertrophy, remodeling, and injury (Harding et al., 2010; Nako et al., 2012; Huang et al., 2014). In the left ventricle, ASK1 is activated by generation of angiotensin II–induced...
reactive oxygen species (ROS) through the angiotensin II type 1 receptor, resulting in cardiac hypertrophy and remodeling (Nako et al., 2012). Yokoi et al. (2006) reported that ASK1 mediates cellular senescence induced by high glucose in endothelial cells. They found that high glucose induces up-regulation of the ASK1 signaling in endothelial cells. However, transfection with a dominant-negative form of the ASK1 gene significantly inhibited SA-β-gal activity induced by high glucose (Yo, 2003) reported that H2O2 induces ASK1 activation. However, VEGF treatment prevented oxidative stress–induced ASK1 activation (Marui et al., 2012).

Protein arginine methyltransferases (PRMTs) are a group of proteins that catalyze the methyl transferal to the nitrogen of the terminal guanidine of arginines (Fisk and Read, 2011; Chen et al., 2014). PRMTs are divided into four types according to the terminal arginine modification. Types I–III all catalyze this first methyl transfer reaction (monomethylation), but, only type II PRMTs can generate symmetric dimethylarginine in target proteins (Fisk and Read, 2011). In this study, we sought to identify ASK1-interacting proteins to clarify new mechanisms of ASK1-mediated signaling. We identify PRMT5 (type II) as a novel ASK1-binding protein and show that it methylates Arg-89 in ASK1, and this methylation positively modulates Akt-mediated ASK1 phosphorylation at Ser-83, which attenuates H2O2-induced ASK1 activation.

RESULTS

Identification of PRMT5 as an ASK1-binding protein
To identify binding partners that might interact with ASK1, we overexpressed FLAG-tagged ASK1 in EAhy926 cells under VEGF stimuli and undertook coimmunoprecipitation. We found that a band at ~75 kDa was significantly enriched by VEGF stimulation (Figure 1A). We cut the slice and then tried to identify the ASK1-binding protein by liquid chromatography–tandem mass spectrometry (LC-MS/MS) after in-gel digestion. Surprisingly, the sequence identity of the peptides of this protein revealed the presence of PRMT5 (Figure 1B). Although it has been reported that when purifying a FLAG-tagged protein using FLAG antibody a major contaminant is the PRMT5/MEP50 protein complex (Chen and Gingras, 2007), we found that VEGF stimuli induced an abundance of PRMT5 in the immunoprecipitation complex (Figure 1, A and C), which indicates that the interaction is specific and functional.

To confirm the interaction between ASK1 and PRMT5, we first examined the physical association between exogenous ASK1 and PRMT5. To this end, we performed coimmunoprecipitation experiments in HEK293T cells transfected with FLAG-ASK1 and Myc-PRMT5 expression vectors. As shown in Figure 2A, immunoprecipitation of Myc-PRMT5 led to coimmunoprecipitation of FLAG-tagged ASK1 when both proteins were cotransfected. As a control, the anti-Myc antibody did not immunoprecipitate FLAG-tagged ASK1 in the absence of Myc-PRMT5. Similarly, immunoprecipitation of FLAG-tagged ASK1 resulted in coimmunoprecipitation of Myc-PRMT5, whereas the anti-FLAG antibody did not immunoprecipitate Myc-PRMT5 in the absence of FLAG-tagged ASK1. To determine a critical region in ASK1 for PRMT5 association, we constructed several deletion mutants of ASK1. As shown in Figure 2B, deletion of the C-terminal region of ASK1 from residues 687 and 946 did not affect its ability to bind to PRMT5. However, deletion of the N-terminal
PRMT5 mediates arginine methylation of ASK1

ASK1 is methylated and modulated by PRMTs. Cho et al. (2012), for example, reported the PRMT1-mediated methylation of ASK1 at Arg-31, Arg-32, Arg-78, Arg-80, Arg-89, and Arg-90 reside in an RG-enriched motif that serves as a methylation motif for PRMT5 (Figure 3A). We next examined which arginine residue of ASK1 is the methylation site. We replaced these six arginine residues with Trp by site-directed mutagenesis and examined whether the mutant proteins were methylated by PRMT5 in vitro. As shown in Figure 3D, the Arg mutant into Trp has no effect on the association of ASK1 and PRMT5; however, replacement of Arg-89 with Trp markedly reduced the extent of PRMT5-mediated methylation of ASK1 (Figure 3D and Supplemental Figure S1). In contrast, replacement of Arg-31, Arg-32, Arg-78, Arg-80, and Arg-90 with Trp did not affect the level of PRMT5-mediated methylation of ASK1. These data suggested that PRMT5 methylates ASK1 preferentially at Arg-89.

VEGF stimulation attenuates H2O2-induced ASK1 activation

Given that PRMT5 mediates the arginine methylation of ASK1, we next examined whether PRMT5 modulates ASK1 activity. VEGF has been reported to increase endothelial resistance to H2O2 (Liu et al., 2002), and thus we used the cell model of HUVECs stimulated with H2O2 and VEGF. As shown in Figure 4A, H2O2 stimulation induces HUVECs apoptosis. As expected, activation of ASK1 induced by H2O2 was observed by determining the phosphorylation of Ser-83, which inhibits the activity of ASK1 (Figure 4B). Intriguingly, H2O2 treatment decreases the interaction between ASK1 and PRMT5 in a coimmunoprecipitation analysis, which results in attenuation of the methylation of ASK1 (Figure 4C). These results suggest that VEGF-induced methylation of ASK1 positively regulates phosphorylation of Ser-83 and thus negatively regulates H2O2-induced activation of ASK1.

PRMT5-mediated methylation of ASK1 at Arg-89 promotes the association between ASK1 and Akt

On the basis of our finding that methylation of ASK1 negatively regulates H2O2-induced activation of ASK1, we next asked whether
methylolation of ASK1 at Arg-89 affects Ser-83 in vitro and in vivo. To address this issue, we used a HEK293T cell line cotransfected with Akt, PRMT5, and ASK1 or mutant ASK1 (R89W). As shown in Figure 5A (top and left), Akt phosphorylates wild-type ASK1 at Ser-83 but does not phosphorylate mutant ASK1 (R89W). In addition, phosphorylation of ASK1 at Ser-83 was inhibited at a basal level. immunoprecipitation analysis revealed that both ASK1 and mutant ASK1(R89W) interact with endogenous Akt, indicating the possibility that mutant ASK1(R89W) competed with endogenous ASK1 for interaction with Akt. PRMT5 enhances the association between ASK1 and Akt; however, this association is significantly inhibited by mutant ASK1 (R89W), further indicating the dominant-negative effect of mutant ASK1(R89W) on the phosphorylation level of endogenous ASK1 at Ser-83 (Figure 5A, bottom) by competing with endogenous ASK1 by inhibiting Ser-83 phosphorylation (Figure 5C). We next examined whether overexpression of PRMT5 attenuates H2O2-induced ASK1 activation in endothelial cells. As shown in Figure 6A, H2O2 treatment resulted in increased HUVEC apoptosis. Moreover, ectopic expression of PRMT5 inhibited H2O2-induced apoptosis in HUVECs. We determined the phosphorylation of ASK1 at Ser-83; the results show that H2O2 treatment inhibits phosphorylation of Ser-83 in ASK1 and activates caspase-3. Overexpression of PRMT5 significantly promotes Ser-83 phosphorylation in both basal and H2O2 treatment conditions, but H2O2-induced ASK1 kinase activity was inhibited. The H2O2-induced activation of caspase-3 was reduced by overexpression of PRMT5 (Figure 6B). We conducted further immunoprecipitation experiments in endothelial cells to study the mechanism(s) of how PRMT5 regulates phosphorylation and activity of ASK1 at Ser-83 mediated by Akt. This effect was also examined in EAhy926 cells transfected ASK1 and mutant ASK1. As shown in Figure 5B, VEGF stimulation resulted in activation of Akt and increased the phosphorylation of ASK1 at Ser-83. However, overexpression of mutant ASK1(R89W) significantly inhibited the phosphorylation of Ser-83 but had no effect on the activity of Akt(pS473-Akt), which was induced by VEGF stimulus. These data imply that posttranslational modification (methylation) of ASK1 at Arg-89 contributes to the phosphorylation of ASK1 at Ser-83 mediated by Akt. We next examined the effects of PRMT5 knockdown on the phosphorylation of ASK1 Ser-83 in endothelial cells stimulated with VEGF. As shown in Figure 5C, VEGF stimulus increases the activity of Akt and phosphorylation of ASK1 at Ser-83; knockdown of PRMT5 substantially suppresses the Ser-83 phosphorylation induced by VEGF but has no effect on the VEGF-induced Akt activity. These results indicate that PRMT5 plays an important role in VEGF-induced phosphorylation of ASK1 at Ser-83. Furthermore, immunoprecipitation analysis shows that VEGF stimulation enhances the interaction of ASK1 with both Akt and PRMT5, resulting in increased arginine methylation and phosphorylation (at Ser-83) of ASK1, as well as decreases the interaction of ASK1 and Akt (Figure 5D). Taken together, these data indicate that PRMT5 is required for VEGF-induced interaction of ASK1 and Akt in endothelial cells and PRMT5-mediated methylation of ASK1 at Arg-89 positively regulates its phosphorylation at Ser-83 by Akt.

PRMT5 suppresses H2O2-induced activation of ASK1

The ASK1 signaling pathway mediates the induction of apoptosis by oxidants such as H2O2. Deletion of PRMT5 by small interfering RNA (siRNA) increased the activation of ASK1 by inhibiting Ser-83 phosphorylation (Figure 5C). We next examined whether overexpression of PRMT5 attenuates H2O2-induced ASK1 activation in endothelial cells. As shown in Figure 6A, H2O2 treatment resulted in increased HUVEC apoptosis. Moreover, ectopic expression of PRMT5 inhibited H2O2-induced apoptosis in HUVECs. We determine the phosphorylation of ASK1 at Ser-83; the results show that H2O2 treatment inhibits phosphorylation of Ser-83 in ASK1 and activates caspase-3. Overexpression of PRMT5 significantly promotes Ser-83 phosphorylation in both basal and H2O2 treatment conditions, but H2O2-induced ASK1 kinase activity was inhibited. The H2O2-induced activation of caspase-3 was reduced by overexpression of PRMT5 (Figure 6B). We conducted further immunoprecipitation experiments in endothelial cells to study the mechanism(s) of how PRMT5 regulates ASK1 phosphorylation and activity of ASK1 at Ser-83.
In the present study, using a proteomics strategy, we successfully identified PRMT5 as an ASK1-binding protein in endothelial cells. The association of PRMT5 and ASK1 was further confirmed in vitro and in vivo. Further investigation indicated that the N-terminal of ASK1 was critical for association with PRMT5. PRMT5 catalyzes the methylation of ASK1 at Arg-89, and this PRMT5-mediated arginine methylation promotes the association between ASK1 and Akt and leads to increased ASK1 phosphorylation at Ser-83.

**FIGURE 4:** VEGF attenuates H$_2$O$_2$-induced ASK1 activation. HUVECs were treated with different concentrations of H$_2$O$_2$ for 24 h. Cell viability (A), phosphorylation of ASK1 at Ser-83 (B), and methylation of ASK1 (C, D) were determined by the indicated antibodies. HUVECs were pretreated with VEGF for 30 min and then treated with 300 μM H$_2$O$_2$ for 24 h. Cell viability (E) and phosphorylation of ASK1 at Ser-83, cleaved caspase-3 (F) and methylation of ASK1 (G) were determined by the indicated antibodies. *p < 0.05 compared with control group.

m ethylation. We verified that H$_2$O$_2$ treatment decreased the interaction between ASK1 and Akt in coimmunoprecipitation analysis in HUEVCs, and H$_2$O$_2$-induced dissociation was reversed by overexpression of PRMT5 (Figure 6B). The inhibition of arginine methylation (at Arg-89) and phosphorylation (at Ser-83) of Ask1 due to H$_2$O$_2$ stimulus was reversed by overexpression of PRMT5 (Figure 6B), suggesting that arginine methylation of ASK1 and interaction with Akt may be involved in the inhibitory effect of PRMT5 on H$_2$O$_2$-induced apoptotic cell death.

**DISCUSSION**

In the present study, using a proteomics strategy, we successfully identified PRMT5 as an ASK1-binding protein in endothelial cells. The association of PRMT5 and ASK1 was further confirmed in vitro and in vivo. Further investigation indicated that the N-terminal of ASK1 was critical for association with PRMT5. PRMT5 catalyzes the methylation of ASK1 at Arg-89, and this PRMT5-mediated arginine methylation promotes the association between ASK1 and Akt and leads to increased ASK1 phosphorylation at Ser-83.
ASK1 mediated H₂O₂-induced apoptosis in endothelial cells (Machino et al., 2003; Zhang et al., 2005). ASK1 is activated during the cellular response to ROS and then activates MAPK kinase 4 (MKK4)/MKK7 and MKK3/ MKK6, which in turn activate JNK and p38 kinase, respectively (Wang et al., 1998; Takeda et al., 2003). ASK1 can be regulated by phosphorylation at several sites, and at least seven phosphorylation sites have been identified. For example, ASK1 is regulated by phosphorylation or dephosphorylation at ASK1 mediated H₂O₂-induced apoptosis in endothelial cells (Machino et al., 2003; Zhang et al., 2005). ASK1 is activated during the cellular response to ROS and then activates MAPK kinase 4 (MKK4)/MKK7 and MKK3/ MKK6, which in turn activate JNK and p38 kinase, respectively (Wang et al., 1998; Takeda et al., 2003). ASK1 can be regulated by phosphorylation at several sites, and at least seven phosphorylation sites have been identified. For example, ASK1 is regulated by phosphorylation or dephosphorylation at
Arginine methylation occurred in the consensus sequences containing an RxRxxS/T motif has been reported in several Akt target proteins, such as FOXO and BAD (Yamagata et al., 2008; Sakamaki et al., 2011), indicating possible cross-talk between arginine methylation and serine phosphorylation in ASK1 and suggest that PRMT5 may be a potential molecular target for developing new antiapoptotic agents.

FIGURE 6: Overexpression of PRMT5 suppresses H2O2-induced activation of ASK1. (A) HUVECs were infected with either Ad-PRMT5 or Ad-GFP for 48 h and then treated with 300 μM H2O2 for 24 h. Cell viability assay (*p < 0.05 compared with control group). (B) Cell lysates obtained from A were subjected to Western blot. Phosphorylation of ASK1 at Ser-83 and cleaved-caspase-3 was determined. Methylation of ASK1 was determined by immunoprecipitation with antibody to ASK1.
antibodies were purchased from Cell Signaling Technology (Danvers, MA).

**Plasmid and site-directed mutagenesis**
Site-directed mutagenesis was performed by PCR with the use of a QuickChange Kit (Stratagene, La Jolla, CA). The arginine mutants of ASK1 were constructed with the following mutagenic primers: R31W, 5'-GGATCCTGCAAGGGAGGAGCCCGG-3'; R32W, 5'-GGATCCTGCAAGGGAGGAGCCCGG-3'; R78W, 5'-GAGCAGTGCCACCGGGGCCGGCGAC-3'; R80W, 5'-GCCCACCGAGGGCCGCGCTTGG-3'; R98W, 5'-GGGGAGGAGCCGGAGCCACGGTGGCA-3'; and R90W, 5'-GGGGAGGAGCCGGAGCCACGGTGGCA-3'. Mutant G367A/R368A pCS2-PRMT5 was constructed with the primer 5'-GTGCTGGAGACGACGGAGACGGACCTGGTGG-3' (mismatches with the template are underlined). All mutagens were verified by automatic DNA sequencing.

**Coimmunoprecipitation assay and Western blotting**
The coimmunoprecipitation assay was carried out as described previously (Daitoku et al., 2004). Protein extract (20 μg) was fractionated on SDS–PAGE gels and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore). The membrane was blocked with 1× PBS/0.3% Tween 20 containing 5% dry milk and incubated with primary antibody overnight at 4°C. The immune complexes were detected by chemiluminescence (ECL; Amersham International).

**Proteomic analysis**
FLAG-tagged ASK1 were overexpressed in EAhy926 cells, after which the cells were treated with 100 ng/ml VEGF for 30 min. Cell lysates were first precleared and then incubated with anti-FLAG antibodies. Next protein A/G-agarose beads were added to the cell lysates and incubated for 4 h. The bound proteins were analyzed by SDS–PAGE and Coomassie brilliant blue staining. Gel slices were excised and subjected to in-gel digestion with trypsin. Peptides were extracted from the gel pieces. Sample was resuspended in 23 μl of 50 mM (NH₄)₂CO₃ and analyzed by LC-MS/MS (Finngan LCQ Deca XP Plus ion trap mass spectrometer). All MS/MS spectra were searched against the NCBInr_20130221 database. Database searching was performed allowing for differential modification on cysteine residues and methionine residues and full tryptic cleavage, with peptide mass tolerance of 1.5 Da and fragment tolerance 0.8 Da.

**siRNA transfection**
The siRNAs used were, for human PRMT5, 5'-UUCGUGAACUCUUCUGUGCCCUUAG-3'; 5'-AAUGGUAUAUGAGCUGGGCUU-3', and for green fluorescent protein, 5'-UAAUGGUAUAUGAGCUGGGCUU-3'. Cells were transfected by using Lipofectamine RNAiMAX reagent according to the manufacturer's instructions (Invitrogen).

**In vitro methylation assay**
FLAG-ASK1 was incubated with Mec-PRMT5 in the presence of SAM (55 Ci/μmol) at 37°C for 6 h. After washing of the beads, the reaction products were analyzed by fluorography and Coomassie brilliant blue staining.

**Adenoviral constructs**
Recombinant adenoviral vectors Ad-GFP and Ad-PRMT5 were amplified in 293 cells and purified by cesium chloride gradient. After vector purification, adenoviral vectors were kept at −80°C in 10 mM Tris containing 20% glycerol.

**Cell viability assay**
Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (Cho et al., 2012).

**Statistical analyses**
Data are expressed as mean ± SE. The statistical significance of differences was assessed by Student’s t test or analysis of variance, as appropriate; p < 0.05 was considered statistically significant.

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