Dimethylarginine Dimethylaminohydrolase 2 Increases Vascular Endothelial Growth Factor Expression Through Sp1 Transcription Factor in Endothelial Cells

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Objectives—Dimethylarginine dimethylaminohydrolase (DDAH) is a degrading enzyme for asymmetrical dimethylarginine, an endogenous NO synthase inhibitor. The molecular mechanism for DDAH-induced vascular endothelial growth factor (VEGF) expression was examined.

Methods and Results—Although the transfection of expression vectors for 2 isoforms of DDAH, DDAH1, or DDAH2 increased DDAH activity in bovine aortic endothelial cells and human umbilical vein endothelial cells, expression and secretion of VEGF were increased only in DDAH2-transfected cells. Knocking down the DDAH2 gene reduced VEGF production, and DDAH2 overexpression enhanced both proliferation and migration of endothelial cells. The VEGF promoter activity was increased by DDAH2 transfection, which was not blocked by an NO synthase (NOS) inhibitor but required the Sp1 sites. DDAH2 overexpression increased nuclear protein levels bound to Sp1 oligonucleotides in endothelial cells. Sp1 small interfering RNA blocked DDAH2-induced upregulation of VEGF. DDAH2 transfection increased nuclear and threonine-phosphorylation levels of Sp1 in a protein kinase A (PKA)–dependent manner. Protein–protein interaction between DDAH2 and PKA was enhanced in DDAH2-transfected cells.

Conclusions—DDAH2 upregulated the expression of VEGF through Sp1-dependent and NO/NOS system-independent promoter activation. DDAH2-increased Sp1 DNA binding activity was PKA dependent. These mechanisms may provide a novel therapeutic strategy for VEGF-related vasculopathies such as atherosclerosis. (Arterioscler Thromb Vasc Biol. 2006;26:1488-1494.)

Key Words: DDAH ■ ADMA ■ Sp1 ■ VEGF ■ endothelial cell

A growing body of evidence has been accumulated that asymmetrical dimethylarginine (ADMA) constitutes an important determinant of the development of cardiovascular disease.1 This substance impairs endothelial function by competing with the substrate for NO synthase (NOS), L-arginine, and is metabolized mainly by dimethylarginine dimethylaminohydrolase (DDAH), a rate-limiting enzyme for plasma ADMA level. DDAH is composed of 2 isoforms, DDAH1 and DDAH2,2 and these 2 isoforms stem from different chromosomes and differ in several aspects. For example, DDAH2, but not DDAH1, is expressed in spleen, thymus, peripheral leukocytes, lymph node, and bone marrow. In cultured human endothelial cells, DDAH1 is uniformly distributed in the cytosol and nucleus, whereas DDAH2 is found only in the cytosol.3 The different characteristics of these isoforms suggest different physiological functions.

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In addition to the role of DDAH in regulating ADMA levels, recent studies have demonstrated that DDAH contributes to angiogenesis. Transgenic mice overexpressing human DDAH1 not only exhibits greater tissue DDAH activity, reduces plasma ADMA levels, increases NOS activity, but also enhances the ability for angioadaptation in response to an ischemic stimulus.4 Although augmented NOS activity is suggested,4 the mechanisms for enhanced ability of angiogenesis have not been elucidated. On the other hand, the transfection with a construct encoding DDAH2 is reported to enhance vascular endothelial growth factor (VEGF) mRNA expression in endothelial cells, and its effect on angiogenesis is mediated at least in part by the release of NO.5 Because the reduction of the ADMA concentration and the increase in NO levels in the DDAH2-overexpressing cells was modest and the study did not demonstrate a direct link between NO and VEGF expression,5 it remains to be elucidated whether DDAH-induced VEGF induction or enhanced angiogenic action of DDAH overexpression is mediated by the increase in NO levels.

In the present study, we examined a molecular mechanism for DDAH2-induced VEGF expression. We demonstrated...
that overexpression of DDAH2 but not DDAH1 induces the mRNA expression and secretion of VEGF in endothelial cells, resulting in the increase in proliferation and migration of endothelial cells. These effects are mediated by the activation of Sp1 transcription and do not depend on the NO/NOS pathway. This novel regulatory mechanism for the VEGF expression may provide a therapeutic strategy for the treatment of VEGF-related vasculopathies.

**Methods**

**Cell Culture and Materials**

Bovine aortic endothelial cells (BAECs) and human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Cell System). Inhibitors of protein kinase A (PKA), H89 and NOS, and N\(^2\)-nitro-L-arginine-methyl ester (L-NAME) were obtained from Calbiochem, Sigma-Aldrich, respectively. The proliferation and migration of endothelial cells were evaluated by Cell Proliferation kit and QCM Chemotaxis 96-well Cell Migration assay kit (both from Chemicon), respectively.

**Constructs and Transfection**

DDAH1 expression plasmid was prepared as described previously.\(^6\) cDNA for DDAH2 was cloned from murine kidney cDNA using the primers ATGGGGAGCCGGGGGA (forward) and GCAGTGGGGGCG TGTCGCTG (reverse), and the polymerase chain reaction (PCR) product was subcloned into the mammalian expression vector pcDNA3.2/V5/GW/D-TOPO (Invitrogen). The mouse VEGF (mVEGF)/pGL2 plasmids (−1207Luc, −927Luc, −100Luc, −81Luc, −81mLuc, or −1Luc) containing the mouse VEGF promoter sequences between −1207, −927, −100, −81, or −1 and +372 fused to a pGL2 vector, a firefly luciferase reporter plasmid (Promega), were prepared as described previously.\(^7\) Transfection was performed using Lipofectamine 2000 (Invitrogen).

**DDAH Immunoblotting and DDAH Activity**

Immunoblotting was performed using a rabbit anti-DDAH antibody,\(^8\) a mouse anti-DDAH2 antibody (Abcam Inc), and a rabbit anti-Sp1 antibody (Santa Cruz Biotechnology). Band intensities were quantified with the Scion Image Software (Scion Corp). DDAH activity was determined as described previously.\(^9\)

**Real-Time PCR**

Real-time PCR was performed using an ABI PRISM-7700 sequence detector (PE Applied Biosystems). SYBER Green I Dye (PE Applied Biosystems) was used to detect the PCR. The sequences used for the forward and reverse primers were: 5′-CGAACAACCATCAAC-3′ for bovine VEGF, 5′-GAACCTCTGCCTCTGTGGG-3′ and 5′-CGCTGCCTGGTGG-3′ for human VEGF, 5′-GGTGTGCTGCTGCGCA-3′ for rat VEGF, 5′-CCUGAGUGAAGCUGAU-3′ for VEGF mRNA expression, and 28SrRNA as an endogenous control.

**VEGF Protein, Luciferase Assay, and Electrophoretic Mobility Shift Assay**

The concentration of VEGF was measured by ELISA kit (Immuno Biological Laboratories Co). DDAH1 or DDAH2 expression plasmid, mVEGF/pGL2 (−1207Luc, −927Luc, −100Luc, −81Luc, −81mLuc, or −1Luc), and pRL-CMV (Renilla Luciferase Reporter Vector; Promega) were prepared\(^7\) and cotransfected into cells. Luciferase activity was measured using the Dual-Luciferase Reporter kit (Promega). Renilla luciferase activity was used for normalization of transfection efficiency. Electrophoretic mobility shift assay (EMSA) was performed using EMSA kit (Panomics) and Nuclear Extraction Kit (Panomics).
Transfection of DDAH2 Expression Vector Induced VEGF Promoter Activity

To test whether DDAH2 transfection increased VEGF promoter activity, -1207Luc, a plasmid containing the mouse VEGF promoter sequence between -1207 and +372 relative to the transcription start site fused to a pGL2 vector, was transiently transfected into BAECs, and promoter activity was assessed by luciferase assay. The luciferase activity was increased by 3.47-fold by the transfection of DDAH2 (Figure 2A). DDAH can increase NO, which might affect the VEGF expression.5 The pretreatment with L-NAME failed to alter the VEGF promoter activity in either basal (control) or DDAH2-transfected conditions (Figure 2A), suggesting that the NOS/NO pathway did not affect the basal VEGF level or mediate the upregulation of VEGF by DDAH2.

Next, we measured the promoter activity of a series of 5'-deletion constructs (-927 Luc, -450Luc, -100Luc, and -81Luc) and showed that the DDAH2-induced increments in the VEGF promoter activity averaged between 3.2- and 3.7-fold compared with control (Figure 2B). However, the induction of promoter activity was abolished by the deletion of the promoter region from -1207 to -1 (-1Luc), which suggested that the sequence between -81 and -1 was requisite for DDAH2-induced expression of the VEGF gene.

DDAH2 response region between -81 and -1 contained consensus Sp1 sites at -77 to -53.10 Transfection of -81(Sp1m)Luc, a plasmid containing mutations within the 2 Sp1 binding sites, revealed that the disruption of the Sp1 sites impaired the responsiveness to DDAH2 stimulation (Figure 2C). These results indicate that the activation of the VEGF promoter in response to DDAH2 expression depends on the integrity of 2 Sp1 sites. Similar results were observed in DDAH2-overexpressing HUVECs (Figure 2D and 2E).

Role of Sp1 Protein in DDAH2-Induced VEGF Induction in Endothelial Cells

To examine the ability of the nuclear protein in DDAH2-expressing cells to interact with Sp1 site, EMSAs were performed with the biotin-labeled Sp1 probe and nuclear extracts prepared from BAECs. As shown in Figure 3A, the binding proteins for Sp1 oligonucleotide were increased in the nuclei of BAECs transfected with DDAH2 compared with those in untransfected control cells (lane 2 versus lane 4). The addition of both unlabeled Sp1 oligonucleotide (lane 5) and antibody against Sp1 protein (lane 6) diminished this binding, indicating that Sp1 was a principal DNA-binding component of this protein–DNA complex.

The contribution of Sp1 to the DDAH2-induced VEGF expression was examined with the use of siRNA selectively
targeting Sp1. As evident in Figure 3B, this oligonucleotide effectively suppressed the Sp1 protein level in DDAH2-transfected HUVECs. We observed that DDAH2 transfection produced a marked increase (ie, 2.1+/−0.3-fold) in the VEGF mRNA expression level in HUVECs cotransfected with control (GFP) oligonucleotides (Figure 3C). The elevation in the VEGF mRNA expression was blunted by the treatment with Sp1 siRNA oligonucleotides. These observations indicate that Sp1 plays a critical role in the induction of VEGF by DDAH2.

DDAH2 Induced Sp1 Protein Expression and Sp1 Phosphorylation

We examined the mechanism for DDAH2-induced upregulation of Sp1 binding activity. The increase in Sp1 binding, as assessed by EMSA, allows us to speculate that DDAH2 increases the Sp1 levels. We therefore conducted immunoblotting analyses using nuclear extracts from DDAH2-transfected BAECs. Thus, Sp1 antibody yielded 2 distinct bands corresponding to molecular masses of 95- and 105-kDa (supplemental Figure IIA), an observation consistent with the previous report.11 Furthermore, DDAH2 transfection upregulated the nuclear Sp1 protein levels by 1.3+/−0.2-fold (supplemental Figure IIA). In contrast, cytoplasmic extracts from cells transfected with DDAH2 showed a similar amount of Sp1 protein abundance compared with control cells (supplemental Figure IIB).

We further evaluated the effects of DDAH2 overexpression on Sp1 protein phosphorylation. As illustrated in Figure 4A, DDAH2 enhanced the phosphothreonine level of Sp1 (1.7+/−0.1-fold induction), whereas the phosphoserine level was unaltered. Because Sp1 has been reported to be phosphorylated at threonine residues in a PKA-dependent manner,12 the effect of the PKA inhibitor on the threonine-phosphorylated Sp1 level was examined. Thus, H89 markedly attenuated threonine phosphorylation of Sp1 (Figure 4B). Consistent with this result, the induction of VEGF promoter activity by DDAH2 was markedly suppressed by H89 (Figure 4C) but not by genistein (a tyrosine kinase inhibitor) or chelerythrine (a PKC inhibitor). Finally, we examined the
protein–protein interaction between endogenous PKA and DDAH2. Immunoprecipitation using cell extracts prepared from BAECs that overexpressed DDAH2 revealed that DDAH2 was coprecipitated with endogenous PKA (Figure 4D).

Discussion

In the present study, we demonstrated that the transfection of DDAH1 or DDAH2 enhances the cellular DDAH activity in endothelial cells (Figure 1C). However, in this setting, only DDAH2 upregulates the VEGF mRNA and protein expression in endothelial cells (Figure 1D and 1E). Furthermore, DDAH2 transfection stimulates the VEGF production in a DNA dose-dependent fashion. Conversely, selective DDAH2 gene silencing decreases the VEGF production (supplemental Figure 1B). Both proliferation and migration of BAECs are increased by DDAH2 overexpression (supplemental Figure IC and ID). In concert, these results suggest that DDAH2 plays a substantial role in the proliferation and migration of endothelial cells as an essential physiological regulator of VEGF and contributes to angiogenesis.

Controversy attends the role of DDAH1/2 in the VEGF expression and angiogenesis. Previous investigations have demonstrated that transgenic mice overexpressing DDAH1 has enhanced ability for angioadaptation in response to an ischemic stimulus. Of note, angiogenesis in DDAH1 transgenic mice is inhibited by L-NAME, suggesting that DDAH1 could enhance angiogenesis by reducing ADMA levels, thereby increasing the synthesis of the proangiogenic factor NO. Furthermore, transfection of DDAH1 into glioma tumor cells promotes angiogenesis and enhances VEGF protein expression. In contrast, it has been demonstrated that transfection with a construct encoding DDAH2 enhances VEGF mRNA expression in endothelial cells, although the role of NO has not been elucidated. Furthermore, the present study clearly demonstrates enhanced endothelial migration and proliferation. Our current findings therefore unveil the formulation that DDAH2 upregulates VEGF expression and possibly angiogenesis without requirement of NO induction.

Although it has been reported that DDAH2 enhances VEGF mRNA expression in endothelial cells, the mechanism of this upregulation remains unclarified. In the present study, we have clearly shown that DDAH2 increases VEGF...
expression via the activation of Sp1 binding site of the promoter (Figure 2). The VEGF promoter contains several potential transcription factor binding sites such as Sp1 and activator protein 2. Thus, the introduction of mutation in the Sp1 site between 77 and 53 dramatically abolished the DDAH2-induced VEGF promoter activation. Furthermore, siRNA directed against Sp1 silenced the induction of VEGF mRNA by DDAH2. Finally, EMSA showed that DDAH2 increased the Sp1 protein bound to Sp1 oligonucleotide. In this regard, several stimuli are capable of increasing VEGF through the activation of Sp1, including prostaglandin E2 and retinoic acid. We have also demonstrated that overexpression of DDAH2 increases the nuclear expression of Sp1, with no appreciable changes in cytoplasmic Sp1 levels (supplemental Figure II). Several factors have been proposed as possible mechanisms for the upregulation of nuclear Sp1 levels, including enhanced Sp1 mRNA transcription, augmented nuclear translocation of Sp1 protein, and inhibition of Sp1 protein degradation. Of interest, it has been demonstrated that the phosphorylation of Sp1 induces the protein stability. It is surmised therefore that DDAH2 elicits the phosphorylation of Sp1 (see below) and then increases the nuclear Sp1 level.

In the present study, we further demonstrated that DDAH2 markedly elicits the phosphorylation of Sp1, particularly at the threonine but not the serine residue (Figure 4A and 4B). Because the Sp1 phosphorylation acquires augmented DNA binding activity, DDAH2-induced phosphorylation of Sp1 would result in an increased activity of Sp1 and subsequent stimulation of VEGF transcription. Indeed, the present study shows that PKA inhibition by H89 markedly prevents the Sp1 promoter activity (Figure 4C). Of interest, prostaglandin E2 is reported to stimulate VEGF production in a cAMP-dependent manner, and this action is blocked by PKA inhibitors. In this regard, a recent study has demonstrated that PKA can phosphorylate Sp1 protein and stimulate its DNA binding and transcriptional activity in vitro and in vivo. Collectively, in our experimental setting, PKA plays a stimulatory role in the DDAH2-induced VEGF transcription. Although several lines of studies, including our current finding, have revealed that Sp1 protein is phosphorylated by PKA, it is also reported that other kinases can phosphorylate Sp1 in vitro, including casein kinase and PKC.
less, H89 used in the present study possesses relatively high selectivity for PKA (IC50 values for PKA 0.048 μmol/L versus 31.7 μmol/L for PKC; 38.3 μmol/L for casein kinase 20). Furthermore, we found enhanced protein–protein interaction among DDAH2 and PKA (Figure 4D). Although our preliminary study failed to show that the DDAH2 overexpression increases the PKA activity, augmented interaction among these molecules would contribute to the enhanced phosphorylation of Sp1. Similar observation has been reported among the PKA, DDAH1, and neurofibromin.  

In concert, available evidence lends support to the formulation that DDAH2 enhances the PKA-dependent phosphorylation of Sp1, which then upregulates the VEGF upregulation.

The role of DDAH2 in mediating angiogenesis merits comment. It is well established that VEGF contributes importantly to angiogenesis.  

Conflicting results have been reported that NO enhances or inhibits the VEGF expression, depending on the experimental condition. Alternatively, we demonstrated that DDAH2 increases VEGF expression via an NO/NOS-independent pathway and have further shown that DDAH2 increases endothelial cell proliferation and migration (supplemental Figure IC and ID), suggestive of stimulatory effects of DDAH2 on angiogenesis. However, a controversy exists on the role of DDAH2 in the development of atherosclerosis. VEGF has been reported to be involved in the progression of atherosclerosis.  

In this sense, DDAH2 is considered to accelerate atherosclerosis through the induction of VEGF expression. On the other hand, DDAH2 can also reduce ADMA levels and elevate local NO levels, which would blunt the progression of atherosclerosis. Further studies are required to delineate the in vivo significance of DDAH2 expression.

In summary, the present study demonstrates that transfection with DDAH2 induces the phosphorylation of Sp1 protein at threonine residues in a PKA-dependent manner, as well as increases nuclear Sp1 protein level. These 2 mechanisms act in concert to upregulate the Sp1 DNA binding activity in endothelial cells and then enhance the VEGF mRNA and VEGF protein expression, independently of NO metabolism. Our current findings may provide a novel strategy for the treatment of VEGF-related vasculopathies, including diabetic microangiopathy and atherosclerosis.
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Figure I, Hasegawa et al.

A. Concentration of VEGF (ng/ml medium/10^6 cells)

B. Concentration of VEGF (ng/ml medium/10^6 cells)

C. Proliferation (% of control)

D. Migration (% of control)

Concentration of DDAH2 expression vector (µg/ml)

Concentration of siRNA

Control, GFP, DDAH2
A. Fold induction over control

B. Fold induction over control

Figure II, Hasegawa et al.
Figure I. The effect of DDAH2 on endothelial cell phenotype.

A. BAECs were transfected with varying concentrations of DDAH2 expression vector (0, 0.1, 0.5, 2.0, and 5.0 µg/ml). VEGF expression levels are normalized to that in cells transfected with 0.1 µg/ml of DDAH2 expression vector. *, p<0.05 vs. untransfected control BAECs, n=3.  

B. SiRNA selectively targeting DDAH2 decreased VEGF production in HUVECs. *, p<0.05, vs. GFP siRNA or untransfected cells, n=3. Both proliferation (C) and migration (D) of BAECs were increased by the overexpression of DDAH2. Data are presented as percentage of the number of proliferating or migrating cells transfected with DDAH2, relative to the cells transfected with control vector. Values are mean ± SD of 3 separate experiments performed in quadruplicates. *, p<0.05 vs. the cells transfected with control vector.
Figure II. Effects of DDAH2 on nuclear and cytosolic protein levels of Sp1

A. Nuclear extracts (8µg) from BAECs were analyzed by immunoblotting using antibody against Sp1. The intensity of double bands corresponding to Sp1 proteins (105 and 95 kDa) were increased significantly in the DDAH2-transfected cells as compared with untreated cells (*p<0.05. n=3). B. The intensity of double bands were not altered in cytosolic extracts (8µg) of the DDAH2-transfected cells as compared with that of untreated cells.