Role of Dimethylarginine Dimethylaminohydrolases in the Regulation of Endothelial Nitric Oxide Production*

Arthur J. Pope‡, Kanchana Karrupiah‡, Patrick N. Kearns‡, Yong Xia‡, and Arturo J. Cardounel‡*†

From the ‡Department of Physiology and Functional Genomics, University of Florida College of Medicine, Gainesville, Florida 32607 and the †Davis Heart and Lung Research Institute, Ohio State University, Columbus, Ohio 43210

Reduced NO is a hallmark of endothelial dysfunction, and among the mechanisms for impaired NO synthesis is the accumulation of the endogenous nitric-oxide synthase inhibitor asymmetric dimethylarginine (ADMA). Free ADMA is actively metabolized by the intracellular enzyme dimethylarginine dimethylaminohydrolase (DDAH), which catalyzes the conversion of ADMA to citrulline. Decreased DDAH expression/activity is evident in disease states associated with endothelial dysfunction and is believed to be the mechanism responsible for increased methylarginines and subsequent ADMA-mediated endothelial nitric-oxide synthase impairment. Two isoforms of DDAH have been identified; however, it is presently unclear which is responsible for endothelial ADMA metabolism and NO regulation. The current study investigated the effects of both DDAH-1 and DDAH-2 in the regulation of methylarginines and endothelial NO generation. Results demonstrated that overexpression of DDAH-1 and DDAH-2 increased endothelial NO by 24% and 18%, respectively. Moreover, small interfering RNA-mediated down-regulation of DDAH-1 and DDAH-2 reduced NO bioavailability by 27% and 57%, respectively. The reduction in NO production following DDAH-1 gene silencing was associated with a 48% reduction in l-Arg/ADMA and was partially restored with l-Arg supplementation. In contrast, l-Arg/ADMA was unchanged in the DDAH-2-silenced cells, and l-Arg supplementation had no effect on NO. These results clearly demonstrate that DDAH-1 and DDAH-2 manifest their effects through different mechanisms, the former of which is largely ADMA-dependent and the latter ADMA-independent. Overall, the present study demonstrates an important regulatory role for DDAH in the maintenance of endothelial function and identifies this pathway as a potential target for treating diseases associated with decreased NO bioavailability.

Endothelium-derived nitric oxide (NO)‡ is a potent vasodilator that plays a critical role in maintaining vascular homeostasis through its antiatherogenic and antiproliferative effects on the vascular wall. Altered NO biosynthesis has been implicated in the pathogenesis of cardiovascular disease, and evidence from animal models and clinical studies suggests that accumulation of the endogenous nitric-oxide synthase (NOS) inhibitors, asymmetric dimethylarginine (ADMA) and \( \text{ADMA}^\text{iso} \), methyl-L-arginine (L-NMMA) contribute to reduced NO generation and disease pathogenesis (1, 2). ADMA and L-NMMA are derived from the proteolysis of methylated arginine residues on various proteins. The methylation is carried out by a group of enzymes referred to as protein-arginine methyltransferases (3). Protein arginine methylation has been identified as an important post-translational modification involved in the regulation of DNA transcription, protein function, and cell signaling (4, 5). Upon proteolysis of methylated proteins, free methylarginines are released and can function as competitive inhibitors of NOS activity. The intracellular levels of these free methylarginines are regulated through their metabolism to citrulline by the activity of dimethylarginine dimethylaminohydrolase (DDAH) (6). Currently, there are two known isoforms of DDAH, each having unique tissue specificity. DDAH-1 is thought to be associated with tissues that express high levels of neuronal NOS, whereas DDAH-2 is thought to be associated with tissues that express eNOS (6–15). Decreased DDAH expression/activity is evident in disease states associated with endothelial dysfunction and is believed to be the mechanism responsible for increased methylarginines and subsequent ADMA-mediated eNOS impairment (6, 9, 16–22). However, the contribution of each isoform to the regulation of endothelial NO production has yet to be elucidated.

The most compelling evidence for DDAH involvement in endothelial dysfunction has come from studies using DDAH gene silencing techniques and DDAH transgenic mice. Specifically, Cooke and co-workers (9, 20) have demonstrated that DDAH-1 transgenic mice are protected against cardiac transplant vasculopathy. Other laboratories have demonstrated that DDAH overexpression inhibits ADMA-mediated endothelial function in cerebral arteries and can enhance insulin sensitivity through modulation of nitric oxide (23, 24). Using in vivo siRNA techniques, Wang et al. (11) demonstrated that DDAH-1 gene silencing increased plasma levels of ADMA by 50%, but this increase had no effect on endothelium-dependent relaxation. Conversely, in vivo DDAH-2 gene silencing had no effect on plasma ADMA but reduced endothelium-dependent relaxation by 40% (11). These latter findings are particularly intriguing and demonstrate that elevated plasma ADMA is not associated with impaired endothelium-dependent relaxation.
whereas loss of DDAH-2 activity is associated with impaired endothelium-dependent relaxation, despite the fact the plasma ADMA levels are not increased (11). These findings are further supported by a recent study demonstrating that down-regulation of DDAH-2 in response to hyperhomocysteinemia was not associated with increased plasma ADMA (25). Given the obvious inconsistencies in the literature regarding the individual roles of DDAH-1 and DDAH-2, the current study establishes the specific role of each DDAH isoform in the regulation of endothelial NO production and its potential role in disease pathogenesis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Bovine aortic endothelial cells (BAECs) were purchased from Cell Systems and cultured in Dulbecco’s modified Eagle’s medium (Sigma) containing 10% FBS, 1% NEAA, 0.2% endothelial cell growth factor supplement, and 1% antibiotic-antimyotic (Invitrogen) and incubated at 37 °C, 5% CO₂, 95% O₂.

**DDAH Overexpression**—Following the 48 h of Ad-DDAH-1 (50 MOI) or Ad-DDAH-2 (50 MOI) transduction, cells were trypsinized and pelleted at 1000 × g for 4 min. The cell pellet was then washed one time with PBS and centrifuged at 200 × g for 4 min. The cell pellet was then resuspended in 1.5 ml of OptiMEM plus 10% FBS, 1% NEAA, 0.2% endothelial cell growth factor. The cells were then added on top of the RNAiMax-siRNA complexes and incubated at 37 °C, 5% CO₂, 95% O₂ for 6 h. After the 6-h incubation period, 1 ml of minimum essential medium containing 10% FBS, 1% NEAA, 0.2% endothelial cell growth factor was added. The medium was removed and replaced with fresh minimum essential medium containing 10% FBS, 1% NEAA, 0.2% endothelial cell growth factor. The transfection was continued for an additional 24 h.

**Assessment of mRNA Levels following DDAH Gene Silencing**—Following the 72-h siRNA transduction period, BAECs were trypsinized and pelleted at 200 × g for 4 min. The cell pellet was then washed one time with PBS and centrifuged at 200 × g for an additional 4 min. The cell pellet was then homogenized in the lysis buffer. Following lysis, RNA was extracted using a Qiagen (Valencia, CA) RNAeasy minikit. CDNA was then isolated using the Invitrogen One Step reverse transcription-PCR kit. Semiquantitative PCR was performed in order to detect changes in mRNA expression following DDAH-1 or DDAH-2 gene silencing. Bovine primers for DDAH-1 forward (GAGGAGGAGGCTGACATGA), DDAH-1 reverse (TTCAGTGCAAGGCATCCAC), DDAH-2 forward (CTAGCCAAAGCTTACAGGGGACAT), and DDAH-2 reverse (TACGTCACACGTGCCATTTGGCCT) were purchased from Invitrogen.

**DDAH Activity**—DDAH activity was measured from the conversion of L-[^3]H]NMMA to L-[^3]H]citrulline. A T-75 flask was used for each measurement. BAECs were trypsinized, pelleted, and resuspended in 150 μl of 50 mM Tris (pH 7.4). The cells were then sonicated three times for 2 s each, and 150 μl of reaction buffer (50 mM Tris, 20 μM L-[^3]H]NMMA, 180 μM L-NMMA, pH 7.4) was added. The samples were then incubated in a water bath at 37 °C for 90 min. Following the 90-min incubation, the reaction was stopped with 1 ml of ice-cold stop buffer (20 mM HEPES with 2 mM EDTA, pH 5.5) (15). Separation of L-[^3]H]citrulline from L-[^3]H]NMMA was performed using the cation exchange resin Dowex AG50WX-8 (0.5 ml, Na⁺ form; Amersham Biosciences). The L-[^3]H]citrulline in the eluent was then quantified using a liquid scintillation counter.

**HPLC Method**—BAECs were collected from confluent 75-mm culture flasks and sonicated in PBS followed by extraction using a cation exchange column. Samples were derivatized with O-phthalaldehyde and separated on a Supelco LC-DABS column (4.6 × 25 cm inner diameter, 5 μm particle size), and methylarginines were separated and detected using an ESA (Chelmsford, MA) HPLC system with electrochemical detection at 400 mV. Homoarginine was added to the homogenate as an internal standard to correct for the efficiency of extraction.

**DDAH Activity and Nitric Oxide**—Following experiments were performed on cells grown in 6-well plates. Attached cells were studied because scraping or enzymatic removal leads to injury and membrane damage with impaired NO generation. The medium from 90% A to 30% A (22, 39).

**EPR Spectroscopy and Spin Trapping**—Spin trapping measurements of NO were performed using a Bruker E-scan spectrometer with Fe²⁺-MGD (0.5 mM Fe²⁺, 5.0 mM MGD) as the spin trap (22, 38). For measurements of NO produced by BAECs, cells were cultured as described above, and spin trapping experiments were performed on cells grown in 6-well plates. Attached cells were studied because scraping or enzymatic removal leads to injury and membrane damage with impaired NO generation. The medium from 90% A to 30% A (22, 39).

**HPLC Method**—BAECs were collected from confluent 75-mm culture flasks and sonicated in PBS followed by extraction using a cation exchange column. Samples were derivatized with O-phthalaldehyde and separated on a Supelco LC-DABS column (4.6 × 25 cm inner diameter, 5 μm particle size), and methylarginines were separated and detected using an ESA (Chelmsford, MA) HPLC system with electrochemical detection at 400 mV. Homoaarginine was added to the homogenate as an internal standard to correct for the efficiency of extraction. The mobile phase consisted of buffer A (50 mMKH₂PO₄,pH 7.0) and buffer B (acetonitrile/MeOH, 70:30) run at room temperature with a flow rate of 1.3 ml/min. The following gradient method was used: 0–10 min, 90% A; 10–40 min, a linear gradient from 90% A to 30% A (22, 39).

**DDAH-1 and -2 Gene Silencing**—21-bp siRNA nucleotide sequences targeting the coding sequences of DDAH-1 (GenBank™ accession number NM_001102201) and DDAH-2 (GenBank™ accession number NM_001034704) were purchased from Ambion. Control cells received scramble siRNA also purchased from Ambion. 400 μl of nuclease-free water was added to the dried oligonucleotides to obtain a final concentration of 100 μM. Transfections were performed using the lipid-mediated transfection reagent RNAiMax (Invitrogen). The procedure was as follows. 240 nm or 5 μl of siRNA per well of a 6-well plate was diluted into 250 μl of OptiMEM (Invitrogen), and 5 μl of RNAiMax was diluted in 250 μl of Opti-MEM. The siRNA and RNAiMax were then combined into one Eppendorf tube and incubated at room temperature for 20 min. Following the 20-min incubation period, the RNAiMax-siRNA complexes were added to each well of a 6-well plate. The mixture was rocked back and forth to allow for coating of the entire well. BAECs were trypsinized and spun down at 200 × g for 4 min and then resuspended in 1.5 ml of OptiMEM plus 10% minimum essential medium containing 10% FBS, 1% NEAA, 0.2% endothelial cell growth factor. The cells were then added on top of the RNAiMax-siRNA complexes and incubated at 37 °C, 5% CO₂, 95% O₂ for 6 h. After the 6-h incubation period, 1 ml of minimum essential medium containing 10% FBS, 1% NEAA, 0.2% endothelial cell growth factor was added. 24 h later, 1 ml of minimum essential medium containing 10% FBS, 1% NEAA, 0.2% endothelial cell growth factor was added. At 48 h, 2 ml of medium was removed and replaced with fresh minimum essential medium containing 10% FBS, 1% NEAA, 0.2% endothelial cell growth factor, and the transfection was continued for an additional 24 h.

**Assessment of mRNA Levels following DDAH Gene Silencing**—Following the 72-h siRNA transduction period, BAECs were trypsinized and pelleted at 200 × g for 4 min. The cell pellet was then washed one time with PBS and centrifuged at 200 × g for an additional 4 min. The cell pellet was then homogenized in the lysis buffer. Following lysis, RNA was extracted using a Qiagen (Valencia, CA) RNAeasy minikit. CDNA was then isolated using the Invitrogen One Step reverse transcription-PCR kit. Semiquantitative PCR was performed in order to detect changes in mRNA expression following DDAH-1 or DDAH-2 gene silencing. Bovine primers for DDAH-1 forward (GAGGAGGAGGCTGACATGA), DDAH-1 reverse (TTCAGTGCAAGGCATCCAC), DDAH-2 forward (CTAGCCAAAGCTTACAGGGGACAT), and DDAH-2 reverse (TACGTCACACGTGCCATTGGCCT) were purchased from Invitrogen.

**DDAH Activity**—DDAH activity was measured from the conversion of L-[^3]H]NMMA to L-[^3]H]citrulline. A T-75 flask was used for each measurement. BAECs were trypsinized, pelleted, and resuspended in 150 μl of 50 mM Tris (pH 7.4). The cells were then sonicated three times for 2 s each, and 150 μl of reaction buffer (50 mM Tris, 20 μM L-[^3]H]NMMA, 180 μM L-NMMA, pH 7.4) was added. The samples were then incubated in a water bath at 37 °C for 90 min. Following the 90-min incubation, the reaction was stopped with 1 ml of ice-cold stop buffer (20 mM HEPES with 2 mM EDTA, pH 5.5) (15). Separation of L-[^3]H]citrulline from L-[^3]H]NMMA was performed using the cation exchange resin Dowex AG50WX-8 (0.5 ml, Na⁺ form; Amersham Biosciences). The L-[^3]H]citrulline in the eluent was then quantified using a liquid scintillation counter.

**DDAH Overexpression**—Following the 48 h of Ad-DDAH-1 (50 MOI) or Ad-DDAH-2 (50 MOI) transduction, cells were trypsinized and pelleted at 1000 × g for 4 min. The cell pellet was then washed one time with PBS and centrifuged at 200 × g.
for an additional 4 min. The cell pellet was then homogenized using radioimmuno precipitation buffer containing sodium orthovanadate (2 mM), phenylmethylsulfonyl fluoride (1 mM), and protease inhibitor mixture (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Following homogenization, the cell pellet was briefly sonicated three times for 2 s each. Protein concentration was quantitated using the Bradford assay. 1x sample buffer containing dithiothreitol was added to 40 μg of protein and boiled at 95 °C for 3 min and then spun down briefly and cooled for 2 min. The samples were then loaded on an SDS Tris-glycine gradient gel, 4–12% (Invitrogen), and run at 130 V for 2 h. The gel was then removed, and the protein was transferred onto a nitrocellulose membrane using the semi-dry transfer blot system (Bio-Rad). Following the transfer, the nitrocellulose membrane was blocked for 1 h in Tris-buffered saline and 0.05% Tween (TBST) with 5% milk powder. After the blocking period, the membrane was washed three times for 5 min each with TBST, and then the respective primary antibody was added and incubated overnight at 4 °C. DDAH was detected by anti-DDAH-1 and anti-DDAH-2 rabbit IgG obtained from Dr. Renke Mass (Hamburg, Germany) and diluted 1:500. Following the overnight incubation with the primary antibody, the membrane was washed for 15 min three times with TBST, and the secondary goat anti-rabbit horseradish peroxidase-tagged antibody diluted 1:2000 was added. After 1 h of incubation at room temperature, detection was performed using an enhanced chemiluminescence kit purchased from Amersham Biosciences.

**eNOS Activity**—eNOS activity was measured from the conversion of L-[3H]arginine to L-[14C]citrulline. A T-75 flask was used for each measurement. BAECs were trypsinized, pelleted, and resuspended in 132 μl of 50 mM Tris (pH 7.4). The cells were then sonicated three times for 2 s each in 28 μl of reaction buffer (50 mM Tris containing 5 μM L-[14C]arginine, 50 μM L-arginine, 500 μM NADPH, 50 μM CaCl2, 10 μM tetrahydrobiopterin, pH 7.4). The samples were then incubated in a water bath at 37 °C for 30 min. Following the 30-min incubation, the reaction was stopped with 1 ml of ice-cold stop buffer (20 mM HEPES with 2 mM EDTA, pH 5.5). Separation of L-[14C]arginine from L-[14C]citrulline was performed using the cation exchange resin Dowex AG50WX-8 (0.5 ml of Na+ form; Amersham Biosciences). The L-[14C]citrulline in the eluent was then quantitated using a liquid scintillation counter.

**RESULTS**

**Effects of DDAH-1 and DDAH-2 Overexpression on Endothelial NO Production**—Previous studies have demonstrated that both DDAH-1 and DDAH-2 are expressed in the vasculature (6, 9, 11, 15). However, it is presently unknown which of the DDAH isoforms is responsible for the regulation of endothelial NO. Therefore, studies were carried out using adenovirus-mediated overexpression of both DDAH-1 and DDAH-2 in order to determine which isoform is responsible for endothelial methylarginine metabolism and NO regulation. Endothelial cells were grown to 90% confluence and then transduced with either Ad-DDAH-1 (10–50 MOI) or Ad-DDAH-2 (10–50 MOI) for 48 h. Western blot analysis demonstrated dose-dependent increases in endothelial expression of both DDAH-1 and DDAH-2 following respective adenoviral treatment (Fig. 1A). Additional studies were performed to assess the effects of DDAH overexpression on DDAH activity. Adenovirus-mediated overexpression of DDAH-1 (50 MOI) resulted in a nearly 2-fold increase in DDAH activity (Fig. 1B) Surprisingly, DDAH-2 overexpression did not result in a statistically significant increase in total DDAH activity despite a robust increase in protein expression. This is probably due to our preliminary observations that DDAH-2 catalytic activity is nearly 10 times less than that of DDAH-1 and is supported by previous studies demonstrating that increased DDAH-2 expression is not associated with an increase in total cellular DDAH activity (26). At the end of the 48-h period, NO production was measured by EPR as described previously. Results demonstrated that following 48 h of transduction, Ad-DDAH-1 (50 MOI)-mediated overexpression resulted in a 24% increase in NO production over basal NO levels (Fig. 2). HPLC analysis of intracellular ADMA and L-Arg demonstrated that although DDAH-1 overexpression increased the L-Arg/ADMA ratio by 27%, DDAH-2 gene silencing had no significant effect (Table 1). It was anticipated that if DDAH-1 overexpression is increasing NO through the metabolism of methylarginines, then L-Arg supplementation should prevent the increase. Endothelial cells were transduced with

3 A. J. Pope, K. Karrupiah, P. N. Kearns, Y. Xia, and A. J. Cardounel, unpublished results.
Ad-DDAH-1 (50 MOI) for 48 h as described previously. After the 48-h exposure, the medium was removed, and BAECs were incubated with l-Arg (100 μM) for 30 min in KREBS-HEPES buffer. Results demonstrated that l-Arg supplementation alone resulted in a 30% increase in basal NO production in control cells (Fig. 2). Moreover, in the presence of DDH-1 overexpression, l-Arg supplementation resulted in an additive effect with a 13% increase in NO compared with l-Arg supplementation alone.

Similar to Ad-DDAH-1, DDAH-2 (50 MOI) overexpression resulted in an 18% increase in endothelial NO production (Fig. 3). Similar results were obtained with l-Arg supplementation of DDAH-2-overexpressing cells in which we observed a 45% increase NO production following the addition of l-Arg compared with a 28% increase with l-Arg supplementation alone (Fig. 3). The observation that the effects of l-Arg supplementation on NO production are not attenuated in the presence of DDH-1 or DDAH-2 overexpression clearly demonstrates that ADMA is not responsible for the “arginine paradox” as has been proposed (27). Moreover, the ability of DDAH-2 overexpression to enhance endothelial NO production appears to be independent of ADMA because levels of this competitive NOS inhibitor were unaltered by DDAH-2 overexpression (Table 1).

**Effects of DDAH overexpression and silencing on endothelial l-Arg, ADMA, and l-Arg/ADMA**

| Group       | l-Arg | ADMA | l-Arg/ADMA |
|-------------|-------|------|------------|
| Control     | 489.5 | 2.3  | 212.8      |
| Ad-DDAH-1   | 511.7 | 1.9  | 269.3      |
| Ad-DDAH-2   | 556.0 | 2.5  | 222.4      |
| siDDAH-1    | 585.5 | 5.1  | 114.8      |
| siDDAH-2    | 471.0 | 2.2  | 214.1      |
| siDDAH-1/2  | 535.1 | 4.5  | 118.9      |

**TABLE 1**

Effects of DDAH overexpression and silencing on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 μM)-stimulated BAECs (1 × 10^6) was measured by EPR spin trapping with the Fe^{2+}-MGD complex. Left, the amplitude of the NO triplicate EPR spectra of 30 consecutive 20-s scans following a 30-min incubation period. Right, characteristic triplicate NO spectra and the effects of Ad-DDAH-1 (50 MOI) overexpression on NO production. Results are means ± S.D. *, significance at p < 0.05 as compared with the control (Ad-GFP, 50 MOI).

**FIGURE 2. Effects of DDAH-1 overexpression on endothelial cell NO production.** NO generation from calcium ionophore A23187 (1 μM)-stimulated BAECs (1 × 10^6) was measured by EPR spin trapping with the Fe^{2+}-MGD complex. Left, the amplitude of the NO triplicate EPR spectra of 30 consecutive 20-s scans following a 30-min incubation period. Right, characteristic triplicate NO spectra and the effects of Ad-DDAH-1 (50 MOI) overexpression on NO production. Results are means ± S.D. *, significance at p < 0.05 as compared with the control (Ad-GFP, 50 MOI).

**DDAH and Nitric Oxide**

Previous studies have suggested that a decrease in DDAH activity, as has been observed in vascular disease, contributes to endothelial dysfunction through a mechanism involving increased cellular ADMA levels. In support, ADMA levels are an independent risk factor for cardiovascular disease, and results from numerous clinical and basic science studies have revealed increased ADMA levels in a variety of diseases, including diabetes, pulmonary hypertension, coronary artery disease, and atherosclerosis (16, 19–22, 28, 29). However, whether loss of DDAH activity is directly responsible for the impaired NO production and which specific isoform is responsible for NO regulation in the endothelium are unknown. Therefore, in order to determine the role of each DDAH isoform in the regulation of endothelial NO, cellular studies were performed using BAECs to assess the effects of DDAH-1 and DDAH-2 gene silencing on NO production. Bovine aortic endothelial cells were cultured in 6-well plates, and using the reverse transfection protocol described under “Experimental Procedures,” DDAH-1 and DDAH-2 genes were silenced with specific siRNAs. The degree of gene silencing was evaluated using both semiquantitative PCR and Western blot analysis. Results demonstrated greater than 70% knockdown in the siDDAH-1 and siDDAH-2 groups compared with control (siGFP). Following the transfection period, the cells were harvested and homogenized. The samples were subjected to cation exchange and separated by HPLC. Peaks were identified based on retention times of standards for l-Arg and ADMA. Results represent the mean ± S.D. of at least three experiments. The results are representative of at least three independent experiments, and results from one experiment are shown.

**Effects of DDAH overexpression and silencing on endothelial l-Arg, ADMA, and l-Arg/ADMA**

**TABLE 1**

Effects of DDAH overexpression and silencing on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 μM)-stimulated BAECs (1 × 10^6) was measured by EPR spin trapping with the Fe^{2+}-MGD complex. Left, the amplitude of the NO triplicate EPR spectra of 30 consecutive 20-s scans following a 30-min incubation period. Right, characteristic triplicate NO spectra and the effects of Ad-DDAH-1 (50 MOI) overexpression on NO production. Results are means ± S.D. *, significance at p < 0.05 as compared with the control (Ad-GFP, 50 MOI).

**FIGURE 2. Effects of DDAH-1 overexpression on endothelial cell NO production.** NO generation from calcium ionophore A23187 (1 μM)-stimulated BAECs (1 × 10^6) was measured by EPR spin trapping with the Fe^{2+}-MGD complex. Left, the amplitude of the NO triplicate EPR spectra of 30 consecutive 20-s scans following a 30-min incubation period. Right, characteristic triplicate NO spectra and the effects of Ad-DDAH-1 (50 MOI) overexpression on NO production. Results are means ± S.D. *, significance at p < 0.05 as compared with the control (Ad-GFP, 50 MOI).

**DDAH and Nitric Oxide**

Previous studies have suggested that a decrease in DDAH activity, as has been observed in vascular disease, contributes to endothelial dysfunction through a mechanism involving increased cellular ADMA levels. In support, ADMA levels are an independent risk factor for cardiovascular disease, and results from numerous clinical and basic science studies have revealed increased ADMA levels in a variety of diseases, including diabetes, pulmonary hypertension, coronary artery disease, and atherosclerosis (16, 19–22, 28, 29). However, whether loss of DDAH activity is directly responsible for the impaired NO production and which specific isoform is responsible for NO regulation in the endothelium are unknown. Therefore, in order to determine the role of each DDAH isoform in the regulation of endothelial NO, cellular studies were performed using BAECs to assess the effects of DDAH-1 and DDAH-2 gene silencing on NO production. Bovine aortic endothelial cells were cultured in 6-well plates, and using the reverse transfection protocol described under “Experimental Procedures,” DDAH-1 and DDAH-2 genes were silenced with specific siRNAs. The degree of gene silencing was evaluated using both semiquantitative PCR and Western blot analysis. Results demonstrated greater than 70% knockdown in the siDDAH-1 and siDDAH-2 groups compared with control (siGFP). Following the transfection period, the cells were harvested and homogenized. The samples were subjected to cation exchange and separated by HPLC. Peaks were identified based on retention times of standards for l-Arg and ADMA. Results represent the mean ± S.D. of at least three experiments. The results are representative of at least three independent experiments, and results from one experiment are shown.

**Effects of DDAH overexpression and silencing on endothelial l-Arg, ADMA, and l-Arg/ADMA**

**TABLE 1**

Effects of DDAH overexpression and silencing on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 μM)-stimulated BAECs (1 × 10^6) was measured by EPR spin trapping with the Fe^{2+}-MGD complex. Left, the amplitude of the NO triplicate EPR spectra of 30 consecutive 20-s scans following a 30-min incubation period. Right, characteristic triplicate NO spectra and the effects of Ad-DDAH-1 (50 MOI) overexpression on NO production. Results are means ± S.D. *, significance at p < 0.05 as compared with the control (Ad-GFP, 50 MOI).
DDAH activity by measuring the conversion of L-[3H]NMMA to L-[3H]citrulline. Results demonstrated that DDAH-1 gene silencing resulted in a 64% decrease in total DDAH activity, and DDAH-2 silencing resulted in a 48% decrease in total DDAH activity (Fig. 5C). Interestingly, silencing of both DDAH-1 and DDAH-2 resulted in only a 50% drop in total DDAH activity, suggesting that other methylarginine metabolic pathways may be invoked as a consequence of loss of DDAH activity (Fig. 5C).

The functional effects of DDAH gene silencing were assessed using EPR spin trapping techniques to measure endothelium-derived NO production. Results demonstrated that DDAH-1 silencing reduced endothelial NO production by 27% (Fig. 6). In order to determine whether the effects of DDAH gene silencing on NO production resulted from increased intracellular levels of ADMA, L-Arg supplementation experiments were carried out to assess the ability of L-Arg to overcome ADMA-mediated eNOS inhibition. Specifically, DDAH gene silencing studies were carried out in the presence of L-Arg (100 μM). Results demonstrated that L-Arg (100 μM) supplementation restored 50% of the siDDAH-1 mediated loss of endothelial NO production (Fig. 6).

In order to determine whether the effects of DDAH gene silencing reduced endothelial NO production by 27% (Fig. 6). DDAH-2 gene silencing resulted in a 57% reduction in endothelial NO production. L-Arg supplementation did not increase endothelial NO production in DDAH-2-silenced BAECs (Fig. 7). These results further support our observation that the effects of DDAH-2 on endothelial NO production are independent of ADMA-mediated eNOS inhibition. Additional studies were performed in which both genes were silenced. Silencing of both the DDAH-1 and DDAH-2 genes resulted in 55% inhibition, which was not increased with L-Arg supplementation (Fig. 8).

These results are surprising, given that L-Arg would be expected to overcome the accumulation of methylarginines following loss of DDAH expression/activity. Therefore, to confirm that L-Arg supplementation can in fact ameliorate ADMA-mediated inhibition, validating studies were carried out with cells treated with exogenous ADMA, and the ability of L-Arg supplementation to overcome eNOS inhibition was measured. These studies were carried out using BAECs stimulated with the calcium ionophore A23187 (1 μM). EPR-based NO measurements were performed in modified Krebs buffer
by 50%, suggesting that the endothelium may possess alternate metabolic pathways for methylarginine metabolism. These are unexpected results, given that DDAH is considered to be the principle metabolic pathway for ADMA metabolism and was previously demonstrated to mediate >80% of cellular methylarginine metabolism in tissues (16). Therefore, studies were carried out using HPLC techniques with radiolabeled NMMA to assess the metabolites of methylarginine metabolism. Results demonstrated that in BAECs that were not DDAH-silenced, three radiolabeled peaks were identified as L-arginine, L-citrulline, and L-NMMA. However, in BAECs that were silenced, an additional unidentified radiolabeled peak was observed, suggestive of induction of an alternate metabolic pathway. Furthermore, following dual gene silencing, the concentration of the unknown metabolite increased 2-fold (Table 2). These results would suggest that BAECs have an alternate inducible pathway for methylarginine metabolism in response to loss of DDAH activity or methylarginine accumulation.

FIGURE 5. Effects of DDAH gene silencing on DDAH mRNA expression. A, DDAH mRNA expression was measured by semiquantitative PCR and run on an agarose gel to assess differences in DDAH expression following siRNA treatment. Experimental groups consist of 60 nM siRNA (siDDAH-1 and siDDAH-2) and 240 nM siRNA (siDDAH-1 and siDDAH-2). B, Western blot analysis was performed on BAEC following siRNA treatment directed against DDAH-1 (240 nM), DDAH-2 (240 nM), or dual silencing (240 nM each). C, DDAH activity was measured from the conversion of L-[14C]NMMA to L-[14C]citrulline using BAEC homogenates following 72 h of DDAH gene silencing. Experimental groups consisted of si-DDAH-1 (240 ng), si-DDAH-2 (240 ng), and dual silencing DDAH-1/2 (240 ng each). Control groups consisted of scrambled siRNA (240 ng). Results are means ± S.D. *, significance at p < 0.05 as compared with the respective control.

FIGURE 6. Effects of DDAH-1 gene silencing on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 μM)-stimulated BAECs (1 × 10⁶) was measured by EPR spin trapping with the Fe²⁺-MGD complex. Experimental groups consisted of scrambled siRNA (control) and siDDAH-1 (240 nM). These experiments were performed both in the presence and absence of L-arginine (100 μM). Results are means ± S.D. *, significance at p < 0.05 as compared with the respective control.

FIGURE 7. Effects of DDAH-2 gene silencing on endothelial cell NO production. NO generation from calcium ionophore A23187 (1 μM)-stimulated BAECs (1 × 10⁶) was measured by EPR spin trapping with the Fe²⁺-MGD complex. Experimental groups consisted of scrambled siRNA (control) and siDDAH-2. Results are means ± S.D. *, significance at p < 0.05 as compared with the respective control.
**DDAH and Nitric Oxide**

**Effects of DDAH-1 and -2 Gene Silencing on eNOS Activity—**The studies on DDAH silencing demonstrate that loss of DDAH-2 expression/activity may elicit ADMA-independent effects, given that L-Arg supplementation was not able to enhance endothelial NO production from DDAH-2-silenced cells. Therefore, studies were carried out in order to determine whether gene silencing has any direct effects on eNOS activity independent of ADMA. Studies were performed measuring the conversion of L-[14C]arginine to L-[14C]citrulline from BAEC homogenates following DDAH gene silencing. Results demonstrated that DDAH-1, DDAH-2, and dual silencing resulted in no change in total eNOS activity based on L-nitro-arginine methylester-inhibitable counts (Fig. 10).

**DISCUSSION**

ADMA plasma levels have been shown to be elevated in diseases related to endothelial dysfunction, including hypertension, hyperlipidemia, diabetes mellitus, and others (16, 28, 30–32). Moreover, it has been shown that ADMA predicts cardiovascular mortality in patients who have coronary heart disease. Recent evidence published in the multicenter Coronary Artery Risk Determination Investigating the Influence of ADMA Concentration (CARDIAC) study (33) has indicated that ADMA is indeed an independent risk factor for coronary artery disease. There is a growing body of evidence implicating ADMA as a key player in endothelial dysfunction and an independent risk factor involved in the pathophysiology of a variety of cardiovascular diseases (21–26). Recently, several groups have demonstrated that modulating DDAH activity can have a profound effect on endothelial NO production (8, 9, 11, 20, 21, 23–25, 34, 35). In this regard, our group and others have shown that overexpression of DDAH-1 results in increased NO production (9, 36). Furthermore, oxidized low density lipoprotein and tumor necrosis factor α have been shown to decrease DDAH activity, leading to decreased endothelial NO production (37). It has also been demonstrated that 4-hydroyxynonenal, the highly reactive oxidant product of lipid peroxidation, inhibits DDAH activity and leads to impaired NO generation through the formation of Michael addition products in the catalytic triad of DDAH (36). Thus, evidence suggests that DDAH-1 activity is under redox control, and loss of enzyme function impairs endothelial NO generation.

Whether the increased risk associated with elevated ADMA is a direct result of NOS impairment is an area of controversy. Significant debate about the contribution of ADMA to the regulation of NOS-dependent NO production has been initiated. In pathological conditions, such as pulmonary hypertension, coronary artery disease, diabetes, and hypertension, plasma ADMA levels have been shown to increase from an average of ~0.4 μM to ~0.8 μM (13, 29, 32, 33, 38, 39). Given that these values are at least 2 orders of magnitude lower than the plasma L-Arg levels, it is unlikely that elevated plasma ADMA can significantly regulate eNOS activity. It is more likely that elevated plasma ADMA levels reflect increased endothelial concentrations of ADMA. In support of this hypothesis, we and others have demonstrated that endothelial ADMA levels increase 3–4-fold in restenotic lesions and in the ischemia reperfused myocardium (18, 40). Based on the kinetics of cellular inhibition, these concentrations of ADMA would be expected to elicit a 30–40% inhibition in NOS activity (18). These studies, however, involve lesion-specific increases in ADMA and are not associated with increased plasma levels of ADMA and would not be expected to contribute to systemic cardiovascular pathology. In this regard, there is little direct evidence that elevated plasma ADMA levels are associated with increased endothelial ADMA; nor is it clear whether ADMA directly contributes to the NOS inhibition observed in chronic cardiovascular diseases.
TABLE 2
Effects of DDAH gene silencing on endothelial cell methylarginine metabolism

| Group        | Arginine | Citrulline | Unknown |
|--------------|----------|------------|---------|
| Control      | 5.5 ± 1.1| 4.1 ± 0.8  | 0 ± 0.0 |
| siDDAH-1     | 5.0 ± 0.9| 1.5 ± 0.5  | 2.2 ± 0.3|
| siDDAH-2     | 4.3 ± 0.4| 2.1 ± 0.5  | 2.8 ± 0.4|
| siDDAH-1/2   | 5.1 ± 0.9| 1.3 ± 0.2  | 4.4 ± 1.0|

The current hypothesis in the field suggests that decreased DDAH activity, as has been observed in cardiovascular diseases, results in impaired endothelial methylarginine metabolism with subsequent elevations in ADMA, leading to NOS inhibition. However, identification of the endothelial DDAH isoform responsible for NOS regulation and direct evidence for its role in modulating endothelial NO production have not been demonstrated. Therefore, the current study was undertaken to evaluate the roles of both DDAH-1 and DDAH-2 in the regulation of methylarginine metabolism and endothelial NO production.

Initial studies were carried out to determine how cellular endothelial NO production is regulated by the DDAH isoforms. DDAH-1 and DDAH-2 overexpression was induced using an adenoviral construct carrying either the human DDAH-1 gene (Ad-DDAH-1) or DDAH-2 gene (Ad-DDAH-2). Activity studies demonstrated that DDAH-1 overexpression resulted in a nearly 2-fold increase in total DDAH activity. However, protein expression was increased nearly 10-fold and suggests that DDAH activity may involve unidentified post-translational modifications. With regard to DDAH-2, despite a robust increase in protein expression, DDAH activity did not increase significantly following Ad-DDAH-2 treatment. We believe that this is probably the result of the low catalytic activity of this isoform and the induction of alternate methylarginine-metabolizing pathway(s). Nevertheless, results demonstrated that adenovirus-mediated overexpression of both DDAH-1 and DDAH-2 increased cellular endothelial NO production. However, although the former was associated with reduced intracellular ADMA levels, the latter was not and indicated that DDAH-2 may elicit its effects on NO through ADMA-independent mechanisms. Importantly, these initial studies were done in the presence of basal methylarginine levels and demonstrate that normal endogenous levels of these NOS inhibitors are present at concentrations sufficient to regulate eNOS activity. It had previously been proposed that ADMA may be responsible for the arginine paradox (27), and these studies would appear to support the hypothesis. However, subsequent studies using L-Arg supplementation with DDAH overexpression demonstrated an additive effect that clearly indicates that ADMA is not involved in the arginine paradox.

It has been estimated that more than 80% of ADMA is metabolized by DDAH (16); however, it is unclear which DDAH isoform represents the principal methylarginine-metabolizing enzyme. PCR and Western blot analysis have revealed that the endothelium contains mRNA and protein for both DDAH-1 and DDAH-2 (8, 11, 14, 15, 37, 41). However, in order to assess the relative contribution of each enzyme, a detailed analysis of the enzyme kinetics of each isoform is necessary. Unfortunately, detailed biochemical studies have only been published for DDAH-1 (19, 42). Using purified recombinant hDDAH-1, we and others have demonstrated the precise enzyme kinetics of this isoform, and results demonstrated $K_m$ values of 68.7 and 53.6 $\mu$M and $V_{max}$ values of 356 and 154 nmol/mg/min for ADMA and L-NMMA, respectively (19, 42). In regard to DDAH-2, previous attempts at purifying the protein have been unsuccessful primarily due to solubility issues with recombinant enzyme. Therefore, to investigate the role of the DDAH isoforms in the regulation of endothelial NO production, studies were performed using siRNA to silence both the DDAH-1 and DDAH-2 genes in BAECs. It was anticipated that silencing of DDAH would lead to increased cellular methylarginines and decreased endothelial NO production. Results supported this prediction and demonstrated that DDAH-1 silencing reduced endothelial NO production by 27%, whereas DDAH-2 silencing reduced it by 57%. These studies were then repeated with L-Arg supplementation in order to establish the ADMA dependence of the DDAH effects. The addition of L-Arg (100 $\mu$M) was able to restore 50% of the loss of endothelial NO generation observed with DDAH-1 silencing. Although it may be predicted that L-Arg supplementation should completely restore NO production given that ADMA is a competitive inhibitor of NOS, these results are consistent with previously published studies and suggest that DDAH-1 silencing may lead to ADMA accumulation in sites that are not freely exchangeable with L-Arg. In support of this hypothesis, it has been demonstrated by Simon et al. (43) that within the endothelial cell there exist two pools of L-Arg, both of which eNOS has access. Pool I is largely made up of extracellular cationic amino acids transported through the cationic amino acid transporter transport system; however, Pool II does not freely exchange with extracellular cationic amino acids. Furthermore, they also demonstrated that Pool II is separated into two components. Pool II A participates in the
recycling of citrulline to L-Arg, whereas Pool II B is occupied by protein-derived by-products. It is within this Pool II B where the methylarginines are likely to accumulate, thus rendering their inhibitory effects on eNOS (43). Alternatively, ADMA and/or DDAH may elicit effects that are independent of NOS; this appears to be the most plausible explanation with regard to DDAH-2, wherein loss of activity reduced endothelial NO production by greater than 50%, and this occurred in the absence of any significant change in endothelial ADMA levels. Moreover, the loss of NO was unaffected by L-Arg supplementation. This is strong evidence that DDAH may elicit effects that are independent of ADMA. Although this may represent an overall paradigm shift with regard to the role of DDAH in the endothelium, it is not without support. The most convincing evidence that DDAH may regulate cellular function through mechanisms independent of ADMA-mediated NOS inhibition comes from data on the DDAH-1 knock-out mouse. Homozygous null mice for DDAH-1 are embryonic lethal, whereas the NOS triple knock-out mice are viable (21). This provides strong evidence that DDAH effects are not limited to ADMA-dependent regulation of eNOS.

It has been widely reported that DDAH-2 is the predominant DDAH isoform in the vascular endothelium; however, these studies have relied on assessing the expression of the DDAH isoforms in various cell and tissue types (11, 15, 21, 41). Consequently, studies were carried out in BAECs to determine which isoform is responsible for the majority of the DDAH activity in the endothelial cell. DDAH-1 and DDAH-2 gene silencing decreased total DDAH activity by 64 and 48%, respectively. Additional studies demonstrated that dual gene silencing only resulted in a 50% loss in total DDAH activity in BAECs, thus suggesting that other methylarginine metabolic pathways may be invoked as a consequence of loss of DDAH activity. To investigate the possibility that loss of DDAH activity may lead to the induction of other methylarginine metabolic enzymes, we used HPLC techniques to measure the metabolic products of [1-3H]L-NMMA metabolism. In control cells, we observed three peaks with radioactive counts, and they were identified as L-NMMA, L-arginine, and L-citrulline. The formation of radio-labeled L-citrulline is probably from the metabolism of L-NMMA by DDAH, whereas radioactive L-Arg is generated from citrulline recycling through argino-succinate lyase and ASL. In contrast, results from DDAH-1- and DDAH-2-silenced cells indicated the presence of four radioactive peaks, including L-NMMA, L-arginine, L-citrulline, and an unidentified peak. The concentration of this unidentified peak increased 2-fold in the dual silencing group as compared with the levels in either the DDAH-1 or DDAH-2 silencing groups alone. Initial mass spectrometry analysis has been unsuccessful in identifying the unknown species and is currently an area of active investigation in our laboratory. Regardless, the results clearly indicate that the endothelium possesses an alternate inducible pathway for metabolizing methylarginines. Together, these results demonstrate that both DDAH-1 and DDAH-2 are involved in the regulation of endothelial NO production. However, although DDAH-1 effects are largely ADMA-dependent, DDAH-2 effects appear to be ADMA-independent.

To determine whether the ADMA-independent effects of DDAH silencing on endothelial NO production involved changes in eNOS protein, we measured eNOS activity from BAEC homogenates following DDAH-1, DDAH-2, and dual silencing. Analysis of eNOS activity demonstrated that DDAH gene silencing had no effect on the enzyme. These experiments were carried out in the presence of saturating concentrations of substrate and cofactors and thus cannot rule out DDAH effects on endothelial substrate/cofactor bioavailability.

Overall, these results demonstrate that loss of DDAH activity, as has been demonstrated in a number of cardiovascular diseases, leads to significant inhibition of endothelial NO production. Moreover, in the bovine endothelium, the effects of DDAH-1 and DDAH-2 appear to manifest through very different mechanisms, the former of which is largely ADMA-dependent and the latter ADMA-independent. In addition, results suggest that the endothelium may possess alternate inducible pathways through which methylarginines can be metabolized.

REFERENCES

1. Palmer, R. M., Ferrige, A. G., and Moncada, S. (1987) Nature 327, 524–526
2. Griffith, T. M., Edwards, D. H., Lewis, M. J., Newby, A. C., and Henderson, A. H. (1984) Nature 308, 645–647
3. Leiper, J., and Vallance, P. (1999) Cardiovasc. Res. 43, 542–548
4. Bedford, M. T., and Richard, S. (2005) Mol. Cell 18, 263–272
5. Boulanger, M. C., Liang, C., Russell, R. S., Lin, R., Bedford, M. T., Wainberg, M. A., and Richard, S. (2005) J. Virol. 79, 124–131
6. Vallance, P., and Leiper, J. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 1023–1030
7. Birdsey, G. M., Leiper, J. M., and Vallance, P. (2000) Acta Physiol. Scand. 168, 73–79
8. Smith, C. L., Birdsey, G. M., Anthony, S., Arrigoni, F. I., Leiper, J. M., and Vallance, P. (2003) Biochem. Biophys. Res. Commun. 308, 984–989
9. Tanaka, M., Sydow, K., Gunawan, F., Jacobi, J., Tsao, P. S., Robbins, R. C., and Cooke, J. P. (2005) Circulation 112, 1549–1556
10. Tran, C. T., Leiper, J. M., and Vallance, P. (2003) Atheroscler. Suppl. 4, 33–40
11. Wang, D., Gill, P. S., Chabrashvili, T., Onozato, M. L., Raggi, J., Mendonca, M., Dennehy, K., Li, M., Modlinger, P., Leiper, J., Vallance, P., Adler, O., Leone, A., Tojo, A., Welch, W. J., and Wilcox, C. S. (2007) Circ. Res. 101, 627–635
12. Wojciak-Stothard, B., Torondel, B., Tsang, L. Y., Fleming, I., Fissthaler, B., Leiper, J. M., and Vallance, P. (2007) J. Cell Sci. 120, 929–942
13. Zakrzewicz, D., and Eckelberg, O. (2009) BMC Pulm. Med. 9, 5
14. Palm, F., Onozato, M. L., Luo, Z., and Wilcox, C. S. (2007) Am. J. Physiol. Heart Circ. Physiol. 293, H3227–H3245
15. Gray, G. A., Patrizio, M., Sherry, L., Miller, A. A., Malaki, M., Wallace, A. E., Leiper, J. M., and Vallance, P. (2009) Acta Histochem., in press
16. Achan, V., Broadhead, M., Malaki, M., Whitley, G., Leiper, J., MacAllister, R., and Vallance, P. (2003) Arterioscler. Thromb. Vasc. Biol. 23, 1455–1459
17. Böger, R. H., Lentz, S. R., Bode-Böger, S. M., Knapp, H. R., and Haynes, W. G. (2001) Clin. Sci. 100, 161–167
18. Cardouel, A. J., Cui, H., Samouilov, A., Johnson, W., Kearns, P., Tsai, A. L., Berka, V., and Zweier, J. L. (2007) J. Biol. Chem. 282, 879–887
19. Hong, L., and Fast, W. (2007) J. Biol. Chem. 282, 34684–34692
20. Jacobi, J., Sydow, K., von Degenfeld, G., Zhang, Y., Dayoub, H., Wang, B., Patterson, A. J., Kimoto, M., Blau, H. M., and Cooke, J. P. (2005) Circulation 111, 1431–1438
21. Leiper, J., Nandi, M., Torondel, B., Murray-Rust, J., Malaki, M., O’Hara, B., Rossiter, S., Anthony, S., Madhani, M., Selwood, D., Smith, C., Wojciak-Stothard, B., Rudiger, A., Sidwill, R., McDonald, N. Q., and Vallance, P. (2007) Nat. Med. 13, 198–203
22. Lin, K. Y., Ito, A., Asagami, T., Tsao, P. S., Adimoolam, S., Kimoto, M., Tsuji, H., Reaven, G. M., and Cooke, J. P. (2002) Circulation 106, 987–992
23. Dayoub, H., Rodionov, R. N., Lynch, C., Cooke, J. P., Arning, E., Bottiglieri, T., Lentz, S. R., and Faraci, F. M. (2008) Stroke 39, 180–184
24. Sydow, K., Mondon, C. E., Schrader, J., Konishi, H., and Cooke, J. P. (2008) Arterioscler. Thromb. Vasc. Biol. 28, 692–697
25. Dayal, S., Rodionov, R. N., Arning, E., Bottiglieri, T., Kimoto, M., Murry, D. J., Cooke, J. P., Faraci, F. M., and Lentz, S. R. (2008) Am. J. Physiol. Heart Circ. Physiol. 295, H816–H825
26. Li Volti, G., Sorrenti, V., Acquaviva, R., Murabito, P., Gullo, A., Barcellona, M. L., Galvano, F., Rodella, L., Rezzani, R., Vanella, L., Tringali, G., Caruso, M., Gazzolo, D., and Di Giacomo, C. (2008) Anesthesiology 109, 1054–1062
27. Cooke, J. P. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 2032–2037
28. Böger, G. I., Rudolph, T. K., Maas, R., Schwedhelm, E., Dumbadze, E., Bierend, A., Benndorf, R. A., and Böger, R. H. (2007) J. Am. Coll. Cardiol. 49, 2274–2282
29. Maas, R., Schulze, F., Baumert, J., Löwel, H., Hamraz, K., Schwedhelm, E., Koenig, W., and Böger, R. H. (2007) Clin. Chem. 53, 693–701
30. Maas, R., Quitzau, K., Schwedhelm, E., Spieker, L., Raffenbeul, W., Steenpass, A., Lüscher, T. F., and Böger, R. H. (2007) Atherosclerosis 191, 211–219
31. Maas, R., Dentz, L., Schwedhelm, E., Thoms, W., Kuss, O., Hiltmeyer, N., Haddad, M., Kloss, T., Standl, T., and Böger, R. H. (2007) Crit. Care Med. 35, 1876–1881
32. Böger, R. H., Bode-Böger, S. M., Sydow, K., Heistad, D. D., and Lentz, S. R. (2000) Arterioscler. Thromb. Vasc. Biol. 20, 1557–1564
33. Schulze, F., Lenzsen, H., Hanefeld, C., Bartling, A., Osterziel, K. J., Goudeva, L., Schmidt-Lucke, C., Kusus, M., Maas, R., Schwedhelm, E., Strodtter, D., Simon, B. C., Mugge, A., Daniel, W. G., Tillmanns, H., Maisch, B., Streichert, T., and Böger, R. H. (2006) Am. Heart J. 152, 493.e1–e8
34. Onozato, M. L., Tojo, A., Leiper, J., Fujita, T., Palm, F., and Wilcox, C. S. (2008) Diabetes 57, 172–180
35. Potena, L., Fearon, W. F., Sydow, K., Holweg, C., Luikart, H., Chin, C., Weisshaar, D., Mocarski, E. S., Lewis, D. B., Valantine, H. A., and Cooke, J. P. (2008) Transplantation 85, 827–833
36. Pope, A. J., Druhan, L., Guzman, J. E., Forbes, S. P., Murugesan, V., Lu, D., Xia, Y., Chicoine, L. G., Parinandi, N. L., and Cardounel, A. J. (2007) Am. J. Physiol. Cell Physiol. 293, C1679–C1686
37. Ito, A., Tsao, P. S., Adimoolam, S., Kimoto, M., Ogawa, T., and Cooke, J. P. (1999) Circulation 99, 3092–3095
38. Zoccali, C., Maas, R., Cutrupi, S., Pizzini, P., Finocchiaro, P., Cambareri, F., Panuccio, V., Martorano, C., Schulze, F., Enia, G., Tripepi, G., and Böger, R. (2007) Nephrol. Dial. Transplant. 22, 801–806
39. Vallance, P., and Leiper, J. (2005) Am. Soc. Nephrol. 16, 2254–2256
40. Stühlinger, M. C., Conci, E., Haubner, B. J., Stocker, E. M., Schwaighofer, L., Cooke, J. P., Tsao, P. S., Pachinger, O., and Metzler, B. (2007) Cardiovasc. Res. 75, 417–425
41. Leiper, J. M., Santa Maria, J., Chubb, A., MacAllister, R. J., Charles, I. G., Whitley, G. S., and Vallance, P. (1999) Biochem. J. 343, 209–214
42. Forbes, S. P., Druhan, L. J., Guzman, J. E., Parinandi, N., Zhang, L., Green-Church, K. B., and Cardounel, A. J. (2008) Biochemistry 47, 1819–1826
43. Simon, A., Plies, L., Habermeyer, A., Martiné, U., Reining, M., and Closs, E. I. (2003) Circ. Res. 93, 813–820