Isoform-Specific Regulation by $N^G,N^G$-Dimethylarginine Dimethylaminohydrolase of Rat Serum Asymmetric Dimethylarginine and Vascular Endothelium-Derived Relaxing Factor/NO

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Abstract—Asymmetric dimethylarginine (ADMA), which inhibits NO synthase, is inactivated by $N^O,N^O$-dimethylarginine dimethylaminohydrolase (DDAH). We tested whether DDAH-1 or -2 regulates serum ADMA ($S_{ADMA}$) and/or endothelium-derived relaxing factor (EDRF)/NO. Small inhibitory (si)RNAs targeting DDAH-1 or -2, or an siRNA control were given intravenously to rats. After 72 hours, EDRF/NO was assessed from acetylcholine-induced, NO synthase–dependent relaxation and 4-amino-5-methylamino-2,7′-difluorofluorescein diacetate for NO activity in isolated mesenteric resistance vessels (MRVs). Expression of mRNA for DDAH-1 versus -2 was 2- and 7-fold higher in the kidney cortex and liver, respectively, whereas expression of DDAH-2 versus -1 was 5-fold higher in MRVs. The proteins and mRNAs for DDAH-1 or -2 were reduced selectively by 35% to 85% in the kidney cortex, liver, and MRVs 72 hours following the corresponding siRNA. $S_{ADMA}$ was increased only after siDDAH-1 (266±25 versus 342±39 [mean±SD] nmol·L$^{-1}$; $P<0.005$), whereas EDRF/NO responses and NO activity were not changed consistently by any siRNA. In conclusion, $S_{ADMA}$ is regulated by DDAH-1, which is expressed at sites of ADMA metabolism in the kidney cortex and liver, whereas EDRF/NO is regulated primarily by DDAH-2, which is expressed strongly in blood vessels. This implies specific functions of DDAH isoforms. (Circ Res. 2007;101:627-635.)

Key Words: RNA interference ■ hypertension ■ kidney ■ blood vessel ■ endothelium
hypothesis that a specific DDAH isoform regulates S_{DMA} and/or EDRF/NO.12

Materials and Methods

Animal Preparation

Studies were approved by the Georgetown University Animal Care and Use Committee. Experiments were performed on male Sprague–Dawley rats weighing 210 to 300 g fed a synthetic casein-based diet with precisely regulated NaCl content (Na^+ 0.3 g/100 g; Tekland Inc, Madison, Wis), as described.13 Groups of rats were euthanized under anesthesia with sodium pentobarbital (50 mg/kg IP) at 72 hours after intravenous injections of siRNA duplexes or vehicle. The abdomen and thorax were opened, and blood was sampled from the heart. Serum was separated without delay and frozen at −70°C for subsequent analysis of ADMA, SDMA, and L-arginine (Arg). A portion of intestine and mesentery was removed. Mesenteric resistance vessels (MRVs) were dissected in cool (4°C) physiologic salt solution (PSS) for EDRF/NO studies or histologic analysis.10 In other groups of rats at 24 and 72 hours after injection, 1 set of MRVs, 1 kidney cortex, and a portion of the liver were removed and frozen in liquid nitrogen for later analysis of mRNA and protein expression for DDAH-1 and -2 to determine the extent and time course of changes in DDAH expression. The investigators performing the studies were blinded to the rat treatment.

A second set of rats (n=7 per group) were anesthetized for 10 minutes with 2% isoflurane for intraarterial placement of cannulae connected to telemetric blood pressure transducers.14 After 10 days of recovery, baseline values for mean arterial pressure (MAP) and heart rate were recorded over 3 days and averaged. Thereafter, rats were weighed, in random order, control, non-targeting siRNA (siCont), or siRNA targeted to DDAH-1 or -2, as in the first set of studies. MAP and heart rate were recorded over 3 days; after which, rats were anesthetized and MRVs were dissected for studies of 4-amino-5-methoxyamino-2,7'-difluorofluorescein diacetate (DAFFM-DA) fluorescence for NO activity and relaxation.

Immunohistochemistry

MRVs were immersed in periodate–lysin–paraformaldehyde solution overnight at 4°C and embedded in paraffin for light microscopic immunohistochemical analysis or in Lowicryl K4M (Electron Microscopy Sciences, Fort Washington, Pa) embedding medium for immunogold studies with electron microscopy. The remaining tissue was processed for the preembedding immunoperoxidase method. The methods used,10,15 and the antibodies to DDAH-1 and -2, have been described in detail.9 The antibodies to DDAH-1 and -2 were used at 1:100 dilution for light microscopy and 1:75 for electron microscopy.

EDRF/NO and EDHF Responses of Isolated MRVs

Vessels were prepared as described previously in a 4-vessel isometric Mulvany–Halpern myograph (J.P. Trading, Science Park, Aarhus, Denmark).2 After equilibration for 30 minutes in PSS at 37°C, measurements of media thickness and internal diameter were performed, the length-tension characteristic was determined, and the internal circumference was set to 90% of that calculated for a relaxed vessel in vivo at a transmural pressure of 100 mm Hg.2 Thereafter, vessels were incubated in PSS for 30 minutes (replaced each 30 minutes), stimulated 3 times with 40 mmol/L K^+ PBS containing 10^{-5} mol/L norepinephrine (NE) (NAK) to produce a standard contraction, followed by 1 minute with buffer alone and 1 minute with PSS containing 10^{-6} mol/L NE. Contractions were maintained for 3 minutes before rinsing with PSS.

Vessel Protocol

MRVs were precontracted with NE (10^{-5} mol/L) and relaxed with acetylcholine (ACh) (10^{-8} to 10^{-3} mol/L) or with the endothelium-independent vasodilator sodium nitroprusside (10^{-8} to 10^{-3} mol/L). The EDRF/NO response was taken as the inhibition of relaxation to 10^{-4} mol/L ACh by a maximally effective dose of 10^{-6} mol/L ADMA. The EDHF response was taken as the inhibition of the relaxation to 10^{-4} mol/L ACh by 40 mmol/L KC1 in the presence of 10^{-5} mol/L ADMA (to inhibit NOS) and 5×10^{-3} mol/L indomethacin (Indo) (to inhibit cyclooxygenase). A similar effect of high K^+ was seen with a combination of 10^{-6} mol/L amin (an inhibitor of small conductance K^+-activated Ca^2+ channels) plus 10^{-2} mol/L charybdotoxin (an inhibitor of large and intermediate conductance KCa channels), as described previously for mouse MRVs.5

Additional rats (n=5 per group) were administered siDDAH-1 or -2 or siCont 72 hours previously. Their MRVs were used to study contractile responses to 10^{-2} mol/L phenylephrine and the effects of bath addition of 10^{-3} mol/L Arg on ACh-induced relaxations.

Vessel NO Activity

4-Amino-5-methoxamino-2,7'-difluorofluorescein diacetate (DAFFM-DA; Invitrogen, Carlsbad, Calif) is taken up into cells, trapped by deesterification, and covalently converted to a fluorescent triazolo-fluorescein analog by NO.16 DAFFM-DA was loaded in a buffer into MRVs mounted on a wire myograph over 30 minutes. It was excited at 495 nm, and the emission was isolated at 515 nm during contraction with 10^{-5} mol/L NE and relaxation with 10^{-5} mol/L ACh, with simultaneous recording of vessel tension. The change with time (ΔF/F_0) was normalized to the response to an NO donor compound, 5×10^{-3} mol/L 2-(N,N-diethylamino) diazenolate-2-oxide sodium salt (NONOate) (Cayman Chemicals, Ann Arbor, Mich).

siRNA Construction, Validation, and Administration

Two sets each of 21-bp siRNA duplexes with 2 overhanging nucleotides targeting the coding regions of DDAH-1 (GenBank accession no. NM_022297) and DDAH-2 (accession no. NM_212532) were constructed (Table 1 in the online data supplement, available at http://circres.ahajournals.org) and validated in vitro by using heman-gioendothelioma cells (CRL-2586; American Type Culture Collection, Manassas, Va) that express both DDAH-1 and -2. Cells were transfected with 10 mmol/L siDDAH-1, siDDAH-2, or siCont with Lipofectamine 2000 (Invitrogen Inc, Rockville, Md) and harvested after 24 hours. The mRNA expression was analyzed using real-time PCR. The siRNA constructs selected produced >85% inhibition of the target mRNA. A control siRNA sequence (siCont) was used that has no homology to any mammalian sequence (QIAGEN Inc, Valencia, Calif) and had no effect on DDAH-1 or -2 expression in the cell system.

For systemic administration, each rat was anesthetized with 1% to 2% isoflurane and received 50 μg of siRNA in 6 mL of PBS via the left jugular vein as a 7-second bolus injection to mimic the protocol of “hydrodynamic stress” developed in the mouse17 and adapted by us for use in the rat.12

Quantitative TaqMan Gene Expression Assays

RNA was isolated using the RNAgous-4PCR kit (Ambion, Austin, Tex) and reverse transcribed with superscript III (Invitrogen Inc), and cDNA was multiplexed with 6-carboxyfluorescein (FAM)-labeled DDAH-1 or DDAH-2 and 6-carboxyfluorescein (VIC)-labeled 185 as an internal control, as described.18 The TaqMan gene expression assay probe sets used were Rn00574200_m1 (DDAH-1) and Rn01525776_m1 (DDAH-2) (Applied Biosystems, Foster City, Calif). TaqMan Universal PCR Master Mix was used for PCR with an ABI Prism Sequence Detection System (Applied Biosystems). The comparative ΔΔCt method was used for relative quantification and statistical analysis.18

Protein Expression

Brieﬂy, 100 μg of lysates were run on 12.5% SDS-PAGE gels (Bio-Rad, Hercules, Calif), transferred to nitrocellulose membranes, and incubated overnight at 4°C while shaking in 5% Blotto with primary antibodies for DDAH-1 and DDAH-2 at 1:20000 dilution as described.12,18 The secondary antibody was peroxidase-labeled
goat anti-rabbit (KPL, Gaithersburg, Md) at 1:20,000 dilution. Blots were probed with tubulin to control for equal loading (Sigma, St Louis, Mo).

Chemical Methods

$S_{\text{ADMA}}, S_{\text{SDMA}},$ and $S_{\text{ARG}}$ were assayed by a modified high-performance liquid chromatography method. Samples were spun at 15,000g, diluted 50:50 with water, applied to a preconditioned CBA Bond-elute cartridge (Varian Inc, Walnut Creek, Calif), washed sequentially with water and methanol, eluted with 10% ammonia:methanol (vol/vol), dried under $N_2$, derivatized with 50 $\mu$L of borate buffer, 50 $\mu$L of water, and 20 $\mu$L of 4-fluoro-7-nitrobenzofurazan in acetonitrile (5 mg/mL), and heated at 60°C for 2 minutes; 100 $\mu$L was transferred to an autosampler vial. A 15-$\mu$L aliquot of the solution was injected onto a high-performance liquid chromatograph (Agilent Technologies Inc, Palo Alto, Calif). A C18 25×4.6 mm cartridge was connected in series to a UV/Vis diode array detector, and an 8HP fluorescence detector (both from Agilent Technologies Inc) set to 470 nm and 470/530 nm. The mobile phase was a gradient of aqueous sulfate buffer (9.5%; 90.5% acetonitrile starting condition) with a ramp to 50% acetonitrile at 12 minutes. The current chromatogram demonstrated clear separation of the 3 peaks within 10 minutes. Samples were quantitated by peak area against samples supplemented with known concentrations of Arg, ADMA, and SDMA. This assay has a coefficient of variation for Arg, ADMA, and SDMA of 3.8, 4.9, and 5.5%, respectively. The limits of detection are <50 nmol/L.

Statistical Analysis

Statistical tests used 2-factor repeated-measures ANOVA. Where appropriate, post hoc comparisons between groups were made with
Results

In a pilot study, hemangioendothelioma cells showed a 10.5-fold (ΔΔCt 3.2±0.3) greater expression of DDAH-2 relative to DDAH-1. Incubation for 24 hours with the selected siDDAH-1 or -2 produced dose-dependent suppression to >85% of the target mRNA. In a second pilot study, there were no significant differences in SADMA or EDRF/NO responses 72 hours after intravenous injection of 50 µg of the siCont or vehicle. Therefore, siCont was selected for comparison with siDDAH-1 or -2. In a third pilot study, bath addition of ADMA or L-nitroarginine produced graded inhibition of ACh-induced relaxation of MRVs. A maximal inhibition of 43±9% was seen at 10⁻⁶ mol/L ADMA, which was not significantly different from an inhibition of 49±5% produced by a supramaximal dose of 10⁻⁴ mol/L L-nitroarginine. Endothelial removal effectively prevented relaxation to 10⁻⁴ mol/L ACh (7±8%; P=NS). Therefore, we selected 10⁻⁶ mol/L ADMA-inhibitable, 10⁻⁴ mol/L ACh-induced relaxation as the index of EDRF/NO responses (Figure 1A).

To assess EDHF responses, MRVs were incubated with 10⁻⁶ ADMA (to inhibit NOS) and 5×10⁻⁵ mol/L Indo (to inhibit cyclooxygenase) for 20 minutes and relaxed with 10⁻⁴ mol/L ACh alone or during bath with the addition of 40 mmol/L KCl (to block K⁺ channels) (Figure 1B). Indo had no effect an ACh-induced relaxation alone or in the presence of ADMA.
Immunohistochemistry

Immunostaining of MRVs for DDAH-1 was uniformly negative (Figure 2). Immunostaining for DDAH-2 was positive in the endothelium, vascular smooth muscle cells and adventitia of MRVs from rats given vehicle, siCont, or siDDAH-1 72 hours previously but was negative in rats given siDDAH-2 (Figure 2).

Preembedding grids were counterstained with only lead citrate. Uranyl acetate, which stains membranes, was omitted to enhance the contrast. This method located DDAH-2 in the apical membrane and cytoplasmic vesicles of endothelial cells (Figure 3A), which was much reduced in MRVs from rats administered siDDAH-2 (Figure 3B). The immunogold method located DDAH-2 in the apical membrane of endothe-
lial cells (Figure 3C and 3D) and the smooth muscle fibrils (Figure 3C and 3F) and nuclei (Figure 3C and 3E) of vascular smooth muscle cells.

The expression of mRNA for DDAH-1 in the kidney cortex and liver was 2.2- and 7.2-fold higher, respectively, than DDAH-2, whereas the expression of DDAH-2 in MRVs was 5.1-fold higher than DDAH-1. The expression of mRNA and protein in the kidney cortex, liver, and MRVs after intravenous injections of vehicle or siCont were not significantly different at either 24 or 72 hours (data not shown). Therefore, data after siDDAH-1 or -2 were compared with that after siCont.

Seventy-two hours after intravenous injection of siDDAH-1, the mRNA and protein expression for DDAH-1 was reduced by 35% to 50% in the kidney cortex, liver, and MRVs without significant changes in DDAH-2 expression (Figure 4A). Similar injections of siDDAH-2 reduced its mRNA and protein expression for DDAH-2 by 40% to 85% without significant changes in DDAH-1 expression (Figure 4B). We conclude that the siRNA methods produced moderate, isoform-specific knockdown of mRNA and protein at 72 hours. The changes were similar, but generally rather more modest, at 24 hours after injection; at which time, there was an unexplained increase in mRNA for DDAH-1 in the MRVs after siDDAH-2 (Figure 4A) and in mRNA and protein for DDAH-2 in the liver after siDDAH-1 (Figure 4B). These latter changes were not investigated further because they were not apparent at 72 hours.

$S_{\text{ADMA}}$ was increased significantly by 25% following either of the 2 siDDAH-1 without significant changes in $S_{\text{Arg}}$ or $S_{\text{SDMA}}$. There were no changes following siDDAH-2 (Figure 5).

ACh-induced relaxations of NE-preconstricted MRVs were similar in vessels from rats that had received vehicle, siCont, or siDDAH-1 72 hours prior but were impaired after siDDAH-2 (Figure 6A). The 50% effective dose for ACh-induced relaxation was 6.0 ± 0.2 in vessels from rats given siCont 5.9 ± 0.2 in those given siDDAH-1 (P = NS) and 4.5 ± 0.3 in vessels from rats given DDAH-2 (P < 0.005). Incubation with $10^{-8}$ mol/L Arg for 20 minutes significantly increased responses of MRVs only from rats administered siDDAH-2, although the relaxations remained significantly less than in vessels from rats given siCont (Figure 6B). Constrictions to $10^{-5}$ mol/L phenylephrine and relaxations to graded doses of sodium nitroprusside were similar in MRVs from each group (data not shown).

The MRVs of rats given siDDAH-1a had a modest reduction in EDHF/NO response, which was not seen after siDDAH-1b (Figure 7A). EDHF/NO responses were reduced profoundly and were not significantly different from 0 in rats given siDDAH-2a or -2b. There were no significant effects of any treatment on the EDHF responses (Figure 7B).

In an additional set of rats, relaxation responses and NO activity were studied in MRVs from groups (n = 7) of rats administered siCont, siDDAH-1, or siDDAH-2 72 hours previously. Relative to siCont, the mRNA expression in MRVs for DDAH-1 was reduced to 30 ± 3% after siDDAH-1 and to 33 ± 3% for DDAH-2 after siDDAH-2. Both DAFFM-DA fluorescence and relaxations of NE-preconstricted vessels administered $10^{-5}$ mol/L ACh were reduced in MRVs from rats given siDDAH-2 but not siDDAH-1 (Figure 8). There were no differences between vessels in NO activity after incubation with an NO donor compound (Figure 8C).

The MAP and heart rate of rats studied by telemetry averaged 107 ± 5 mm Hg and 360 ± 9 min$^{-1}$ respectively. There were no significant changes over 3 days after siRNA administration. ($\Delta$MAP with siCont, +1 ± 1; with siDDAH-1 to 1 ± 1; and with siDDAH-2, +7 ± 4 mm Hg; P = NS).

**Discussion**

The main new findings are that rat MRVs express abundant DDAH-2 but little DDAH-1. DDAH-2 is located in the apical membrane, cytoplasmic vesicles, and nuclear envelope of...
endothelial cells and among the myofibrils and nuclei of vascular smooth muscle cells. In contrast, the mRNA for DDAH-1 is expressed 2- and 7-fold more than that for DDAH-2 in the kidney cortex and liver. An intravenous injection of siRNA targeted to DDAH-1 or -2 produces 35% to 85% inhibition of the mRNA and protein expression for the specific isoform in the kidney cortex, liver, and MRVs at 72 hours, without confounding effects on the expression of the other isoform. Silencing of the mRNA for DDAH-1 increases $S_{\text{ADMA}}$ by 25% with only an inconsistent effect to blunt EDRF/NO, whereas silencing of the mRNA for DDAH-2 does not alter $S_{\text{ADMA}}$ but greatly reduced ADMA-inhibitable, ACh-induced relaxation and NO activity without changes in EDHF or contractions to phenylephrine. These impaired relaxation responses are partially, but incompletely, restored by incubating the MRV with Arg. Neither siRNA affects the MAP or heart rate significantly over 3 days. Thus $S_{\text{ADMA}}$ is regulated predominantly by DDAH-1, whereas EDRF/NO is regulated predominantly by DDAH-2. MAP is not changed, consistent with the finding that even an intravenous infusion of ADMA that raises plasma levels 40-fold does not raise MAP in human subjects because a rise in systemic vascular resistance is offset by a fall in cardiac output. Although this study did not measure tissue ADMA concentrations or cardiac output, which are limitations, they have shown that DDAH-2 expression is required for EDRF/NO responses in normal resistance vessels and have largely dissociated the effects of DDAH on EDRF/NO from circulating ADMA concentrations or MAP.

We adapted the mouse “hydrodynamic injection” method of siRNA administration to rats and found a >60% knockdown of mRNA and protein for $p22^{phox}$ in the kidney 72 hours after siRNA injection. This did not activate the expression of the Toll-like receptor 3 or Stat-1 and, therefore, apparently did not induce interferon. In the present study, we detected no significant differences in mRNA or protein expression or in $S_{\text{ADMA}}$, EDRF/NO responses between rats that had received a control nontargeted siRNA or a vehicle. The specificity of the responses was shown further by similar effects of 2 siRNAs targeted to distinct regions of the gene and the isoform-specific effects of the siRNAs.

Figure 6. A, Relaxation responses to ACh of preconstricted MRVs from rats after vehicle, siCont, siDDAH-1, or siDDAH-2. Compared with siCont: ***$P<0.005$. B, Relaxation of preconstricted MRVs to $10^{-4}$ mol/L ACh before and after bath addition of $10^{-3}$ mol/L Arg from rats after siCont, siDDAH-1, or siDDAH-2. Compared with corresponding value in siCont: **$P<0.01$, ***$P<0.005$.

Figure 7. EDRF/NO responses (A) from the ADMA-inhibitable, ACh-induced relaxations and EDHF response (B) from the high KCl-inhibitable, ACh-induced relaxations in the presence of ADMA and Indo of MRVs after vehicle, siCont, siDDAH-1, and siDDAH-2. Compared with siCont: *$P<0.05$, ***$P<0.005$. 

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The observation that DDAH-2 is expressed strongly in vascular endothelial cells is consistent with our finding that it regulates EDRF/NO. The small and inconsistent effects of siDDAH-1 to reduce EDRF/NO may be secondary to the modest increase in SADMA.

Transgenic mice globally overexpressing DDAH-1 have reduced SADMA, increased NOS activity and excretion of NO2/NO3, and a lower vascular resistance. Because the liver and kidneys extract ADMA from the circulation, our observation of a 2.2- and 7.2-fold greater mRNA for DDAH-1 than DDAH-2 in the kidney cortex and liver, respectively, may explain why blunting DDAH-1 expression increased SADMA in this study. This is consistent with the finding that DDAH-1 mice also have increased SADMA and the conclusion that DDAH-2 does not affect circulating ADMA. However, the DDAH-1/− mouse has endothelial dysfunction, in contrast to the preserved EDRF/NO responses in rats administered siRNA to DDAH-2. This may relate to the greater duration of gene knockdown in the mouse model. Moreover, the siRNA method was rather more effective in reducing DDAH-2 than -1 in our study.

Excess Arg can reverse the acute inhibitory effect of NOS antagonists on ACh-induced relaxation of isolated blood vessels. However, incubation of NOS with L-monomethyl arginine over 2 hours causes largely insurmountable inhibition of the enzyme. This may explain why EDRF/NO responses could not be restored in full in vessels from rats given siRNA to DDAH-2 72 hours previously by incubation with Arg for 20 minutes. Thus, the siRNA to DDAH-2 may have increased endothelial ADMA and led to a partially insurmountable inhibition of eNOS. Prolonged infusion of ADMA impairs blood vessel function even in eNOS knockout mice.

Perspective
Defects in EDRF/NO and/or elevations in SADMA accompany many of the major cardiovascular diseases or risk factors. The expression of DDAH-2 in human endothelial cells depends on an insertion–deletion polymorphism in the promoter region. The finding that DDAH-2 expression is required for EDRF/NO responses in normal resistance vessels despite normal values for SADMA demonstrates that defects in DDAH-2 could contribute to cardiovascular disease even in subjects with normal SADMA because circulating levels of ADMA do not reflect spillover from endothelial sources. The present studies extend the use of in vivo RNA interference in the rat to blood vessels, liver, and kidneys and demonstrate its utility in probing physiologic regulation of a system by isoform-specific gene knockdown.

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Disclosures
A.L. is founder and director of Oxonon BioAnalysis, Emeryville, Calif, and developed the assays for ADMA, SDMA, and Arg that are described in this article. The author undertook them at Oxonon BioAnalysis.

References


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### Online Table I: Sequences for siRNA constructs used

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