Specific Lowering of ADMA by Pharmacological DDAH Improves Endothelial Function, Reduces Blood Pressure and Ischemia-Reperfusion Injury

Young Lee, Purvi Mehrotra, David Basile, MD Mahbub Ullah, Arshnoor Singh, Nicholas Skill, Subhi Talal Younes, Jennifer Sasser, Anantha Shekhar and Jaipal Singh

Indiana Center for Biomedical Innovation, Indianapolis, IN (Y.L., A.Singh., J.S.); Indiana University School of Medicine, Indianapolis, IN (P.M., D.B., M.U., N.S., A.Shekhar., J.S.); University of Mississippi Medical Center, Jackson, MS (S.Y., J.S.); Vasculonics LLC, Indianapolis, IN (J.S.)
Running Title

Therapeutic Potential of Pharmacological DDAH

Corresponding Author

Jaipal Singh, 1800 N. Capital Ave, Noyes Building 5th floor, Indianapolis, IN 46202, singh52@iupui.edu

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Abbreviations

ADMA, asymmetric dimethylarginine;
rDDAH, recombinant dimethylarginine dimethylaminohydrolase;
M-DDAH, modified DDAH by PEGylation;
NOS, nitric oxide synthases;
NO, nitric oxide;
TGFβ, transforming growth factor beta;
PA, pseudomonas Aeruginosa;
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Abstract
Multiple clinical and preclinical studies have demonstrated that plasma levels of asymmetric dimethylarginine (ADMA) are strongly associated with hypertension, diabetes, cardiovascular and renal disease. Genetic studies in rodents have provided evidence that ADMA metabolizing dimethylarginine dimethylaminohydrolase (DDAH-1) plays a role in hypertension and cardiovascular disease. However, it remains to be established whether ADMA is a bystander, biomarker or sufficiently contributes to the pathogenesis of hypertension, and cardiovascular and renal disease. The goal of the present investigation was to develop a pharmacological molecule to specifically lower
ADMA and determine the physiological consequences of ADMA lowering in animal models. Further, we sought to determine if ADMA lowering will produce therapeutic benefits in vascular disease in which high ADMA levels are produced. A novel long acting recombinant DDAH (M-DDAH) was produced by post-translational modification which effectively lowered ADMA in vitro and in vivo. Treatment with M-DDAH improved endothelial function as measured by increase in cGMP and in vitro angiogenesis. In a rat model of hypertension, M-DDAH significantly reduced blood pressure (vehicle: 187±19 mmHg vs M-DDAH: 157±23 mmHg; p<0.05). Similarly, in a rat model of ischemia-reperfusion injury, M-DDAH significantly improved renal function as measured by reduction in serum creatinine (vehicle: 3.14±0.74 mg/dL vs M-DDAH: 1.1±0.75 mg/dL; p<0.01), inflammation and injured tubules (vehicle: 73.1±11.1% vs M-DDAH: 22.1±18.4%; p<0.001). These pharmacological studies have provided direct evidence for a pathological role of ADMA and the therapeutic benefits of ADMA lowering in preclinical models of endothelial dysfunction, hypertension and ischemia-reperfusion injury.

**Significance Statement**

High levels of ADMA occur in patients with cardiovascular and renal disease. A novel M-DDAH effectively lowers ADMA, improves endothelial function, reduces blood pressure and protects from ischemia-reperfusion renal injury.

**Introduction**
Asymmetric dimethylarginine (ADMA), also known as a cardiotoxin exhibits a strong association with cardiovascular and renal disease. ADMA levels correlate with increased cardiovascular morbidity and mortality (Boger et al., 2005). High plasma concentrations of ADMA are found in a number of disease states involving vascular dysfunction including, coronary artery disease (Krempl et al., 2005), hypertension (Goonasekera et al., 1997; Perticone et al., 2005), heart failure (Zoccali et al., 2001), peripheral arterial disease (Boger et al., 1997), chronic kidney disease (Ravani et al., 2005) and pregnancy related hypertension (Pettersson et al., 1998). In diabetic hypertensive patients, high ADMA was associated with chronic kidney disease progression (Triches et al., 2018). In large prospective studies, plasma ADMA was independently associated with cardiovascular mortality in patients with ischemic heart disease (Meinitzer et al., 2007) and increased incidence of myocardial infarction and stroke in women (Leong et al., 2008). These studies have suggested that ADMA is a biomarker and perhaps a risk factor contributing to microvascular dysfunction and cardiovascular disease.

Initially identified as an endogenous inhibitor of nitric oxide synthases (NOS) (Leiper and Nandi, 2011; Schwedhelm and Boger, 2011), ADMA also inhibits arginine transport and increases oxygen free radicals, mitochondrial dysfunction and transforming growth factor beta (TGFβ) expression (Druhan et al., 2008; Fabrice et al., 2011; Sun et al., 2013). Further, nitric oxide (NO)-independent cellular actions of ADMA have been identified (Jarzebska et al., 2019). The major pathway for ADMA metabolism is by dimethylarginine dimethylaminohydrolase (DDAH) enzymes (Leiper and Nandi, 2011; Schwedhelm and Boger, 2011). Two isoforms of DDAH (DDAH-1 and DDAH-2)
encoded by distinct genes exist (Leiper and Nandi, 2011). In preclinical models of cardiac or renal ischemia-reperfusion, DDAH-1 is reduced with a concomitant increase in tissue ADMA (Stuhlinger et al., 2007; Nakayama et al., 2014). DDAH gene knockout and DDAH overexpression, either in transgenic mice or by adenoviral gene delivery in rodents, have provided evidence of a pathological role of ADMA (Leiper et al., 2007; Xu et al., 2017). Although, these DDAH gene knockout and over expression studies have produced strong evidence for the pathological role of ADMA in cardiovascular and renal disease, the results of other genetic studies have shown contradictory phenotype (Nakayama et al., 2014; Tomlinson et al., 2015; Rodionov et al., 2019; Wetzel et al., 2020). Thus, modification of pathological state by pharmacological lowering of ADMA would provide a direct evidence for its role in cardiovascular and renal disease. To-date specific lowering of ADMA by pharmacological treatment has not been achieved. Previous attempts to discover small molecule modulators of DDAH activity have not been successful (Ghebremariam et al., 2012; Linsky and Fast, 2012). Thus, at the present time, pharmacological agents that can specifically lower pathological ADMA are not available. The goal of the present study was to develop a pharmacological molecule to specifically lower ADMA and test the hypothesis that ADMA lowering would reduce pathological state. We here show, for the first time, that a novel M-DDAH with drug-like pharmacological properties effectively reduced ADMA in preclinical models. ADMA lowering improved endothelial function ex-vivo and significantly reduced blood pressure in hypertensive rats. In a rat model of ischemia-reperfusion injury, M-DDAH protected from renal injury. These data show that pharmacological lowering of ADMA may provide a new mechanism for targeting disease states associated with high ADMA, including
cardiovascular disease, heart failure, vascular complications of diabetes and kidney disease.

**Methods**

**Cloning, Expression and Purification of DDAH and Mutants**

Pseudomonas Aeruginosa-DDAH (PA-DDAH), PA-DDAH mutants and human DDAH-1 were cloned and expressed in E. coli. Coding sequence of DNA was synthesized and ligated into a pE-SUMO vector. PA-DDAH mutants were prepared by individually replacing lysine residue # 3, 25, 103 and 159 with cysteine. Plasmid containing DDAH or mutant genes were transformed into BL21 (DE3) E. coli cells (EMD Millipore, USA), plated on LB agar plate with 50 µg/mL kanamycin and grown overnight. The colonies expressing highest levels of DDAH were then used for large scale production. For preparation of purified DDAH, 50 mL culture were inoculated in 1 L LB with 50 µg/mL kanamycin and grown to 0.8 OD at 600 nm and then induced with IPTG. After 16 h, cells were collected by centrifugation, resuspended and lysed by sonication. DDAH in the supernatant was then purified using a Ni-sepharose column. DDAH activity was determined by generation of L-citrulline from ADMA using a colorimetric assay (Knipp and Vasak, 2000).

**Site Specific PEGylation of DDAH Mutant and Generation of M-DDAH**

The 159Lys-Cys mutant cloned and expressed as above was selected for site specific PEGylation. 159Lys-Cys DDAH protein was dialyze against 0.1 M phosphate buffer, pH 6.3. mPEG<sub>40k</sub>-Mal (Nanocs, USA) (10x molar excess) was added to the dialyzed protein
and incubated for 4 h at room temperature. The PEGylated protein was purified using HiTrap Q FF anion exchange chromatography (Sigma-Aldrich, USA). The column was equilibrated with 20 mM Tris pH 8. PEGylation reaction mixture was applied to the column. The PEGylated protein was eluted in 175 mM NaCl. The PEGylated protein was evaluated for purity, molecular weight and DDAH activity.

For the generation of M-DDAH, PA-DDAH was PEGylated at the lysine residues using mPEG_{10K}-NHS (Nanocs, USA). PEGylation was initiated after extensive dialysis of DDAH against 0.1 M phosphate buffer, pH 8 to remove amine containing substances. mPEG_{10K}-NHS (Nanocs, USA) (20x molar excess) was then added and incubated for 4 h at room temperature. The reaction mixture was purified using 50 kD Amicon® ultra centrifugal filter unit (EMD Millipore, USA) and centrifugation at 4500 rpm for 15 min at 4 °C. Excess and unreacted mPEG_{10K}-NHS was removed by 3 cycles of purification using the 50 kD Amicon filter. SDS gel electrophoresis showed multiple bands of PEGylated DDAH after one cycle of PEGylation. In order to achieve maximum PEGylation and homogenous preparation, the partially PEGylated protein was subjected to additional cycles of PEGylation as above until the protein was fully PEGylated. The optimized PEGylation reaction protocol achieved PEGylation of all accessible lysine residues yielding a protein band at 250 kD in SDS gels. The purified M-DDAH was then evaluated for molecular weight and DDAH activity.

**ADMA Measurement**

ADMA in plasma was analyzed by modification of previously published HPLC based method (de Jong and Teerlink, 2006; Teerlink, 2007). Test samples were prepared by
solid-phase extraction (SPE) using an Oasis MCX Cartridge (Waters, USA). Briefly, the 
column was conditioned with 1 mL of elution buffer consisting of 30% ammonium 
hydroxide:water:methanol (10:40:50 by volume) followed by 1 mL of ultrapure water. 
Plasma (50 µL) was mixed, vortexed, and spinned with cold methanol (100 µL) for 
extraction and 100 µL was recovered, mixed with 200 µL of PBS and loaded on to the 
column. The column was lightly centrifuged and rinsed with 1 mL of 0.1 M HCl and 1 mL 
of methanol two times. Analytes were eluted with 400 µL elution buffer and dried under 
nitrogen. Samples were reconstituted in 50 µL of ultrapure water and derivatized with 
ortho-phthaldialdehyde (OPA) reagent (Sigma-Aldrich, USA) (4:1 by volume). After 
mixing, the analytes were heated at 30 °C for 1 min and 10 µL was injected into the 
HPLC system, equipped with a fluorescent detector RF-10AXL (ex: 340, em: 455) and 
Chromolith® performance RP-18e column (100 x 4.6 mm) (EMD Millipore, USA). Mobile 
phase A consisted of 25 mM potassium phosphate buffer (pH 6.5) and mobile phase B 
was methanol/THF (97/3 by volume). Chromatographic separation was performed at 
room temperature at a flow rate of 2 mL/min (10-20% solvent A).

cGMP Measurement
The effect of ADMA and ADMA + M-DDAH on NO generation in endothelial cells was 
determined using cGMP, the downstream marker of NO responses. HUVECs 
(1,000,000/well) in 6-well plate were incubated in medium containing 0.5 mM IBMX 
(Control) or IBMX +100 µM ADMA for 5 min at 37 °C. The medium was aspirated and 
cells were incubated in fresh medium containing 0.5 mM IBMX or 0.5 mM IBMX + 10
\( \mu g/mL \) M-DDAH for 60 min. Cells were collected using trypsinization and cGMP was analyzed using enzyme immunoassay kit (Arbor Assay, USA).

**In Vitro Angiogenesis**

Matrigel coated plates were prepared by placing 200 \( \mu \)L Matrigel (Corning, USA) into the 24-well plate using a method previously described (DeCicco-Skinner et al., 2014). The plates were incubated at 37 °C for 45 min. HUVEC (60,000 cells/well) in 0.5% FBS medium or experimental groups were added and incubated at 37 °C for 6 h. The cells were imaged using 4x objective were analyzed using imageJ (Carpentier G, 2012).

**Animals**

All animal studies were approved by local IUCAC at Indiana University and the University of Mississippi. Sprague Dawley (SD) rats (Envigo, Indianapolis) were maintained on normal chow (TD7034, 0.3% NaCl, Harlan Teklad) and water adlibitum on a 12 h light-dark cycle. Dahl salt-sensitive hypertensive female rats were obtained from Dr. Michael Garrett at the University of Mississippi Medical Center (Rapp and Dene, 1985). Rats were euthanized in accordance with Guidelines of the American Veterinary Medical Association.

**Pharmacodynamic Studies**

Male SD rats (~250-300 g) were catheterized in the Jugular vein to collect blood. DDAH or M-DDAH (2 mg/kg) was administered by single intravenous injection. Blood was drawn at various times and plasma prepared by centrifugation. DDAH activity was
assayed by modification of previously published method (Tain and Baylis, 2007). Plasma (70 µL) was incubated with 20 µL urease for 15 min before adding ADMA (10 µM). The mixture was incubated for 3 h at 37 °C. Samples were deproteinized by addition of 50 µL of 8% sulfosalicylic acid and centrifuged. Supernatant (80 µL) was used for determination of citrulline.

**Blood Pressure Measurement**

Dahl salt-sensitive female rats were anesthetized using isoflurane (5% induction, 2% maintenance) and carprofen. Carotid artery and jugular vein catheters were inserted one day before the measurements (Younes et al., 2020). M-DDAH (1 mg/kg) or PBS vehicle was infused intravenously to conscious restrained rats. An exteriorized arterial and venous catheters for arterial pressure measurement and arterial blood sampling and venous infusions was used for these acute studies. The rats were placed in restrainers, and the measurements were taken after the rats were acclimated and quietly resting in the restrainer. The carotid catheter was connected to a Deltran pressure transducer (Utah Medical) and mean arterial pressure in control and treated groups was measured over a 4 h. Data were collected using an amplifier and PowerLab from ADInstruments.

**Renal Ischemia-Reperfusion Model**

Male SD rats (~250-300 g) were anesthetized with Ketamine (100 gm/kg) and Xylazine (5 mg/kg) subjected to bilateral ischemia-reperfusion injury by clamping the renal pedicle for 30 min similar to procedures previously described (Phillips et al., 2010). M-
DDAH or vehicle was first administered by i.v. injection at the time of reperfusion and 3 h after the first administration. Renal function was determined by measuring plasma creatinine using a Pointe Scientific Analyzer. Blood pH and analytes were measured with a GEM Premier 4000 (Instrumentation Laboratories). Animals were euthanized with Fatal Plus and kidneys were removed and processed for immune cells and histology.

**Immune Cells Analysis**

Harvested kidneys from the ischemia-reperfusion study were minced and digested in liberase (2 µg/ml) (Roche, Switzerland) for 15 min at 37 °C with the help of Gentle MACs (Miltenyi, Germany). The digested tissue was filtered through a 100 µm filter mesh and washed with tissue culture medium. The lymphocytes were separated by Percoll (Sigma-Aldrich, USA) and counted by hemocytometer. To evaluate T lymphocytes, the cells were stained with antibodies against rat CD4 (PE-Cy7), CD8a (Alexa 647). To evaluate the cytokines secreted by T cells, the cells were stained for CD4 surface marker and then permeabilized and stained with antibodies against rat IL-17 (FITC). Macrophages were stained using anti-CD11b/c (PE). All antibodies were obtained from BD Bioscences. Cells were scanned using flow cytometry (FACSCalibur, BD Biosciences) and scans were analyzed using Flowjo software (Tree Star, USA). Representative gating for cell populations identified by flow cytometry were exactly as described previously (Mehrotra et al., 2015). The data is expressed as the presence of total number of specific cell population per gram of kidney tissue.

**Histological Studies**
One half of the kidney from the ischemia-reperfusion study was fixed in 10% formalin, embedded in paraffin and 5 µm sections prepared. Sections were stained with hematoxylin and eosin (H&E) and imaged with a Nikon Optiphot-2 microscope using a 20x objective, equipped with a Leica DMC 2900 camera using Lieca LAS Software. At least 3 random images through the renal outer medulla were obtained from each animal. Sections were assessed for tubular injury as described previously (Basile et al., 2013). The degree of injury is expressed as the percentage of damaged tubules that display cellular necrosis, loss of brush border, cast formation and tubular dilation. Both the individual who acquired the images and who scored the slides were blinded to the treatment groups.

**Statistical Analysis**

Statistical significance was evaluated by student t test or one-way analysis of variance (ANOVA), followed by Dunnett’s test for multiple comparison between the experimental groups. All data presented as mean ± SD, P value of < 0.05 were considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001.

**Results**

**Cloning, Expression and Purification of Recombinant DDAH**

The translated DNA sequences of human and pseudomonas aeruginosa (PA) DDAH genes were synthesized and cloned in pE-SUMO plasmid, and then expressed in E. coli. To facilitate purification, DDAH was expressed as a protein containing His-tag at the C-terminal. Recombinant protein was purified using Ni-sepharose column. Figure 1A
shows that the two step Ni-sepharose purification produced a single PA-DDAH band with greater than 90% purity when analyzed by SDS gel electrophoresis. The DDAH activity was determined by hydrolysis of ADMA to citrulline using a colorimetric assay. Previous studies have reported discrepant values of the Michaelis–Menten constant (Km) of PA-DDAH using different assay systems. Stone et al reported a Km of 310±20 µM for recombinant PA-DDAH using a less sensitive colorimetric assay (Stone et al., 2005), whereas a Km of 39±2 µM was reported by Hong and Fast (Hong and Fast, 2007) using a more sensitive assay. In order to confirm the Km, we used an HPLC based sensitive assay to determine ADMA degradation to citrulline. The Km of PA-DDAH was 76 µM (Figure 1B), a value close to that previously reported using a sensitive assay. The recombinant PA-DDAH, designated as rDDAH dose-dependently (Figure 1C) and rapidly (Figure 1D) reduced ADMA in plasma when incubated in vitro. These data demonstrated that rDDAH produced as described has the anticipated biological activity and is a suitable molecule for investigation of its actions in vivo.

**In Vivo ADMA Lowering by rDDAH**

The effect of rDDAH on ADMA in vivo was determined by administration of single dose (2.0 mg/kg) intravenously to normal rats. Blood was collected at various times for measurement of ADMA and DDAH activity in plasma. Figure 1E shows that ADMA in plasma was reduced within 5 min of rDDAH administration but returned to baseline levels by 40 min. Measurement of rDDAH in plasma showed that DDAH activity in the circulation was rapidly reduced (Figure 1F). Western blotting confirmed that rDDAH protein was similarly reduced in plasma within 30 min (Figure 1G). These data
suggested that exogenous native rDDAH was rapidly eliminated from the blood. To test if the blood constituents inactivated or sequestered DDAH, rDDAH was incubated with whole blood for varying lengths of time. As shown in figure 1H, the DDAH activity was stable in blood, suggesting that in vivo loss of rDDAH was not simply due to its inactivation or sequestration in the blood. These data also suggested that continuous presence of DDAH activity is required to achieve sustained ADMA lowering.

**Generation of Long Acting M-DDAH**

Several approaches to increase the duration of rDDAH activity in vivo and to achieve extended reduction of ADMA were investigated. We first tested if filtration by the kidney can be reduced by increasing the molecular size using site specific PEGylation of a mutant rDDAH. We cloned and expressed PA-DDAH mutants in which lysine residues (residue # 3, 25, 103 or 159) were individually replaced with cysteine. Adding a single cysteine allows site specific PEGylation using mPEG_{40K}-maleimide reaction with the cysteine residue. As shown in figure 2A, all Lys-Cys mutants were active in vitro with varying potency. Based on the solubility characteristics, enzyme activity and PEGylation efficiency, 159Lys-Cys mutant was selected for further studies. The 159Lys-Cys mutant was PEGylated using mPEG_{40K}-maleimide. As shown in figure 2B, the 159Lys-Cys PEGylated protein with MW of 75 kD was active in metabolizing ADMA. However, the 159Lys-Cys PEGylated protein was also rapidly reduced in circulation (Figure 2C). Since a protein with a MW of ~75 kD likely filtered only minimally in the glomerulus, these data suggest that the clearance of the PEGylated 159Lys-Cys if via a different
mechanism and a different approach to enhance the in vivo duration of DDAH activity would be required.

We then PEGylated the rDDAH using mPEG$_{10k}$-NHS which conjugated at the lysine residues. The PEGylation conditions were optimized to PEGylate all available lysine residues to produce a rDDAH with an average MW of 250 kD. The maximum PEGylated rDDAH, designated as M-DDAH maintained 90% activity in vitro (Figure 2D). Intravenous administration to rats showed that the activity of M-DDAH remained in circulation significantly longer as compared to rDDAH (Figure 2E). Consistent with the prolonged activity in vivo, a significant reduction in plasma ADMA was observed up to 6 h (Figure 2F). These data showed that as compared to the native rDDAH, M-DDAH exhibited more than 15-fold duration of activity in vivo, suggesting that it is suitable for pharmacological studies in animal models.

**Effect of M-DDAH on Endothelial Function**

Plasma ADMA levels in human subjects are strongly correlated with endothelial dysfunction (Cooke John, 2000). DDAH-1 gene deletion in mice resulted in high plasma ADMA, endothelial dysfunction and increased blood pressure (Leiper et al., 2007). We first determined if M-DDAH treatment reduced the intracellular levels of ADMA in endothelial cells. Figure 3A shows that incubation of cells with ADMA resulted in 3-fold increase in intracellular ADMA which was reduced by addition of M-DDAH. Thus, extracellular M-DDAH can effectively reduce ADMA levels in the cells. We then determined the effect of M-DDAH on inhibition of NO responses induced by ADMA. HUVECs were treated with ADMA or ADMA + M-DDAH and the effect on cGMP, a
marker of NO pathway, was determined. Figure 3B shows that ADMA treatment significantly reduced cGMP levels in HUVECs which was reversed by treatment with M-DDAH. These data demonstrate that extracellular M-DDAH attenuated inhibition of NO synthesis within the cells by reducing ADMA.

**M-DDAH Promotes In Vitro Angiogenesis**

Effect of M-DDAH on endothelial function was assessed using in vitro angiogenesis assay. Human umbilical vein endothelial cells (HUVEC) in Matrigel were treated with ADMA in the presence or absence of M-DDAH and branch formation was determined by image analysis (Figure 3C). Figure 3D shows that treatment of HUVEC with M-DDAH without addition of exogenous ADMA significantly enhanced angiogenesis measured by number of nodes, segments and mesh. Addition of ADMA significantly inhibited in vitro angiogenesis and treatment with M-DDAH reversed the effect of ADMA (Figure 3C and 3D). These results suggest that reduction of ADMA by M-DDAH may improve endothelial function and their regenerative function.

**Effect of M-DDAH on Blood Pressure**

Because ADMA levels are associated with elevation in blood pressure, we investigated whether reductions in ADMA by M-DDAH might directly lower blood pressure in an animal model of hypertension. Dahl salt-sensitive (DSS) hypertensive rats have been shown to exhibit higher levels of ADMA and spontaneous hypertension with age (Rapp and Garrett, 2019). Single intravenous administration of M-DDAH to DSS hypertensive rats showed sustained enzymatic activity in plasma (Figure 4A) and produced
significant reduction in ADMA (Figure 4B) and blood pressure for up to 4 h post-injection (Figure 4C). These data suggest that a targeted reduction of ADMA by M-DDAH manifest physiologically relevant blood pressure lowering in a model of hypertension.

**M-DDAH Protects Kidney Function in Response to Ischemia-Reperfusion Injury**

ADMA plays an important role in the kidney by inducing microcirculatory dysfunction which may contribute to accelerated renal disease. Ischemia-reperfusion (I/R) reduces DDAH resulting in the accumulation of ADMA in the target organ (Nakayama et al., 2014). In order to assess the effect of M-DDAH on I/R injury, we first determined the effect of I/R on DDAH expression and ADMA using a model of renal I/R injury. Consistent with previous data (Nakayama et al., 2014), renal I/R produced a time-dependent reduction in DDAH-1 mRNA (Figure S1A) and increased ADMA in the kidney (Figure S1B). To test the effect of M-DDAH, rats were subjected to 30 min of ischemia followed by reperfusion and then treated with vehicle or M-DDAH. The renal function, immune response and injury score were assessed after 24 h of reperfusion. Rats treated with M-DDAH were significantly protected from I/R injury as determined by serum creatinine which was significantly lower in M-DDAH treated animals (Figure 5A). M-DDAH treatment significantly reduced the number of inflammatory cells in the kidney (Figure 5B) and tubular injury (Figure 5C & D) in renal outer medulla. Metabolic acidosis due to lowered bicarbonate levels was also observed in the setting following I/R. Post-ischemic rats showed significantly lower levels of serum bicarbonate and pH relative to the post-ischemic M-DDAH-treated animals (Table S1). These results showed that reduction of ADMA by M-DDAH produced a significant reduction in inflammatory
response and injury score in the kidney, and improvement in renal function in response to I/R injury.

Discussion

In this first study of its kind, we have developed a novel long-acting modified DDAH (M-DDAH) and investigated the physiological and therapeutic significance of modulation of ADMA in vitro, ex vivo and in vivo. The major findings of this study are that (1) therapeutic levels of M-DDAH can lower plasma ADMA in vivo and it is suitable for pharmacological studies in animal models, (2) M-DDAH can reverse ADMA induced inhibition of cGMP generation, (3) lowering of ADMA can increase angiogenic function in endothelial cells, (4) reduction in ADMA by M-DDAH lowers blood pressure in hypertensive rodents and (5) reduction of ADMA by M-DDAH attenuates inflammatory response and kidney injury, and improves renal function following ischemia-reperfusion injury.

Multiple clinical studies have established an association of plasma ADMA with hypertension, cardiovascular and renal disease, and mortality. Whether ADMA is a bystander, a biomarker or a contributor to the mechanism of disease is yet to be established. The evidence for the physiological role of ADMA and its metabolizing enzyme DDAH has been largely derived from genetic studies in rodents. DDAH-1 gene deletion in mice increased plasma ADMA, endothelial dysfunction and blood pressure (Leiper et al., 2007). DDAH-1 gene overexpression improved angiogenesis in the ischemic hind limb model (Jacobi et al., 2005), and protected from myocardial and renal ischemia-reperfusion injury (Stuhlinger et al., 2007). However, the results of other
genetic studies have been contradictory. For example, DDAH-1 gene deletion did not affect the development of diabetic nephropathy in the streptozotocin-induced diabetes mellitus (STZ) model (Rodionov et al., 2019), whereas adenoviral mediated DDAH-1 gene delivery demonstrated improvement in diabetic nephropathy in STZ model (Wetzel et al., 2020). In another study, DDAH-1 gene deletion in the proximal tubule of mouse kidney increased tubular ADMA but was protective in kidney injury, collagen deposition and profibrotic cytokine expression (Tomlinson et al., 2015). These results were contrary to the reported protection of kidney injury in DDAH-1 transgenics (Nakayama et al., 2014). The results of gene manipulation can be confounded by developmental compensatory or redundant mechanisms. Since two isoforms of DDAH that catalyze ADMA degradation exist (Leiper and Nandi, 2011), the complexity of genetic models are particularly relevant to the ADMA metabolism pathway. In addition, glyoxylate aminotransferase 2 (AGXT2) can also play a role in metabolizing ADMA (Rodionov et al., 2014). Therefore, single DDAH gene deletion or overexpression may not produce a clear phenotype. Therefore, specific modulation of ADMA by a pharmacological molecule can provide a unique opportunity to investigate its pathophysiological role and determine if ADMA can be a target for therapy.

In order to specifically lower ADMA, we have developed a novel pharmacologically viable DDAH. rDDAH expressed and purified from E. coil effectively reduced ADMA in plasma in vitro even while the concentration of ADMA was 100-200 fold below its Km. rDDAH rapidly lowered ADMA in rat plasma in vivo. However, the ADMA lowering was short lived due to the elimination of rDDAH from the circulation. Further, a higher molecular weight (75 kD) PEGylated DDAH mutant was also short
lived in vivo, suggesting that kidney filtration may not be the mechanism of DDAH clearance. A DDAH molecule produced by multiple PEGylation at the lysine residues (M-DDAH) demonstrated more than 15-fold longer duration of activity in vivo. M-DDAH reduced ADMA levels in rat plasma by greater than 80% within 5 min after administration. The rapid and large magnitude of ADMA reduction was unexpected. First, the Km of rDDAH (76 µM) is about 150-fold greater than the ADMA concentration in rat plasma. Second, previous studies have shown that ADMA remains bound to plasma proteins and not removable by dialysis (Kielstein et al., 2004). Based on the DDAH protein structure and localization of the active site within the 3D structure (Frey et al., 2006), it is unlikely that the protein bound ADMA is accessible to the active site for catalysis. The rapid and effective lowering of ADMA by M-DDAH suggest that there may be an efficient dynamic equilibrium between protein bound and free ADMA such that the bound ADMA molecules can readily dissociate and become available for hydrolysis. These studies showed that M-DDAH is highly suitable for specific modulation of ADMA in vivo and therefore it offers a unique opportunity to investigate the physiological impact of ADMA lowering in disease states.

The effect of M-DDAH was investigated in vitro and in vivo under conditions involving high ADMA. In vitro, addition of M-DDAH to endothelial cells reduced intracellular ADMA. Concordantly, M-DDAH treatment increased NO pathway responses as measured by increase in cGMP in endothelial cells. The improvement in endothelial function was further validated by the reversal of the inhibitory effects of ADMA on in vitro angiogenesis. Importantly, reduction in endogenous ADMA by M-DDAH enhanced endothelial activity to undergo branching. Collectively, these results
showed that specific reduction of ADMA by M-DDAH significantly improved endothelial function. The abrogation of ADMA effects on cGMP suggest that improvement in NO bioavailability is an important contributing mechanism to its effects on endothelial function. Since ADMA has been shown to induce other cellular responses including the generation of reactive oxygen species (ROS) by NOS uncoupling, the use of M-DDAH may also allow investigation of the other potential mechanisms contributing to the cellular actions of ADMA.

Two different disease models were used for the investigation of the physiological response to ADMA lowering. The DSS hypertensive rats are known to exhibit high levels of circulating ADMA and endothelial dysfunction (Matsuoka et al., 1997). Administration of M-DDAH to DSS hypertensive rats significantly reduced ADMA in plasma and reduced blood pressure. These data showed that ADMA plays a pathological role in inducing high blood pressure and M-DDAH produces a positive therapeutic outcome. These studies have also provided direct evidence that ADMA is not simply a biomarker. Rather, ADMA acts as a pathological molecule and its lowering may provide a new therapeutic approach to manage hypertension.

Ischemia-reperfusion (I/R) injury is another pathological condition in which high ADMA levels have been implicated. In models of I/R injury to the heart (Stuhlinger et al., 2007) and kidney (Nakayama et al., 2014), ADMA levels are significantly increased, and DDAH expression and activity are reduced. The renal I/R injury may parallel with the acute kidney injury (AKI) that commonly occurs during the cardio-pulmonary bypass and aortic cross clamping (Thakar et al., 2005; Lannemyr et al., 2017). In the rat model of renal I/R injury, M-DDAH significantly attenuated the loss of renal function as measured
by reduced serum creatinine. Concurrently, M-DDAH significantly improved serum bicarbonate and acidosis, and reduction in inflammatory cells in the kidney. These effects may be due to the improved renal perfusion and endothelial function which play critical role in AKI. The modulation of injury response by M-DDAH is particularly relevant to cardiac surgery associated AKI in which high levels of ADMA are produced (Plicner et al., 2014). Cardiac surgery associated-AKI is the second most common cause of kidney injury in hospitalized patients (Uchino, 2006). In addition, contrast media is associated with a high incidence of AKI (Hennessy et al., 2010). Thus, M-DDAH may represent an important therapeutic molecule for the preservation of microcirculatory flow, and prevention and treatment of postoperative AKI.

A limitation of the current M-DDAH molecule is the duration of its in vivo activity. ADMA lowering by DDAH which may be suitable for investigating its efficacy in acute settings such as during the cardiothoracic and heart valve replacement surgery. However, the duration of M-DDAH action is still not adequate for chronic diseases. Also, in the current studies, intravenous delivery of M-DDAH was used. Further development of a molecule that can be delivered by subcutaneous administration could provide a more convenient method of treatment. The primary goal of the current studies was to test the hypothesis that specific lowering of ADMA can produce physiological response. Our experimental approach has clearly demonstrated efficacy of ADMA lowering which could be clinically relevant for patients undergoing cardiac surgery. In future studies, will focus on improving the duration of DDAH action in vivo which will enable testing of prevention as well as treatment approaches for acute and chronic conditions.
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Authorship Contributions

Participated in research design: Singh, Lee, Basile, Mehrotra, Sasser, Skill, Shekhar

Conducted experiments: Lee, Mehrotra, Ullah, A. Singh, Younes, Skill

Contributed new reagents or analytic tools: Singh, Lee, A. Singh

Performed data analysis: Lee, Mehrotra, Ullah, Sasser, Younes, Skill

Wrote or contributed to the writing of the manuscript: Singh, Lee, Basile

References


**Footnotes**

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**Figure Legends**

**Figure 1.** In vitro and in vivo activity of recombinant DDAH. PA-DDAH with His-tag was cloned in pE-SUMO vector and then expressed in E. coli. Purified rDDAH shows
activity and purity in SDS gel (inset lane 2) (n=4) (A). Km determination using high sensitivity citrulline assay (B). In vitro ADMA lowering in rat plasma containing 2.7 µM ADMA after 1 h incubation (C) and ADMA lowering with 10 µg rDDAH/mL incubated for different times (n=3) (D). In vivo activity of rDDAH after intravenous administration of a single dose of 2 mg/kg rDDAH to rats. Blood was drawn, at the indicated time points and ADMA levels (E) and DDAH activity in plasma (F) were determined (n=4). Plasma samples collected at 5 min and 30 min post rDDAH administration were analyzed by western blotting to detect DDAH protein (G). Stability in blood was determined by incubation of rDDAH with heparinized blood for the indicated time at room temperature or PBS at 4 ºC and then assayed for DDAH activity (n=3) (H). All data are given as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 2. Generation of site specific PEGylated and long acting DDAH. DDAH mutants were generated by site directed replacement of Lys #3, 25, 103, or 159 with cysteine. Mutant genes were cloned and expressed in E. coli. Purified proteins were assayed for activity of rDDAH (●), 3Lys-Cys (■), 25Lys-Cys (▲), 103Lys-Cys (▼) and 159Lys-Cys (◆) mutants (n=3) (A). Mutant 159Lys-Cys was PEGylated at cysteine using mPEG₄₀K-maleimide. Activity of 159Lys-Cys mutant before (●) and after PEGylation (■) and SDS gels comparing molecular size (inset gel 1 before and gel 2 after PEGylation) are shown (n=3) (B). In vivo activity of PEGylated 75 kD protein after single administration to rats (C). Long acting M-DDAH was generated by maximum PEGylation protocol using mPEG₁₀K-NHS (n=4). Shown are in vitro activity of rDDAH before (●) and after PEGylation (M-DDAH) (■), and SDS gels (inset lane 2 before and
lane 3 after PEGylation) with 250 kD protein (D). In vivo activity of rDDAH-1 (●) M-DDAH (◼) (E) and ADMA lowering in rat plasma after M-DDAH administration (n=7) (F). All data are given as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 3. Effect of M-DDAH on endothelial function. Effect of M-DDAH on cellular ADMA in endothelial cells was determined using HUVECs culture (A). Triplicate wells were treated with vehicle or 10 µM ADMA. After 30 min cells were washed to remove exogenous ADMA and then incubated in the absence or presence of 100 µg/mL M-DDAH for 1 h. Medium was removed and cells were extracted for measuring ADMA remaining in the cells using HPLC (n=3). Effect on cGMP production in HUVECs was determined in control, ADMA and ADMA + M-DDAH treated cells (B). Triplicate wells were treated with 0.5 mM IBMX (Control) or 0.5 mM IBMX + 100 µM ADMA for 5 min. The medium was aspirated and cells were incubated in fresh medium containing 0.5 mM IBMX or 0.5 mM IBMX + 10 µg/mL M-DDAH for 60 min. Cells were collected and cGMP was analyzed using enzyme immunoassay kit (n=3). In vitro angiogenesis was determined by incubation of HUVECs in Matrigel in the presence of control, ADMA, M-DDAH or ADMA + M-DDAH (C) (n=3 wells per treatment). Angiogenesis was quantified using image J and expressed as number of nodes, segments and mesh (n=3 in each figure) (D). All data are given as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 4. Effect of M-DDAH on blood pressure. M-DDAH (1 mg/kg) (◼) or vehicle (●) was intravenously administered to conscious restrained Dahl salt sensitive hypertensive female rats (n=4). Blood was collected at the indicated time points and plasma DDAH
activity (A) and ADMA levels (B) were determined. Mean arterial blood pressure was determined by Deltran pressure transducer (Utah Medical) connected to the catheter in the carotid artery (C). Data were collected using an amplifier and PowerLab from ADInstruments. All data are given as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.

**Figure 5. Effect of M-DDAH on renal ischemia-reperfusion injury.** Sprague Dawley rats were subjected to 30 min of bilateral ischemia by clamping the renal arteries and then-reperfusion. M-DDAH (2 mg/kg) or vehicle was administered by i.v. injection at the time of reperfusion and second injection 3 h after reperfusion. Plasma and kidney samples were collected after 24 h (n=5). Renal function was determined by measuring plasma creatinine (A). Inflammatory cells were identified by flow cytometry and number of CD11/b/c positive cells, IL17 positive cells, CD4 positive cells and CD8 positive cells, were calculated per gram kidney weight for vehicle treated (black bar) or M-DDAH treated rats (gray bar) (B). Panel C shows representative hematoxylin/eosin stained sections through renal outer medulla demonstrating significant necrosis in vehicle-treated group (arrows) vs minimal tubular damage in M-DDAH treated group. Higher magnification image is shown in figure C inset. Panel D shows quantification of percent damaged tubules. All data are given as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 3

Figure 4
Figure 5