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Deposited on: 22 November 2019

Regulation of fluid reabsorption in rat or mouse proximal renal tubules by asymmetric

dimethylarginine (ADMA) & dimethylarginine dimethylaminohydrolase (DDAH) 1

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Short title: DDAH-1, ADMA and PT reabsorption

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20 **ABSTRACT**

21 **Background:** Nitric oxide prevents hypertension yet enhances proximal tubule Na^+ reabsorption.

22 Nitric oxide synthase is inhibited by asymmetric dimethylarginine (ADMA) that is metabolized

23 by dimethylarginine dimethylaminohydrolase (DDAH) whose type 1 isoform is expressed

24 abundantly in the PT.

25 **Hypothesis:** That ADMA metabolized by DDAH-1 inhibits fluid reabsorbtion (Jv) by the

26 proximal tubule.

27 **Methods:** S2 segments of the PT were microperfused between blocks *in vivo* to assess Jv in

28 anesthetized rats.

29 **Results:** Compared to vehicle, microperfusion of ADMA or N^{ω} -nitro-l-arginine methyl ester

30 (L-NAME) into the proximal tubule reduced Jv dose-dependently. At 10^{-4} mol·l^{-1} both reduced

31 Jv by ~ 40% (vehicle: 3.2 ± 0.7 vs ADMA: 2.1 ± 0.5; P<0.01; vs L-NAME: 1.9 ± 0.4 nl·min^{-}

32 ^{1}·mm^{-1}; P<0.01; n=10). Selective inhibition of DDAH-1 in rats with intravenous L-257 (60

33 mg·kg^{-1}) given 2 hours before and L-257 (10^{-5} mol·l^{-1}) perfused into the proximal tubule for 5

34 minutes reduced Jv by 32±4% (vehicle: 3.2 ± 0.5 vs L-257: 2.2 ± 0.5 nl·min^{-1}·mm^{-1}; P<0.01)

35 and increased plasma ADMA by $\simeq$ 50% (Vehicle: 0.46 ± 0.03 vs L-257: 0.67 ± 0.03 µmol·l^{-1}; P

36   < 0.0001) without changing plasma symmetric dimethylarginine. Compared to non-targeted

37   control small interference RNA, knock down of DDAH-1 in mice by 60% with targeted siRNA

38   reduced Jv by 29±5% (nontargeted SiRNA: 2.8 ± 0.20 vs DDAH-1 knockdown: 1.9 ± 0.31

39    $nl \cdot min^{-1} \cdot mm^{-1}$; $P<0.05$).

40   **Conclusions**: Fluid reabsorption in the proximal tubule is reduced by tubular ADMA or by

41   blocking its metabolism by DDAH-1. L-257 is a novel regulator of proximal tubule fluid

42   reabsorption.

43

INTRODUCTION

Nitric oxide (NO) relaxes blood vessels, prevents salt sensitivity (23), reduces

sympathetic nervous system tone (11), reduces or prevents hypertension and protects blood

vessels, the heart, the kidney and other organs from hypertensive damage (26). Indeed, a reduced

renal expression of nitric oxide synthase (NOS)1 has been related to progression of kidney

disease in a wide range of animal models (3). NO causes vasodilation of renal afferent arterioles

(13), inhibits the vasoconstrictive tubuloglomerular feedback response (24) and inhibits Na^+

reabsorption in the thick ascending limb of the loop of Henle and the collecting ducts (7).

Although, blockade of NOS has variable effects on Na^+ excretion, genetic deletion of NOS1 and

3 sharply reduce the reabsorption of fluid in the mouse proximal tubule in most (21), but not all

studies (18). The differences may relate to the effects of the NOS 1 alpha and NOS 1 beta splice

variants (14).

Asymmetric dimethylarginine (ADMA) is a cellular and circulating inhibitor of NOS (2)

that is metabolized by dimethylaginine dimethylaminohydrolase (DDAH) whose type 1 isoform

is heavily expressed in the proximal tubule (19). Although the function of DDAH in the kidney

has not been studied, there are compelling clinical data linking it to CKD. Thus, circulating

levels of ADMA have been considered to be a uremic toxin and predict the progression of CKD

61 (12, 28). However, activating polymorphism of DDAH-1 (5) that reduce circulating ADMA are

62 associated with protection from progression of CKD (5) and salt sensitivity (6). Thus, DDAH-

63 1/ADMA/NO in the kidney may have effects independent of circulating ADMA. We tested the

64 hypothesis that DDAH-1 regulates proximal tubule fluid reabsorption by regulating ADMA.

65 First, rat renal proximal tubules were perfused with artificial tubular fluid (ATF) with graded

66 addition of N^w-nito-l-arginine methyl ester (L-NAME) to inhibit nitric oxide synthase isoforms

67 or ADMA. Second, the effects of DDAH-1 were tested in rats administered with L-257 that is a

68 specific DDAH-1 inhibitor (22). The findings were extended to a study in the mouse by gene

69 silencing of DDAH-1 (19).

70 **MATERIALS AND METHODS**

71 *Animals*:

72   The experiments were conducted under protocols approved by the Georgetown

73 University Animal Care and Use Committee and performed according to the National Institutes

74 of Health guidelines for the conduct of experiments in animals. Male Sprague-Dawley rats and

75 C57Bl/6 mice were housed in cages kept in temperature-controlled units (25°C) with a 12 h

76 light/dark cycle and maintained on a standard chow with free access to food and water.

5

*Surgical Preparation***:**

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Rats were prepared for renal micropuncture under anesthesia with thiobarbital (Inactin,

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80 mg.kg^{-1} IP; Research Biochemicals, Inc.) and infused with isotonic saline containing1%

79

bovine serum albumin (Sigma Chemical St. Louis, MO) at 1.5ml·hr^{-1} to maintain euvolemia as

80

described (24).

81

Mice were anesthetized by with isoflurane (1.0% in room air, delivered by a pump,

82

Univenter, Malta) and prepared for micropuncture as described (1, 4). Cannulae were placed in a

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jugular vein for infusion of isotonic saline containing 1.5% bovine serum albumin at 0.35 ml·hr^{-1}

84

to maintain euvolemia (4). In both rats and mice, a femoral artery was cannulated for recording

85

of mean arterial pressure (MAP; Powerlab, AD Instruments Inc), the left ureter was cannulated

86

to collect urine from the left experimental kidney that was exposed by a flank incision and

87

stabilized in a Lucite cup (Vestavia Scientific, Birmingham, AL) for micropuncture (1, 4, 24).

88

The study commenced 60 minutes after surgery.

89

Microperfusion of proximal tubules (PTs) of rats and mice:

90

As described previously for rats (24) and mice (1, 4), a surface proximal tubule loop (S2

91

segment) was identified by injection from a "finding" pipette (8-µm outer diameter) containing

92

93   artificial tubule fluid stained with the Fast Green FCF dye (Sigma, 0.1%). An immobile grease

94   block (Apiezon T, Manchester, UK) was injected into the tubule at the puncture site to stop

95   tubular fluid flow. A perfusion pipette (8-to 10-µm outer diameter) was inserted immediately

96   downstream from the block. The perfusion pipette was filled with artificial tubular fluid

97   containing (mmol.l^{-1}): 125 NaCl, 20 NaHCO$_3$, 5 KCl, 1 MgSO$_4$, 2 CaCl$_2$, 1 NaH$_2$PO$_4$, 5 Glucose,

98   4 urea) and [^{14}C]- inulin. It was connected to a calibrated nanoliter perfusion pump (Vestavia

99   Scientific, Birmingham, AL) to perfuse the segment of the proximal tubule for 2 to 4 minutes

100   before timed fluid collections. The collections were made at a downstream site with a

101   micropipette (8-to 10µm outer diameter) after placement of a column of oil to block downstream

102   flow. The samples were collected for 4 minutes and transferred into a constant-bore capillary

103   tube whose length was measured with a micrometer to calculate the tubular fluid volume.

104   Thereafter, the samples were injected into scintillation fluid and the ^{14}C activity counted.

105   Collected samples with <95% and >105% of microperfused [^{14}C]- inulin were discarded. The

106   amount of microperfused inulin was estimated by the average of ^{14}C-activity in 3 samples

107   perfused into a vial over 4 minutes. To determine the lengths of the perfused segments, tubules

108   were filled with high-viscosity microfil (Flow Tech, Inc.), the kidney was partially digested in

109   20% NaOH, and the length of the cast was measured under a dissecting microscope. The Jv was

110   calculated by the difference in the rate of fluid perfusion and the rate of fluid collection factored

111   by the length of the perfused nephron segment (1, 4, 24).

112   *Construction and administration of small interference RNAs (siRNAs)***:**

113   These studies were performed in mice. RNAi duplexes of 21 nucleotides targeting the

114   coding region of DDAH-1 (siDDAH-1) (Qiagen) were validated *in vitro* as described previously

115   (19). The target site in the mouse DDAH-1 cDNA (GenBank accession no. NM_026993) of the

116   construct selected was 673 to 693 (TGGCCGATTCTTTGCATTTAA). The non-silencing

117   control SiRNA (catalogue # 1027280; Qiuagen) had no homology to any sequence in the

118   mammalian genome. Under brief anesthesia with 1% to 2% isoflurane, cannulae were inserted

119   into the femoral vein of the mouse for rapid injection of 25ug siRNA constructs diluted in 1 ml

120   of TransIT-QR Hydrodynamic Delivery Solution (Mirus: ZL) injected within 5 seconds. The

121   effects of this hydrodynamic DDAH-1 silencing were assessed by RNA analysis in the harvested

122   kidney cortex after 48 hours.

123   *Protocols***:**

124   *Protocol 1. Microperfusion of ADMA or L-NAME into the proximal tubule of rats***:**

125   ADMA or L-NAME were dissolved in artificial tubular fluid at 10^{-7} M to 10^{-4} mol·l^{-1} and

126   perfused into a rat proximal tubule between blocks. Alternate tubules were perfused with

127   artificial tubular fluid + vehicle or ADMA or L-NAME.

128   ***Protocol 2. Blockade of DDAH-1 with L-257 in rats*:**

129   For each series, Jv was measured in a perfused proximal tubule of a rat after

130   administration of vehicle or L-257. The optimal method for delivery of L-257 was assessed from

131   three protocols:

132   A. Proximal tubule perfusion of L-257 (10^{-5}mol·l^{-1}) or vehicle;

133   B. IV injection of L-257 (60 mg.kg^{-1}) or vehicle two hours previous, followed by tubular

134   perfusion with vehicle, and

135   C. IV injection of L-257 or vehicle two hours previous followed by tubular perfusion with L-257

136   or vehicle.

137   In separate groups, blood was collected for two hours following bolus IV injection of

138   vehicle or L-257 for measurements of plasma ADMA and symmetric dimethylarginine (SDMA)

139   with a fully validated gas chromatography- mass spectrometry method (GC/MS) and quantitated

140   relative to deuterated standards (17).

141

Protocol 3. Gene silencing of DDAH-1 in mice:

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The Jv of the perfused proximal tubule of C57/BL6 mice was assessed after iv injection 48

143

hours previously of siRNA directed to DDAH-1 or non-targeted control siRNA, as described in

144

detail previously (19). The kidney cortex was harvested to measure mRNA expression of DDAH-

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1.

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*RNA extraction, cDNA synthesis, and real-time PCR***:**

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RNA was extracted from harvested tissues using a RNeasy Mini Kit (Qiagen ZL). The

148

cDNA was synthesized using iScript^{TM} cDNA Synthesis kit (Biorad ZL) . The gene expression

149

for DDAH-1 was assessed with real-time PCR (StepOnePlus Real-time PCR System, ABI ZL),

150

using a FAM (6-carboxy-fluorescine dye)-labeled DDAH-1 Taqman probe assay

151

(Mm01319453_ml, ABI) multiplexed with a VIC (fluorescine dye) –labeled 18S control probe.

152

Relative amounts of mRNA, normalized by 18S rRNA, were calculated from threshold cycle

153

numbers (CT, ie, $2^{-\Delta\Delta CT}$).

154

*Drugs***:**

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156   ADMA (N^G, N^G – dimethylarginine dihydrochloride) and L-NAME (N^ω-nitro-L- arginine

157   methyl ester hydrochloride) were purchased from Sigma Chemical (St. Louis, MO). L-257 is a

158   fully validated DDAH-1 inhibitor that was synthesized in the laboratory of James Leiper of the

159   MRC Clinical Sciences Center, London (22).

160   *Statistical Analysis***:**

161   Data are presented as means $\pm$ SE. The significance of differences within and between

162   groups was evaluated using ANOVA followed by a Fisher's *post hoc* test where appropriate.

163   Results were considered significant at *P*<0.05.

164   **RESULTS**

165   *Proximal tubule fluid reabsorption in rats during luminal microperfusion of L-NAME or*

166   *ADMA***:**

167   Microperfusion of L-NAME or ADMA reduced Jv similarly and dose-dependently (**Figure**

168   **1**). At the maximum dose tested of 10^{-4} mol·l^{-1}, ADMA reduced Jv by 41 $\pm$ 5% (Vehicle: 3.3 $\pm$ 0.5

169   vs ADMA: 1.9$\pm$0.4 nl·min^{-1}·mm^{-1}, P<0.01; n=10 tubules) and L-NAME by 38 $\pm$ 6% (Vehicle: 3.3

170    $\pm$ 0.5 vs L-NAME: 2.1 $\pm$ 0.5 nl·min^{-1}·mm^{-1}; P<0.01, n=10 tubules).

171   *Proximal tubule fluid reabsorption in rats after inhibition of DDAH-1 with L-257***:**

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172   L-257 delivered by IV injection 2 hours prior to experimentation and by direct perfusion

173   of the proximal tubule reduced the Jv by $32 \pm 4\%$ (Vehicle: 3.2 ± 0.4 vs L-257: 2.2 ± 0.5 nl·min$^-$

174   ^{1}·mm^{-1}; $P<0.01$; n=8 tubules). (**Figure 2**). Direct tubular perfusion of L-257 or sole IV

175   administration of L-257 did not change Jv consistently (data not shown). The IV administration of

176   L-257 to rats 2 hours previously increased plasma ADMA by 50% (vehicle: 0.46 ± 0.03 vs L-257:

177   0.67 ± 0.03 µmol·l^{-1}; $P< 0.0001$) without changing SDMA (**Figure 3**).

178   **Proximal tubule fluid reabsorption in mice after knockdown of DDAH-1 with siRNA:**

179   Injection of siRNA for DDAH-1 in rats produced quite variable knockdown of DDAH-1

180   mRNA in the kidney whereas the knockdown in mice was more consistent. Therefore, mice were

181   selected for this protocol. Rapid bolus IV injections of siRNA directed to DDAH-1, compared to

182   non-targeted siRNA, given 48 hours prior to experimentation to mice reduced the expression of

183   mRNA to DDAH-1 in the renal cortex by $55 \pm 5\%$ and reduced Jv in the PT by $43 \pm 5\%$ (siControl:

184   2.3 ± 0.4 vs siDDAH-1: 1.3 ± 0.3 nl·min^{-1}·mm^{-1}; $P<0.01$, n=6) (**Figure 4**).

185   **DISCUSSION**

186   We confirm that L-257 is an effective inhibitor of DDAH-1 and increases plasma levels

187   of ADMA by 50% (22). The main new findings are that ADMA is as effective as L-NAME in

188   reducing Jv of the rat perfused proximal tubule. Maximal concentrations of each drug reduced Jv

189   by $\simeq$ 40% that was similar to the reduction of Jv of 32% following pharmacological inhibition of

190   DDAH-1 with L-257 in rats or reduction of Jv of 43% following gene silencing of DDAH-1 in

191   mice.

192   ADMA is produced by hydrolysis of methyl arginine moieties in proteins after

193   methylation by protein arginine methyl transferases (PRMT) (15). Its plasma levels are primarily

194   regulated by metabolism by DDAH (2, 10). ADMA also can be metabolized by alanine-

195   gluyoxilate amino transferase II (AGXT II) (2). However, the finding that the proximal tubule

196   fluid reabsorption was inhibited similarly by direct microperfusion of ADMA or by

197   pharmacological inhibition of ADMA metabolism by DDAH-1 or silencing of the DDAH-1 gene

198   demonstrates the importance of DDAH-1 for regulation of ADMA in the proximal tubule.

199   DDAH-1 is heavily expressed in the liver and the proximal tubule (9, 16). Measurements

200   of ADMA extraction across organs *in vivo* has shown that the kidney and the liver are the prime

201   sites for clearance of plasma ADMA (2, 10, 15). The large increase in plasma ADMA after

202   knockdown of DDAH-1 in this study is consistent with these findings. SDMA is not metabolized

203   by DDAH (2). Therefore, the finding that plasma levels of ADMA were increased, but SDMA

204   were unchanged, after L-257 provides further evidence of the specific effect of L-257 to inhibit

13

205   DDAH (22). The failure of L-257 to reduce Jv significantly when perfused directly into the PT

206   may relate to the limited time of tubular exposure of ~ 5 minutes. This may have been

207   insufficient for ADMA to accumulate effectively after inhibition of its metabolism by blocking

208   DDAH-1. In contrast, DDAH-2 is expressed in the vascular endothelium and the distal nephron

209   and macula densa of the kidney. Unlike DDAH-1, knockdown of DDAH-2 in the rat impairs

210   endothelial function and reduces the expression of endothelial NOS but does not change plasma

211   levels of ADMA (19). However, the DDAH-1 knockout mouse has endothelial dysfunction (27),

212   suggesting differences between rats and mice in the specific roles of the DDAH isoforms. In this

213   study, DDAH-1 was inhibited by two distinct means (pharmacological and gene knockdown) in

214   rats and in mice. The similar effects of these to reduce proximal tubule fluid reabsorption by 30

215   to 45% suggest an important role for DDAH-1 in the proximal tubules of both species. The

216   expression of DDAH-1, and consequently the plasma and tissue levels of ADMA, are regulated

217   by reactive oxygen species both in vitro (8) and *in vivo* (20, 25) and thereby have been linked to

218   pathophysiology of hypertension and CKD (25).

219   We acknowledge some limitations of our study. DDAH-1 was blocked

220   pharmacologically in rats and by gene knockout in mice. However, the similar effects on

221   proximal fluid reabsorption suggest no major species difference. Inhibition or silencing of

222   DDAH-1 will have systemic effects that could influence proximal tubule reabsorption. However,

223   DDAH-1 silencing over 2 hours does not change BP significantly (19). Moreover, ADMA

224   perfused into the nephron had a similar effect to reduce proximal tubule reabsorption as did

225   systemic inhibition of its metabolism by DDAH-1 blockade or gene knockdown.

226   In conclusion, ADMA and its metabolism by DDAH-1, are important determinants of

227   proximal tubule fluid reabsorption in rats and mice.

228   **PERSPECTIVE**

229   Luminal ADMA inhibited rat proximal tubule fluid reabsorption at 10^{-7} mol·l^{-1} which is

230   at the plasma level of ADMA of 3×10^{-7} mol·l^{-1} recorded in this study. A 55% knockdown of

231   DDAH-1 in the mouse reduced proximal tubule reabsorption significantly. This reduction in

232   DDAH-1 expression is equivalent to that reported in earlier studies in rats infused with

233   angiotensin II that was attributed to reactive oxygen species (8, 20, 25). Thus, these findings

234   establish ADMA as a physiological inhibitor of proximal tubule reabsorption and DDAH-1 as an

235   important physiological regulator of proximal tubule function *in vivo* in rats and mice. Moreover,

236   DDAH-1 and ADMA may contribute to the pathophysiology of hypertension and conditions

237   associated with oxidative stress.

DISCLOSURES

There are no conflicts of interest.

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324

325

FIGURE LEGENDS

Figure 1

L-NAME and ADMA inhibit fluid reabsorption (Jv) in the rat perfused proximal tubule **in vivo**

during microperfusion and recollection of artificial tubular fluid +Vehicle; (open circle), + L-

NAME (solid triangles and continuous lines) or + ADMA (solid squares and broken lines).

Compared with ATF + Vehicle *, P < 0.05.

Figure 2

Blockade of DDAH-1 with L-257 in the rat reduces absolute proximal tubule fluid reabsorption

(Jv) 2 hours after IV injection of L-257 (60 mg·kg^{-1}) and during tubule perfusion of L-257 (10^{-5}

mol·l^{-1}; solid boxes) compared to corresponding administration of vehicle (open boxes)

Figure 3

Intravenous injection of L-257 increases asymmetric dimethylarginine (ADMA) selectively.

Plasma levels of ADMA or symmetric dimethylarginine (SDMA) two hours after IV injection of

vehicle (open boxes) or L-257 (solid boxes).

Figure 4

Knockdown of DDAH-1 with siRNA reduces absolute proximal tubule fluid reabsorption (Jv) in

the mouse proximal tubules 48 hours after IV injections of siRNA to DDAH-1 (solid boxes)
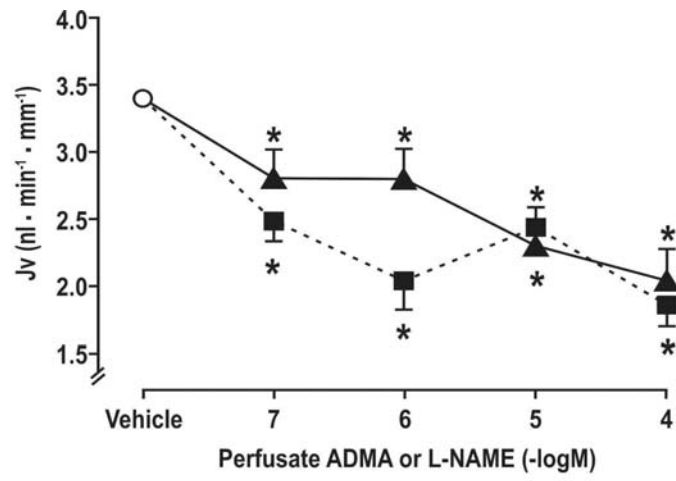
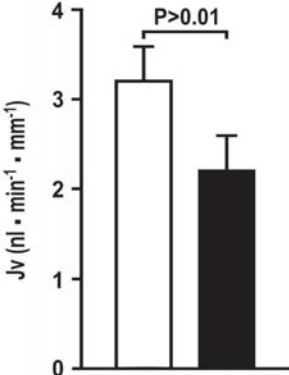compared to non-targeted siRNA (open boxes).
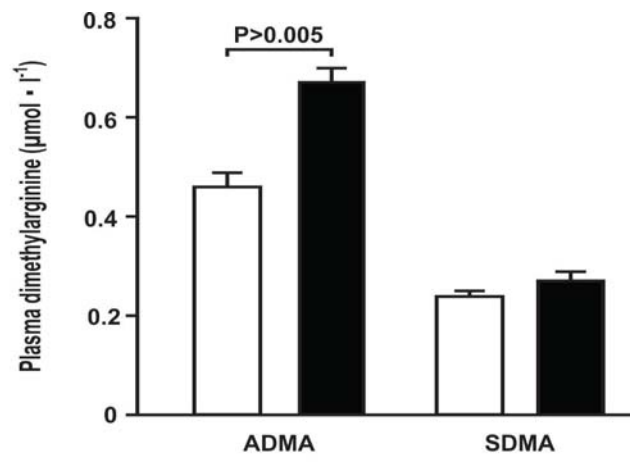
Figure 1

Figure 2

Figure 3

Figure 4

11-14-16B