Role of alanine:glyoxylate aminotransferase 2 in metabolism of asymmetric dimethylarginine in the settings of asymmetric dimethylarginine overload and bilateral nephrectomy

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ABSTRACT

Background. Asymmetric and symmetric dimethylarginines (ADMA and SDMA) predict complications and mortality in cardiovascular and renal diseases. Alanine:glyoxylate aminotransferase 2 (AGXT2) can metabolize both ADMA and SDMA; however, this metabolic pathway is still poorly understood. The goal of our study was to test the hypothesis that AGXT2 is compensatory upregulated in the settings of ADMA overload and bilateral nephrectomy.

Methods. ADMA was infused for 3 days using osmotic minipumps in mice. Half of the mice underwent bilateral nephrectomy 24 h before the end of the infusion.

Results. Infusion of ADMA caused a 3- to 4-fold increase in plasma and urine ADMA levels and a 2- to 3-fold increase in plasma and urine levels of the ADMA-specific metabolite of AGXT2 α-keto-δ-(N,N-dimethylguanidino)valeric acid (DMGV). Bilateral nephrectomy led to an ~4-fold increase of plasma SDMA levels, but did not change plasma ADMA levels. Interestingly, plasma levels of DMGV were elevated 32-fold in the mice, which underwent bilateral nephrectomy. Neither bilateral nephrectomy nor ADMA infusion caused upregulation of AGXT2 expression or activity.

Conclusions. Our data demonstrate that short-term elevation of systemic levels of ADMA leads to a dramatic increase of DMGV formation without upregulation of AGXT2 expression or activity, which suggests that AGXT2-mediated pathway of ADMA metabolism is not saturated under normal conditions and may play a major role in the maintenance of ADMA homeostasis in the setting of local or systemic elevation of ADMA levels.

Keywords: acute renal failure, ADMA, AGXT2, DMGV, nephrectomy

INTRODUCTION

Various epidemiological and experimental studies have demonstrated that endogenously formed methylarginines, such as asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA), can serve as markers and possibly mediators of cardiovascular disease [1–3]. Moreover, plasma
ADMA levels are predictors for graft failure, renal and cardiovascular events and all-cause mortality in kidney transplant patients [4]. The main proposed mechanism of the pathological effects of ADMA is competitive inhibition of nitric oxide (NO) production by nitric oxide synthases (NOS). An increase in ADMA levels in human subjects or animal models leads to endothelial dysfunction, decreased renal blood flow, increased renovascular resistance, renal sodium retention and elevated systemic blood pressure [5]. Even though SDMA cannot directly inhibit NOS [6], it has been proposed to affect NO production indirectly by competing with l-arginine for their common transporter [7, 8]. Plasma SDMA levels correlate with the extent of coronary artery disease and independently predict cardiovascular and all-cause mortality [8–10]. On the other hand, a month-long infusion of SDMA in mice had no effect on renal function, renal histology, blood pressure or cardiac function [11]. Therefore, it is still not clear, whether SDMA plays a direct causative role in vascular damage at least in some cardiovascular pathologies or it is simply a marker of some other underlying processes.

The major pathway of ADMA degradation is catabolism to methylamines and citrulline catalysed by dimethylarginine dimethylaminohydrolases (DDAHs). There are two isoforms of this enzyme in mammals (DDAH1 and DDAH2), which are widely expressed in a variety of vascular and non-vascular tissues [12, 13]. Even though experimental data strongly support a protective role of DDAHs in cardiovascular diseases, no therapeutical approaches for selective upregulation of DDAHs could have been developed so far. One potential explanation for this might be that both DDAH isoenzymes are relatively small in size (31 and 29 kDa, respectively—Uniprot Database, www.uniprot.org) and do not require cofactors, so it is unlikely that they have endogenous allosteric regulatory mechanisms, which could be therapeutically upregulated.

An alternative pathway of ADMA catabolism is its transamination to α-keto-δ-(N,N-dimethylguanidino)valeric acid (DMGV) by alanine:glyoxylate aminotransferase 2 (AGXT2) [14, 15]. We and others have shown the in vivo importance of this pathway for regulation of systemic ADMA levels [16, 17], while the study by Caplin et al. [17] has also suggested that endogenous AGXT2 might be involved in regulation of systemic blood pressure. AGXT2 is larger than DDAHs (49 kDa) and requires pyridoxalphosphate as a cofactor, which increases the chances that activity of this enzyme could be allosterically modulated. Furthermore, in contrast to DDAHs, AGXT2 can metabolize not only ADMA, but also SDMA [18], which makes it a perspective pharmacological target for treatment of both ADMA- and SDMA-mediated cardiovascular pathologies.

Recently, we have developed a method for detection of DMGV in plasma and urine [19] and a method for estimation of AGXT2 activity in biological samples [20]. These methods can serve as powerful tools for identification of the endogenous mechanisms of regulation of expression and activity of AGXT2 with the aim of subsequent therapeutic modulation of those identified mechanisms. The goal of the current study was to investigate the changes in AGXT2 expression, protein levels and activity in the setting of acute elevation of systemic ADMA levels and lack of urinary excretion of ADMA. We have specifically hypothesized that both ADMA infusion and bilateral nephrectomy will lead to upregulation of AGXT2 expression and activity in the settings of an acute experiment.

MATERIALS AND METHODS

ADMA infusion in mice
Animal protocols were approved by the ethical committee of the Technical University of Dresden. ADMA (250 µmol × kg⁻¹ × d⁻¹) was infused in C57/BL6 mice (10 mice per group) using osmotic minipumps (Alzet, model 1003D) implanted intraperitoneally (i.p.) as described previously [21]. The control group, five mice that underwent nephrectomy and five mice that underwent the sham procedure, received infusion of saline. The duration of infusion was 3 days. Bilateral nephrectomy was performed 24 h before completion of the infusion.

Bilateral nephrectomy
Mice were anaesthetized using 2% isoflurane in oxygen inhalation. First, a small flank incision was made to prepare the left kidney. After removal of the kidney capsule, the ureter and vessels were ligated and the left kidney was removed followed by sequential wound closure with single button suture. Subsequently, the right kidney was removed in analogue fashion via a right-sided flank incision. All mice survived the surgery.

Collection of urine, plasma and tissues
Twenty-four hour urine was collected in metabolic cages during the last day of ADMA infusion and stored at −80°C. At the last day of the experiment, mice were sacrificed and blood was collected by cardiac puncture into ethylenediaminetetraacetic acid-containing tubes (final concentration 5 mmol/L). Plasma was separated by centrifugation and stored at −80°C. Liver and kidney samples were collected and flash frozen immediately after the sacrifice of the animals and stored at −80°C until further analysis.

Measurement of ADMA, SDMA, DMGV and creatinine in plasma and urine
Plasma and urine levels of ADMA and SDMA were measured by isotope dilution liquid chromatography-tandem mass spectrometry (LC–MS–MS) as described previously [22]. The DMGV levels in plasma were determined also by LC–MS–MS, applying the procedure specific for this analyte [19]. Creatinine was measured by high-performance liquid chromatography as described previously [23].

Real-time polymerase chain reaction
Messenger RNA (mRNA) levels of mouse Agxt2 gene were determined by real-time reverse transcription-polymerase chain reaction (RT–PCR). The RNA isolation from kidney and liver tissue was carried out using the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany). Reversed transcription of the isolated RNA to cDNA was achieved by applying the RevertAid First Strand cDNA Synthesis kit (Fermentas, Fisher Scientific; Schwerte, Germany). The amplification of the cDNA templates for quantification was carried out using the SensiMix SYBR & Fluorescein kit (Bioline; Luckenwalde, Germany). All
kits were applied according to the manufacturer’s instructions. The primer pairs used in the real-time RT-PCR for amplification of the respective cDNA sequences were designed using the Internet tool Primer-BLAST provided by the United States National Center for Biotechnology Information. The selected primer pair sequences for mouse Agxt2 were 5’-cag ata gac cgc ctc tgg cat a-3’ and 5’-GCC ttc tgc act ctt cac c-3’. As housekeeping genes, Rpl13a and β-actin were selected. The respective primer pair sequences were 5’-GCA GCC TGG CCT CTC TTG GTC-3’ and 5’-GGC ttc tga tcc act gtt cac c-3’, and 5’-GCA GCC TGG CCT CTC TTG GTC-3’ and 5’-GGC ttc tga tcc act gtt cac c-3’. These primer pairs are selective for their respective genes and span exon junctions in order not to detect accidentally genomic DNA. The primer oligomers were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

**Western blot**

Preparation of tissue and cell lysates as well as development of immunoblot analysis was performed as described previously [16]. Total protein concentrations were determined using a standard assay ('BCA Protein Assay' Reagent; Rockford, IL) according to manufacturer’s instructions. For immunoblot analysis, tissue homogenates (30–60 µg of total protein) from liver and kidney of mice were prepared and diluted with Laemmli buffer [62 mM Tris–HCl, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 0.01% bromophenol blue and 0.4 mM dithiothreitol]. After incubation at 95°C for 5 min, proteins were separated by SDS-polyacrylamide gel electrophoresis.

![Figure 1](https://example.com/figure1.png) **FIGURE 1:** Plasma (A) and urine (B) levels of ADMA and DMGV after i.p. infusion of ADMA (250 µmol × kg\(^{-1}\) × d\(^{-1}\)) for 3 days using osmotic minipumps in wild-type mice. *P < 0.005 versus saline infusion.

![Figure 2](https://example.com/figure2.png) **FIGURE 2:** Plasma concentration of ADMA, SDMA and DMGV after i.p. infusion of ADMA (250 µmol × kg\(^{-1}\) × d\(^{-1}\)) for 3 days using osmotic minipumps in wild-type mice. Half of the mice underwent bilateral nephrectomy 24 h before the samples collection. *P < 0.05 versus sham, saline infusion, #P < 0.05 versus sham, ADMA infusion, ¶P < 0.05 versus nephrectomy, saline infusion.
under reducing conditions on 10% polyacrylamide gels and transferred to nitrocellulose membranes (Protran Nitrocellulose Transfer Membrane; Whatman, Dassel, Germany) using a tank blotting system from Bio-Rad (Munich, Germany). Membranes were probed with a polyclonal rabbit anti-mouse AGXT2 antibody (Eurogentec; Seraing, Belgium) at a dilution of 1:250 (target amino acid sequence: VQDKISRQPLPK-TEV). After incubation at 4°C overnight, a horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma-Aldrich; Munich, Germany) was used as secondary antibody at a dilution of 1:7500. Immunoreactive bands were visualized using 'ECL Western Blotting Detection Reagents' from Amersham (GE Healthcare; Buckinghamshire, UK) and a 'Molecular Imager ChemiDoc XRS System' (Bio-Rad). To control sample loading, membranes were incubated for 20 min with ‘Restore Western Blot Stripping Buffer’ (Pierce Biotechnology; Rockford, IL) at 37°C and after washing reprobed with a monoclonal mouse anti-human β-actin antibody (Sigma-Aldrich) at a dilution of 1:15 000. As secondary antibody, a horseradish peroxidase-conjugated goat anti-mouse antibody (Dianova; Hamburg, Germany) was used at a dilution of 1:4000.

**Enzyme activity of AGXT2 and DDAH**

The enzyme activity of AGXT2 was measured by adding stable isotope-labelled ADMA as substrate for this enzyme to kidney or liver tissue homogenates, incubating them for 60 min and determining the amount of the enzyme reaction product.

**FIGURE 3:** Liver and kidney tissue concentrations of ADMA, SDMA and DMGV after i.p. infusion of ADMA (250 µmol × kg⁻¹ × d⁻¹) for 3 days using osmotic minipumps in wild-type mice. Half of the mice underwent bilateral nephrectomy 24 h before the samples collection. All values are normalized for tissue wet weight. *P < 0.05 versus sham, saline infusion; †P < 0.05 versus sham, ADMA infusion; ‡P < 0.05 versus nephrectomy, saline infusion.
(i.e. stable isotope-labelled DMGV) by LC–MS-MS. The experimental and analytical details were reported previously [20]. The total DDAH activity (i.e. the combined activities of DDAH 1 and 2) was determined in a similar way as the AGXT2 activity. The preparation of the tissue homogenates and the incubation buffer was identical to the previously described procedure [20]. The substrate for DDAH was D7-ADMA (Cambridge Isotope Laboratories; Andover, MA) in the final concentration of 0.8 mmol/L in the incubation buffer. After incubation for 60 min at 37°C, the reaction was stopped by adding 750 µL acetonitrile to 100 µL of the reaction mixture. After addition of the internal standard 13C6-arginine (Cambridge Isotope Laboratories; 20 µL of a 0.46 mmol/L solution in water), the amount of the product of the enzymatic reaction, D7-citrulline, was determined by LC–MS-MS. The LC–MS-MS procedure was identical to the one for the determination of ADMA and SDMA in plasma [22]. The specific quantification of D7-citrulline was achieved by the observation of the fragment ion m/z 183 → 77. Calibration was carried out using unlabelled citrulline in the concentration range of 2–50 µmol/L.

The activities of AGXT2 and DDAH were expressed in the unit picomole product per minute reaction time per milligram protein (pmol/min/mg). The total protein contents of the tissue lysates were determined using the BCA Protein Assay kit (Pierce Biotechnology).

Statistical analysis
Statistical analysis was performed using SigmaPlot 12.0. Data were assumed to be normally distributed. Comparisons between the groups were done using unpaired Student’s t-test (two groups) or analysis of variance (more than two groups). Statistical significance was defined as a P-value of <0.05. Values are reported as mean ± SE.

RESULTS

Infusion of ADMA using minipumps
We infused ADMA in C57/BL6 mice for 3 days using osmotic minipumps. This approach resulted in an ~3-fold increase in plasma and a 4-fold increase in urine levels of ADMA compared with the control mice, which received saline infusion (Figure 1A and B, P < 0.05). Elevation of ADMA levels coincided with an ~2-fold increase in plasma and a 3-fold increase in urine levels of DMGV (Figure 1A and B, P < 0.05).

Effect of bilateral nephrectomy and ADMA infusion on ADMA, SDMA and DMGV levels in plasma
Bilateral nephrectomy was performed in order to determine the consequence of lack of urinary excretion on plasma levels of ADMA, SDMA and DMGV. The levels of all the three compounds in the liver were increased as a result of nephrectomy both in the mice with saline and ADMA infusion (Figure 3A, C and E) (P < 0.05). ADMA infusion led to an increase in ADMA and DMGV levels in the liver and kidneys compared with the mice with saline infusion (Figure 3A, B, E and F) (P < 0.05); however, it did not change the tissue levels of SDMA (Figure 3C and D).

Effect of bilateral nephrectomy on AGXT2 expression
In order to determine whether ADMA infusion or bilateral nephrectomy leads to a compensatory increase in Agxt2 expression, we assessed the tissue Agxt2 mRNA levels by real-
time PCR and AGXT2 protein levels by western blot. Neither ADMA infusion nor bilateral nephrectomy affected Agxt2 expression in the liver and kidneys (Figures 4 and 5).

Effect of bilateral nephrectomy and ADMA infusion on activity of ADMA-metabolizing enzymes

Because nephrectomy did not cause any changes in AGXT2 mRNA and protein levels, in a next step we looked at the activity of the enzyme. There were no changes caused by nephrectomy in the AGXT2 activity levels in the kidney and in the liver both in the mice with saline and ADMA infusion (Figure 6A and B). Following this observation, we measured the activity of DDAH in the liver and kidney. There were no changes activity of this enzyme as a result of nephrectomy both in the mice with saline and ADMA infusion (Figure 7A and B).

**DISCUSSION**

In this study, we tested the hypothesis that acute elevation of systemic ADMA levels by either ADMA infusion or bilateral nephrectomy would lead to changes in AGXT2 expression or activity. The main observations of our work are (i) infusion of ADMA led to a several-fold increase in DMGV in plasma and urine, (ii) complete loss of renal function for 24 h due to bilateral nephrectomy caused an ∼4-fold increase of plasma SDMA levels, no significant changes in plasma ADMA levels and a dramatic increase in DMGV plasma levels and (iii) neither ADMA infusion nor bilateral nephrectomy led to upregulation of AGXT2 or DDAHs.

AGXT2 was recognized as an ADMA-metabolizing enzyme of >20 years ago [14]. In contrast to the considerable amount of work done on the DDAH pathway of ADMA metabolism, the physiological significance of AGXT2 is still poorly characterized and importance of this pathway in vivo is not widely accepted [24]. In 2010, we showed that overexpression of human AGXT2 in mice leads to a significant decrease in both plasma and tissue ADMA levels [15]. Caplin et al. [17] reported that AGXT2 knockout mice have increased plasma ADMA and SDMA levels, suggesting that endogenous AGXT2 is required for regulation of systemic levels of methylarginines. Consistent with this observation, we have demonstrated that systemic infusion of another substrate of AGXT2 β-aminoisobutyrate also leads to elevation of plasma ADMA and SDMA concentrations, presumably via competitive inhibition of AGXT2 activity towards these methylarginines [16]. Furthermore, we have developed an isotope-based AGXT2 activity assay and detected AGXT2 activity towards ADMA in mouse liver and kidney lysates [20]. The finding from this study that infusion of ADMA leads to a several-fold increase in plasma and urine DMGV levels is another strong evidence for the in vivo relevance of AGXT2 in ADMA metabolism. The observation that AGXT2 is involved in regulation of plasma levels of...
methylarginines indicates that AGXT2 can have an important role in the pathogenesis of cardiovascular disease. This suggestion is supported by our previous results that overexpression of AGXT2 in cultured endothelial cells ameliorates impairment of NO production caused by high levels of ADMA [15]. This hypothesis is also in line with the hypertensive phenotype of the AGXT2-deficient mice reported by Caplin et al. [17]. Furthermore, it is also supported by the potential association between an AGXT2 polymorphism (rs37369) and increase in diastolic blood pressure, reported in the same paper [17].

One of the most intriguing findings of our study is that complete loss of renal function for 24 h did not lead to elevation of plasma ADMA levels in our experimental model. This phenomenon was also reported by the group of Carello [25], who did not observe any elevation of plasma ADMA in the rat model of bilateral nephrectomy. It is also in line with the finding that unilateral nephrectomy in human kidney donors not only did not result in ADMA elevation, but even caused a transient decrease in plasma ADMA levels coinciding with the elevation of inflammatory cytokines [26]. These reports suggest that plasma ADMA concentration is very tightly regulated in vivo, which is consistent with the proposed important physiological role of ADMA. Interestingly, in our study, the plasma levels of DMGV increased to a remarkable extent (>30-fold) in response to bilateral nephrectomy. We speculate that a shift from renal excretion of ADMA to its conversion to DMGV by AGXT2 might have played a major role in protection from elevation of systemic ADMA levels after complete loss of renal function. We do not believe, however, that compensation for complete loss of renal ADMA excretion was carried out entirely by the AGXT2-mediated pathway, because in that case we would have seen unchanged levels of both plasma ADMA and SDMA, whereas in our study only plasma ADMA levels remained unchanged, while plasma SDMA levels were ∼4-fold increased. These data suggest that both DDAH- and AGXT2-mediated pathways of ADMA clearance contributed to the maintenance of ADMA homeostasis in our experimental settings, while only AGXT2-mediated pathway of metabolism contributed to homeostasis of SDMA. Even though bilateral nephrectomy did not lead to upregulation of either DDAH or AGXT2 activity, these metabolic pathways were nevertheless able to compensate for complete loss of renal ADMA excretion. This suggests that DDAH- and AGXT2-mediated pathways of ADMA clearance.
are not saturated at the normal conditions. Unsaturation of the AGXT2-mediated pathway of ADMA catabolism under normal conditions is also suggested by an observed increase in DMGV production in response to ADMA infusion without upregulation of either AGXT2 expression or activity. Our data did not support the initial hypothesis that either ADMA infusion or bilateral nephrectomy would lead to compensatory upregulation of AGXT2 activity. Further studies are needed to determine whether AGXT2 is a constitutively expressed enzyme with constant activity or whether its activity or expression could be modulated by some other stimuli. These studies are essential to better understand the role of AGXT2 in human diseases and to determine whether its activity could be therapeutically modulated.

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CONFLICT OF INTEREST STATEMENT
None declared.

REFERENCES

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