Effects of chronic SDMA infusion on glomerular filtration rate, blood pressure, myocardial function and renal histology in C57BL6/J mice

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Keywords: blood pressure, ejection fraction, glomerular filtration rate, SDMA

ABSTRACT

Background. Symmetrical dimethylarginine (SDMA), the structural isomer of the nitric oxide synthase inhibitor asymmetrical dimethylarginine, has long been regarded as an inert substance. Recent epidemiological and preclinical data suggest that it might be involved in the pathophysiology of renal and cardiovascular diseases. Therefore, we aimed to investigate the effect of chronic SDMA infusion on renal and cardiac function in mice.

Methods. Eight-week-old male C57Bl/6 mice received vehicle-controlled infusion of SDMA (250 µmol/kg/days) for 28 days using osmotic minipumps (n = 24/group). The following parameters were monitored: glomerular filtration rate (GFR; fluoresceinyl thiocarbamoyl-inulin excretion kinetic), cardiac function (echocardiography) and blood pressure (tail cuff). Blood samples for SDMA determination were obtained at baseline, 2 and 4 weeks. Mice were euthanized at 4 weeks to obtain tissue for renal histology.

Results. Chronic SDMA infusion led to a significant increase of SDMA levels from 0.26 ± 0.10 to 3.49 ± 1.66 µmol/L (P < 0.001) at 4 weeks. Despite this SDMA increase, the GFR did not change (1224 ± 351 versus 1017 ± 345 mL/min/g body weight, n.s.) at 4 weeks, when compared with baseline. We did not find any histological changes, particularly no effect on fibrosis or endothelias nitric oxide synthase expression. There was neither an effect of SDMA on systolic blood pressure (106 ± 12 versus 111 ± 18 mmHg, n.s.) nor on ejection fraction (54.2 ± 1.7 versus 58.4 ± 1.9%, n.s.).

Conclusions. Based on our experiments, it seems unlikely that chronically elevated SDMA alone has an effect on renal and cardiac function in otherwise healthy mice. Future studies have to clarify the potential pathophysiological role of SDMA in cardiovascular disease.

INTRODUCTION

In 1970, Kakimoto and Akazawa were the first to isolate and describe symmetrical dimethylarginine (SDMA) and asymmetrical dimethylarginine (ADMA) in human urine [1]. In 1992, Vallance et al. [2] first reported markedly elevated plasma levels of the nitric oxide synthase (NOS) inhibitor ADMA in
chronic haemodialysis patients. While ADMA correlates with traditional and non-traditional cardiovascular risk factors [3] and predicts cardiovascular events and death in selected patient populations [4–6], little attention has been paid to SDMA. SDMA is almost exclusively eliminated via renal excretion [7, 8], explaining the strong correlation between SDMA and different parameters of renal function [9]. Plasma SDMA levels increase in parallel with creatinine and are sometimes even more sensitive to detect renal dysfunction than creatinine itself [10]. This intimate relationship could explain why SDMA has been shown to be related to advanced age [11]. A clinical study in living related kidney donors showed that SDMA is an early indicator of change in the glomerular filtration rate (GFR) in men, comparable with cystatin C [12]. Interestingly, this also holds true for several animal species in which a strong correlation between SDMA and parameters of renal function has been found [13]. In addition to being an excellent marker of renal function, SDMA may indirectly interfere with nitric oxide (NO) synthesis by competing with the L-arginine uptake into cells as well as with the tubular re-absorption of L-arginine in the kidney. So far, there are only a few studies pointing to a clinical relevance of SDMA beyond being a marker of renal function. In 97 patients with coronary artery disease, SDMA correlated tightly with the coronary calcification score [14]. SDMA is strongly associated with adverse clinical outcome during the first 30 days after ischaemic stroke and also independently with total long-term (7.4 years) mortality after acute stroke, even irrespective of renal function [15, 16]. The potential pathophysiological mechanism by which SDMA might be involved in vascular pathology is not understood. Schepers et al. [17], however, showed in vitro that it increases the production of reactive oxygen species via store-operated calcium influx in monocytes and, thus, is a trigger for endothelial dysfunction. This hypothesis could very recently be supported by clinical findings showing that the inflammatory process of chronic kidney disease (CKD), activating NF-κB and resulting in enhanced expression of IL-6 and TNF-α, are related to high SDMA levels [18]. Hence, SDMA could not only be of clinical significance as an independent cardiovascular risk factor, but might play a pathophysiological role in this process. In the first long-term study infusing SDMA, we aimed to elucidate whether infusion of SDMA in mice could have any physiological effects on the cardiovascular or renal system by triggering endothelial dysfunction via inflammatory processes. Our second aim was to investigate the potential effect of chronic elevated SDMA levels on GFR, to better assess its potential limitations as a marker of renal function. Lastly, we wanted to investigate whether chronic elevated SDMA levels would alter the plasma levels of ADMA and ADMA excretion.

**Materials and Methods**

All applicable institutional and governmental regulations concerning the ethical use of animals were followed during this research. All research and animal care procedures were approved by the Lower Saxony district government (Oldenburg, Germany; ref. no.: 33.9-42502-04-08/1608).

Eight-week-old male C57Bl6/J mice with a mean body-weight of 24 g were purchased from Charles River Laboratories (Sulzbach, Germany) and received SDMA (250 µmol/kg/d; n = 24) or NaCl 0.9% (n = 24) via implanted osmotic minipumps (Alzet, Cupertino, CA) over 4 weeks. We collected blood samples to measure the SDMA levels at baseline, after 2 and 4 weeks.

All species were fed with standard nutrition for laboratory animals. The systolic blood pressure measurements and the echocardiography were performed at baseline, after 2 and 4 weeks. The measurement of the GFR was done before the implantation of the minipumps and at the end of our study.

**Blood pressure measurement**

Systolic blood pressure from the C57Bl6/J mice was monitored by the tail cuff technique with the aid of a computerized system (BP 2000 Blood Pressure Analysis System, Visitech System, Apex, NC). Measurements were performed at the baseline, after 2 and 4 weeks of SDMA infusion with previous 3 days of training. On each day of blood pressure determination, 10 measurements were obtained and averaged for each mouse. All animals were tested by an investigator blinded to the treatment of the animals.

**Echocardiography**

Echocardiography was performed in mice anesthetized with 1.5% isoflurane by inhalation as previously described [19]. In brief, short-axis M-mode images were recorded at the papillary muscle level using the VisualSonic 707 Imaging System (Toronto, Canada) with the cardiovascular scan head (Model RMV 707B). Ejection fraction (EF) was calculated as follows: 

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EF = \frac{\text{end-diastolic}}{\text{end-systolic}} \times 100 \%
\]

All animals were tested by investigator blinded to the treatment of the animals.

**GFR measurement**

The GFR was measured by single-injection fluoresceinyl thiocarbamoyl (FITC)-inulin clearance as described by Qi et al. [20], modified to minimize plasma volume. FITC-inulin (5%), dialysed overnight against 0.9% NaCl (final concentration approximately 3%), was injected at 3.7 µL/g body weight into the left retro-orbital plexus during brief isoflurane anaesthesia (recovery within approximately 20 s). At 3, 7, 10, 15, 35, 55 and 75 min, the mice were placed in a restrainer, the tail vein was punctured with a 30-G atraumatic needle, and approximately 2 µL blood was collected by capillarity into heparinized 10 µL microcaps (Eppendorf, Hamburg, Germany). A total of 500 nL plasma was diluted 1:10 in 500 mmol HEPES (pH 7.4) and measured against a standard curve (1 µL of approximately 3% FITC-inulin diluted 1:50, 1:100 and 1:500 in 500 mmol HEPES). Fluorescence was determined in 2 µL in a Nanodrop-ND-3300 spectrometer (Nanodrop Technologies, Wilmington, DE). The GFR was calculated using a two-compartment model of two-phase exponential decay [20]. After every 75 min, the mice got a brief isoflurane anaesthesia again to get approximately 30 µL of blood from the right
retro-orbital plexus for measuring SDMA and ADMA. All animals were tested by investigator blinded to the treatment of the animals.

**Histology and immunohistochemistry**

The kidneys were perfused with 3% paraformaldehyde in 0.1 M Soerensen’s phosphate buffer. Further, it was fixed for an additional 20 h in 3% paraformaldehyde in Soerensen’s phosphate buffer, then embedded in paraffin and sectioned at 2 μm. For morphological analysis it was stained with Masson-Goldner using a standard procedure. Indirect immunofluorescence for the expression of endothelial nitric oxide synthase (eNOS) was performed with a rabbit polyclonal anti-eNOS antibody (ABR, Affinity Bio reagents, CO). Paraffin-embedded sections were dewaxed, dehydrated and antigen-demasked with 0.05% trypsin. Non-specific binding sites were blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, PA) for 30 min in room temperature. Thereafter, sections were incubated with the primary antibody for 1 h. All incubations were performed in a humid chamber at room temperature. For fluorescent visualization of bound primary antibody, sections were further incubated with Cy3-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories) for 1 h in the dark. Specimens were analysed using a Zeiss Axioplan-2 imaging microscope with the digital image-processing programme AxioVision 4.3 (Zeiss, Jena, Germany).

**Measurements and calculation**

Plasma concentrations of SDMA and ADMA were measured applying a recently developed liquid chromatography–mass spectrometry method described elsewhere [21]. All other measurements were done with routine laboratory tests using certified assay methods.

**Statistical analysis**

We used GraphPad Prism 5 for statistical analysis. The normality of data distribution was confirmed with the Shapiro-Wilk test. Analysis of variance and t-tests as well as Turkey’s multiple comparison test were used. The significance level was set at P < 0.05.

**RESULTS**

Chronic SDMA infusion led to a significant increase of SDMA levels from 0.26 ± 0.10 to 3.49 ± 1.66 μmol/L (P < 0.001) at 4 weeks (Figure 1). ADMA levels remained unchanged in both groups (Figure 1). Urinary SDMA concentration at 2 weeks showed a higher SDMA level in the SDMA group (309.45 ± 163.78 μmol/L) when compared with the control group (31.42 ± 6.42 μmol/L) (P < 0.0001). In contrast to SDMA, the urine levels of ADMA did not differ between both groups.

In the SDMA group, mean systolic blood pressure after 4 weeks was 111 ± 18 mmHg and did not change significantly compared with baseline (106 ± 12 mmHg; Figure 2). The same holds true for the control group.

Although ejection fraction tended to increase after 4 weeks of SDMA administration (54.2 ± 1.7 versus 58.4 ± 1.9%), this difference did not reach the level of significance (Figure 3).
Interestingly, the left ventricular endsystolic diameter (LVESD) did change significantly in the SDMA group from 2.7 ± 0.3 to 3.9 ± 0.2 mm after 4 weeks compared with baseline, whereas it did not change in the control group.

Despite the 10-fold increase in SDMA serum concentration, there was no effect on GFR detectable after 4 weeks when compared with baseline parameters (1224 ± 351 versus 1017 ± 345 µL/min/g body weight; Figure 4. Renal histology revealed neither a difference in expression of eNOS (Figure 5) nor a difference in fibrosis (Figure 6).

**DISCUSSION**

The pertinent finding in our study was that chronically elevated SDMA in healthy mice had neither an effect on renal function and renal histology nor on blood pressure and cardiac function, even though the SDMA levels were increased by an order of magnitude, i.e. they were comparable with patients in CKD5D [11].

**SDMA and the kidney**

Several studies have shown a tight correlation between SDMA and several parameters of renal function. The finding that SDMA had no effect on renal function is not surprising, as SDMA seems to be almost exclusively eliminated by renal excretion. Indeed, SDMA infusion leads to a dramatic increase in urinary SDMA concentration. At least in theory, SDMA could have indirectly interfered with renal function by inhibiting L-arginine uptake. Yet even using the gold standard of GFR measurement in mice, FITC-inulin clearance, we could not detect even minor changes.

Chronic ADMA infusion had been shown to cause microvascular fibrosis in the heart [22]. SDMA did not lead to any changes in renal histology. We particularly investigated eNOS as SDMA can interfere with NO synthesis—at least in vitro [23, 24]. Our data suggest that, in healthy animals, even long-term
elevated SDMA levels neither lead to changes in renal function nor in renal pathology, strengthening its role as a marker of renal impairment that plays no pathophysiological role.

**SDMA and the systemic circulation**

SDMA has been shown *in vitro* to inhibit NO synthesis indirectly [23, 24]. Despite the SDMA increase of more than an order of magnitude that was documented over the 4-week infusion period, we did not see any effect on blood pressure and cardiac function except a significant increase of the heart rate. There are several explanations for this finding.

SDMA was not high enough to inhibit NO production in the presence of stable (and increasing) L-arginine concentrations. Although one could have speculated that SDMA might lead to an increase in ADMA by interfering with its excretion via cationic transporters [25], we did not see any significant changes of ADMA during the time of observation. Blood pressure could have been stable in the presence of increased peripheral resistance due to a decrease in cardiac output. However, we did not find any echocardiographic evidence for decreased cardiac function. Although the increased LVESD would rather point to a systolic dysfunction in the SDMA treated group, the overall ejection fraction was not significantly affected.

**Limitations of our study**

Our results by no means exclude the possibility that SDMA might be involved in vascular and renal pathology. Maybe the absence of confounding factors like inflammation, decreased renal function or hypertension abrogated the potential effect of SDMA. We have chosen a relatively robust mouse strain with a healthy and young species. The observation period might also have been too short. It is possible that we could have obtained more reliable results after 6 or 8 weeks of SDMA infusion, yet ADMA infusion shows pathophysiological effects already after a short period of time.

**ACKNOWLEDGEMENTS**

H.V. is supported by the StrucMed programme of the Medical School Hannover.

**FUNDING**

J.T.K. is supported by a grant of the Else-Kröner-Fresenius Foundation (P63/06/EKMS 06/03).

**CONFLICT OF INTEREST STATEMENT**

Dr Kielstein owns and hosts the website www.adma.com.

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Toll-like receptor 3 ligand, polyIC, induces proteinuria and glomerular CD80, and increases urinary CD80 in mice

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Keywords: animal models, CD80, minimal change disease, nephrotic syndrome, proteinuria

ABSTRACT

Background. We have reported that children with biopsy-proven minimal change disease (MCD) express CD80 (also known as B7.1) in their podocytes and excrete high levels of CD80 in their urine during active nephrotic syndrome. We also reported that polyIC, a Toll-like receptor 3 ligand, increases CD80 mRNA and protein expression in cultured...