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Symmetric dimethylarginine alters endothelial nitric oxide activity in glomerular endothelial cells

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ABSTRACT

Circulating symmetric dimethylarginine (SDMA) is increased in patients with chronic kidney disease. SDMA is considered an inert metabolite, but because it can transported into cells, we studied the effect of SDMA on glomerular endothelial cells. SDMA suppressed VEGF-induced endothelial nitric oxide synthase (eNOS) phosphorylation and nitric oxide production, but not VEGFR2 activation and signaling leading to eNOS activation. SDMA caused eNOS uncoupling and increased superoxide anion production in response to VEGF. All these effects were blocked by preventing cellular uptake of SDMA with a molar excess of arginine. These data show that SDMA interferes with nitric oxide production by uncoupling eNOS and leads to oxidative stress in glomerular endothelial cells.

In conclusion, our data show that SDMA is not an inert metabolite and that it could contribute to oxidative stress in the renal endothelium.

1. Introduction

Arginine methylation (asymmetric and symmetric) is a common post-translational modification of intracellular proteins that alters their localization and/or function [1]. Unlike other post-translational modifications, arginine methylation is not reversed by demethylases. The proteolysis of arginine methylated proteins yields asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) [1]. ADMA, but not SDMA, undergoes hydrolytic degradation to citrulline and dimethylamine by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) [2]. SDMA is not metabolized and is entirely excreted in the circulation [2].

Plasma levels of SDMA is elevated in patients with kidney diseases and correlates strongly with glomerular filtration rate [3]. In addition, plasma SDMA is associated with increased mortality in the general population [4], although the mechanism of this association is unclear.

Unlike ADMA, SDMA is not a direct inhibitor of nitric oxide synthase (NOS) [5] and has been considered an inert metabolite. SDMA is however taken up by cells by the same transport mechanism that transports circulating arginine into cells [6]. Arginine and arginine metabolizing enzymes, such as NOS, seem to have distinct subcellular localization [7]. Consequently, arginine concentration could be the limiting factor for nitric oxide (NO) synthesis, and it appears that it is arginine imported from the circulating pool that serves as a substrate for NO synthesis [8].

It has been hypothesized that SDMA could indirectly affect NOS activity by interfering with arginine uptake [9].

We tested this hypothesis in glomerular endothelial cells and found that not only did SDMA inhibit endothelial NOS (eNOS) by competing with arginine uptake via the cationic amino acid transporter CAT1 but also converted eNOS to its "uncoupled" state that produces superoxide anion.

Our data suggest that SDMA is not an insert metabolite that is merely excreted by the kidney but could cause oxidative stress in the renal vasculature. Our data could also provide an explanation for the correlation with high circulating SDMA and mortality in the general population.

2. Materials and methods

2.1. Glomerular endothelial cells

Glomerular endothelial cells isolated from tsA58 immorto mice were kindly provided by Dr. N. Madaio (Medical College of Georgia, Augusta, GA) [10]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5 mM glucose, 25% Nutrient Mixture F-12 HAM, and 10% fetal bovine serum. Seventy percent confluent cells were serum-starved for 24 h before experiments were performed.

2.2. Nitric oxide

Nitric oxide was measured in 50 μl of conditioned medium using a kit from Calbiochem.

Abbreviations: SDMA, symmetric dimethylarginine; CAT1, cationic amino-acid transporter-1; VEGF, vascular endothelial growth factor; eNOS, endothelial nitric oxide; ROS, reactive oxygen species.

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2.3. Immunoblots

Immunoblots were performed as previously described [11,12]. Briefly, 10–20 μg of whole-cell lysates were separated on SDS–PAGE, transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen) and probed with various primary antibodies, and IRDye800- or IRDye700-coupled secondary antibodies were used for detection using Odyssey Infrared Imaging System (LiCor Biosciences, Lincoln, NE).

2.4. Assay of eNOS dimer/monomer

SDS-resistant eNOS dimers and monomers were assayed by using low temperature SDS–PAGE as described previously [13–15]. Briefly, after being washed twice with ice-cold phosphate-buffered saline, cells were lysed as described above and protein lysates were mixed with loading buffer and loaded on gels without boiling. Proteins were separated with low temperature SDS–PAGE under reducing conditions (with β-mercaptoethanol). Gels and buffers were kept at 4 °C during the whole procedure.

2.5. Detection of intracellular ROS in MCs

ROS generation was also assessed in live cells with DHE (Invitrogen/Molecular Probes) as previously described [14–16]. Cells were loaded with 10 μM DHE in phenol-free DMEM for 30 min at 37 °C. Cells were washed with warm buffer. DHE fluorescent intensity was determined at 520-nm excitation and 610-nm emission and visualized on Olympus FV-500 confocal laser scanning microscope. The brightness intensity of DHE signal was semi-quantified by using either the Image-Pro Plus 4.5 software (Media Cybernetics) or NIH Image/ImageJ software as described [14,15]. The data shown represent three separate experiments, and are expressed as relative fluorescence intensity.

2.6. Statistics

Data from a minimum of 3 experiments were expressed as mean ± SEM and analyzed by ANOVA for comparison among multiple groups using the Tukey post-test analysis (GraphPad Prizm®) or by Student's *t*-test; $p < 0.05$ was considered significant.

3. Results

3.1. SDMA reduces VEGF-induced nitric oxide synthesis

Quiescent glomerular endothelial cells were treated with 20 ng/ml of VEGF for up to 30 min, and NO synthesis was measured as nitrite/nitrate ratio. Fig. 1 shows that VEGF stimulates NO synthesis in a time-dependent manner, confirming our previously published data [17]. Co-incubation with 100 nM SDMA, a dose that is compatible with circulating levels in patients with chronic kidney disease [18], completely abolished VEGF-induced NO synthesis.

3.2. SDMA does not affect VEGF-R2 activation

We have previously shown that VEGF-induced NO synthesis in glomerular endothelial cells required VEGF-R2-dependent Akt activation and Akt-dependent phosphorylation of eNOS on Ser1177 [17]. VEGF-R2 activation, assessed by autophosphorylation on Tyr1054, was increased by VEGF treatment and not affected by SDMA (Fig. 2A). VEGF increased Akt phosphorylation on Ser473, that is required for its activity, in a time-dependent manner; SDMA co-incubation did not inhibit VEGF-induced Akt activation (Fig. 2B). eNOS activation was assessed by its phosphorylation on Ser1177: Fig. 2C shows that co-incubation with SDMA inhibited VEGF-induced eNOS activation. Since Ser1177 phosphorylation is a direct target of Akt and SDMA did not affect Akt activation induced by VEGF, our data suggest that SDMA does not affect

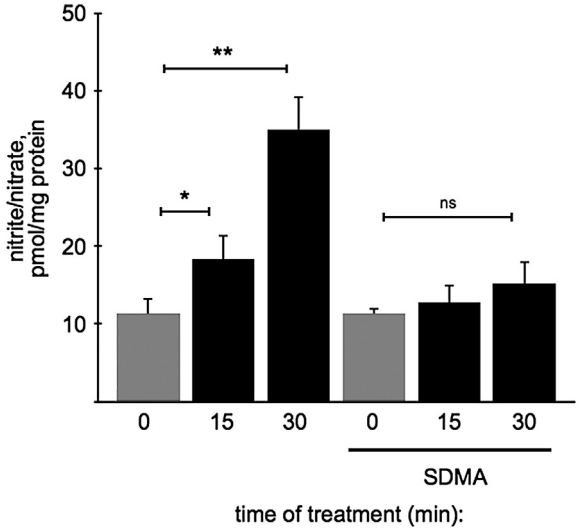

Fig. 1. Nitric oxide synthesis in GEndos treated with VEGF and SDMA. NO was measured on the conditioned medium from GEndos treated with VEGF for the indicated times. Where indicated, the cells were simultaneously treated with VEGF and SDMA. Histogram represents mean ± SEM from 3 independent experiments. $^{*}p < 0.05$ and $^{**}p < 0.01$ vs untreated cells by ANOVA; ns: not significant.

VEGF signaling but somehow alters eNOS structure that prevents its phosphorylation by Akt.

3.3. SDMA reduces arginine uptake by glomerular endothelial cells

Nitric oxide synthesis by eNOS requires arginine uptake from the circulating pool [8,19]. We tested the possibility that SDMA interferes with arginine uptake in glomerular endothelial cells, by competing for

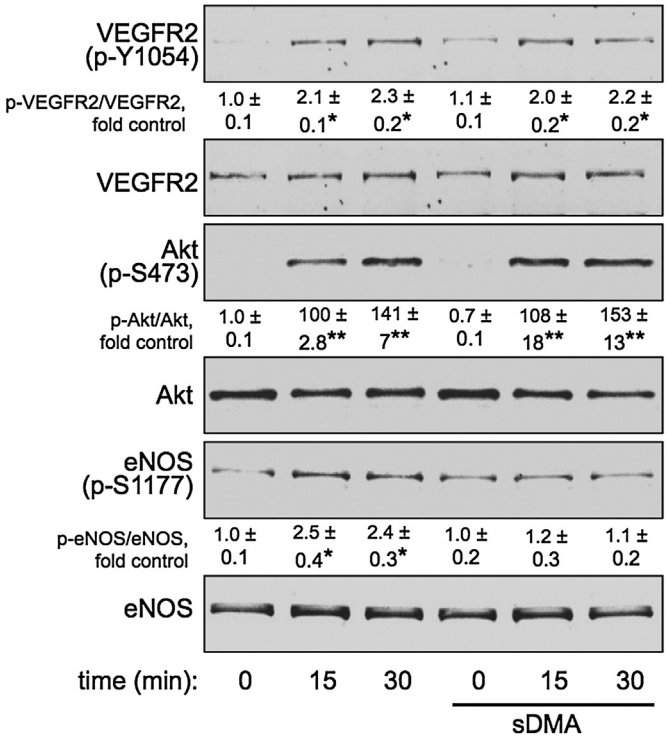

Fig. 2. Signaling pathway leading to eNOS activation in GEndos treated with VEGF and SDMA. Where indicated, the cells were simultaneously treated with VEGF and SDMA. Immunoblots are representative of 3 independent experiments. Numbers between blots represent the result of the densitometric analysis, expressed as fold control (untreated cells). $^{*}p < 0.05$ and $^{**}p < 0.01$ vs untreated cells by ANOVA.