*Am J Physiol Heart Circ Physiol* 294: H1058–H1066, 2008.
First published December 21, 2007; doi:10.1152/ajpheart.01103.2007.

# Role of asymmetric dimethylarginine for angiotensin II-induced target organ damage in mice

**Johannes Jacobi,[1] Renke Maas,[2] Nada Cordasic,[1] Kilian Koch,[1] Roland E. Schmieder,[1] Rainer H. Böger,[2] and Karl F. Hilgers[1]**

[1]*Department of Nephrology and Hypertension, Friedrich Alexander University Erlangen-Nuremberg, Erlangen; and* [2]*Clinical Pharmacology Unit, Institute for Experimental and Clinical Pharmacology and Toxicology, University Hospital Hamburg-Eppendorf, Hamburg, Germany*

Submitted 22 September 2007; accepted in final form 19 December 2007

**Jacobi J, Maas R, Cordasic N, Koch K, Schmieder RE, Böger RH, Hilgers KF.** Role of asymmetric dimethylarginine for angiotensin II-induced target organ damage in mice. *Am J Physiol Heart Circ Physiol* 294: H1058–H1066, 2008. First published December 21, 2007; doi:10.1152/ajpheart.01103.2007.—The aim of the present study was to investigate the role of the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine (ADMA) and its degrading enzyme dimethylarginine dimethylaminohydrolase (DDAH) in angiotensin II (ANG II)-induced hypertension and target organ damage in mice. Mice transgenic for the human DDAH1 gene (TG) and wild-type (WT) mice (each, $n = 28$) were treated with 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, or phosphate-buffered saline over 4 wk via osmotic minipumps. Blood pressure, as measured by tail cuff, was elevated to the same degree in TG and WT mice. Plasma levels of ADMA were lower in TG than WT mice and were not affected after 4 wk by either dose of ANG II in both TG and WT animals. Oxidative stress within the wall of the aorta, measured by fluorescence microscopy using the dye dihydroethidium, was significantly reduced in TG mice. ANG II-induced glomerulosclerosis was similar between WT and TG mice, whereas renal interstitial fibrosis was significantly reduced in TG compared with WT animals. Renal mRNA expression of protein arginine methyltransferase (PRMT)1 and DDAH2 increased during the infusion of ANG II, whereas PRMT3 and endogenous mouse DDAH1 expression remained unaltered. Chronic infusion of ANG II in mice has no effect on the plasma levels of ADMA after 4 wk. However, an overexpression of DDAH1 alleviates ANG II-induced renal interstitial fibrosis and vascular oxidative stress, suggesting a blood pressure-independent effect of ADMA on ANG II-induced target organ damage.

dimethylarginine dimethylaminohydrolase; hypertension; transgenic

ASYMMETRIC DIMETHYLARGININE (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), is increasingly recognized as a potential risk factor and prognostic biomarker in cardiovascular disease (38). Thus far, elevated ADMA levels have been associated with all established cardiovascular risk factors (38). Furthermore, recent data from genetic mouse models indicate a causal role for ADMA in the pathophysiology of vascular disease (7, 19). ADMA derives from the posttranslational methylation of L-arginine residues within proteins catalyzed by enzymes called protein arginine methyltransferases (PRMTs). Upon proteolysis, methylarginines are released into the circulation. Although ~15% of ADMA are excreted via the urine, the major elimination occurs through enzymatic degradation (~85%) (1). The enzyme dimethylarginine dimethylaminohydrolase (DDAH), of which two isoforms with distinct tissue distribution have been described, catalyzes the hydrolysis of ADMA to L-citrulline and dimethylamine (38).

Thus far, clinical studies have mostly addressed the prognostic value of ADMA in cardiovascular or kidney disease as well as the impact of pharmacological interventions aimed at lowering ADMA levels under such conditions. Pharmacological agents that have been tested for their ability to modify ADMA levels in humans include drugs that interfere with the renin-angiotensin system (RAS) by either blocking the formation [angiotensin-converting enzyme (ACE) inhibitors] or pharmacological action [angiotensin receptor blockers (ARBs)] of angiotensin II (ANG II). Although some investigators found significant reductions of ADMA levels in patients treated with RAS-blocking agents (2, 4, 9, 15, 16, 27, 42), other studies have failed to confirm these findings (10, 40). Sound interpretation of the aforementioned clinical trials is complicated due to differences in study design, study cohorts, treatment regimen, and the use of different bioanalytical methods to determine ADMA levels. Recent in vitro studies indicate that short-term incubation (24 h) of human umbilical vein endothelial cells with ANG II (1 $\mu$M) more than doubles ADMA levels in the conditioned medium, whereas coincubation with either losartan or L-arginine almost completely abolished these effects (5).

Upon completion of our studies, Hasegawa et al. (13) reported that ANG II infusions in wild-type (WT) mice (1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ over 2 wk) elicited a 100% increase in plasma ADMA levels, whereas in DDAH2 transgenic mice this increase was only ~50%.

Here we investigated the effects of ANG II infusion on ADMA levels in vivo in WT animals and DDAH1 transgenic (TG) mice that overexpress the human isoform of this enzyme (7). We hypothesized that ANG II-induced hypertension and target organ damage are associated with an elevation of plasma ADMA levels and that, if this holds true, mice overexpressing DDAH should be, in part, protected from ANG II-induced target organ damage.

## METHODS

### *Animals*

DDAH1 TG mice (C57Bl/6J background) were kindly provided by John Cooke (Dept. of Cardiovascular Medicine, Stanford University). After acclimatization, animals were transferred to our transgenic core facility to rederive the colony using embryo transfer. Offsprings were

Address for reprint requests and other correspondence: K. F. Hilgers, Nephrologische Forschungslabors, Loschgestrasse 8, Erlangen D-91054, Germany (e-mail: karl.hilgers@uk-erlangen.de).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

genotyped by PCR of DNA obtained from tail-snip biopsies using transgene-specific oligonucleotide primers as described earlier (7).

All experiments were conducted in male, 4-mo-old, heterozygous DDAH1 TG mice, and age and weight matched WT littermates housed in a temperature-controlled animal facility with a 12-h:12-h light-dark cycle and free access to tap water and rodent chow. The study protocol was approved by the Animal Research Ethics Committee of the local government (Bezirksregierung Mittelfranken, AZ 54-2531.31-1/06).

*ANG II Infusion*

ANG II (Bachem) at a dose of 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (each, $n = 13$) was delivered over 4 wk via unprimed osmotic minipumps (model 2004; Alzet) that were subcutaneously implanted into the back of WT or TG mice. The dose of 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II is most commonly used in rodents and causes relevant elevations of blood pressure as measured by tail-cuff plethysmography or telemetric devices (13, 41).

Based on our initial results, we decided to also study a higher dose of ANG II, namely 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (WT, $n = 9$; and TG, $n = 10$). For control experiments, WT or TG mice were treated with phosphate-buffered saline (PBS) solution delivered via osmotic minipumps (each, $n = 7$).

*Blood Pressure Measurements*

Noninvasive blood pressure was measured by tail-cuff plethysmography (TSE 209000; TSE Systems) as previously described (11). Briefly, all animals were trained on alternate days over a period of 2 wk to get accustomed to the device. Final measurements were performed before pump implantation and on *days 2*, *3*, *7*, *14*, *21*, and *28* after surgery. A total of 20 consecutive readings of systolic blood pressure (SBP) and heart rate were recorded and averaged.

*Metabolic Cages*

Before pump implantation and 3 days before being euthanized, animals were housed in metabolic cages over 24 h for urine collection. Body weight, food intake, water consumption, and urine output were monitored. Albuminuria was measured and expressed as urinary albumine excretion per gram creatinine.

*Hormone Measurements*

Plasma levels of aldosterone were measured by radioimmunoassay as previously described in detail (14). Samples were run in duplicate, and mean values were computed.

*Measurement of ADMA*

Plasma ADMA levels were measured with an enzyme-linked immunosorbent assay (DLD Diagnostika) (32). Optical density was measured at 450 nm using a microtiter plate reader (Tecan Sunrise). All samples were measured in duplicate, and mean values were computed. The intraassay coefficient of variation for ADMA was 8.7%. In mice treated with 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, ADMA levels were measured before and after treatment both by ELISA as well as by liquid chromatography-tandem mass spectrometry (LC-MS) as described elsewhere (33). The interassay accuracy and precision for the determination of ADMA, symmetric dimethlyarginine (SDMA), and L-arginine in plasma using spiked and native samples were in the range of −0.6–1.8% and −4.4–4.8%, respectively, for all analytes.

Baseline blood samples were obtained via retroorbital phlebotomy under inhalation anesthesia with 2% isoflurane, and at death animals were exsanguinated via arterial lines placed in the carotid artery under inhalation anesthesia as described earlier (11). To avoid the possible degradation of ADMA through blood cell components, all blood samples were immediately centrifuged and plasma was stored at −20°C until further processed.

*Histomorphology and Immunohistochemistry*

The degree of glomerulosclerosis was determined in methylcarnoy-fixed periodic acid Schiff (PAS)-stained paraffin sections (2 μm) using a previously described semiquantitative scoring system ranging from 0 to 4 (12). Scoring was performed in a blinded fashion, and a total of 100 glomeruli were analyzed. Based on only mild signs of glomerulosclerosis in ANG II-treated animals (*score 0* to *2*), the results were expressed as percentages of injured glomeruli. The expansion of interstitial collagen I (1:1,000; Biogenesis) was measured using a Leitz Aristoplan microscope (Leica) equipped with an integrated ocular grid (10 × 10 fields). A positive staining within 100 fields was counted in 20 nonoverlapping cortical views excluding glomeruli and expressed as a percentage. Glomerular collagen IV staining (1:500; Southern Biotechnology) was measured using image analysis software (MetaVue version 4.6r9; Molecular Devices) in 30 randomly chosen glomeruli per cross section. The stained area was expressed relative to the total area of the glomerular tuft. Macrophage infiltration (F4/80, clone CI:A3-1, 1:100; Serotec) within kidneys (cortical fields) was analyzed using light microscopy and immunofluorescence, and heat-induced antigen retrieval in citrate buffer was used before incubation. A biotinylated (light microscopy) or Alexa 555-labeled (immunofluorescence) goat anti-rat IgG was used as secondary antibody. Macrophages were counted in 20 randomly chosen high-power fields.

Frozen cross sections of the descending aorta were incubated with monocyte/macrophage antibody (MOMA)-2 antibody (BMA Biomedicals), followed by Alexa 555-labeled anti-rat IgG secondary antibody (Molecular Probes). Sections of the descending aorta were also probed with a polyclonal inducible NOS (iNOS) antibody (Lab-Vision), followed by incubation with a biotinylated secondary antibody and subsequent development using a streptavidin horseradish peroxidase and diaminobenzidine detection kit (Linaris). Controls included the omission of primary antibodies. The area-based wall-to-lumen ratio was determined in PAS-stained cross sections of the descending aorta using image analysis software (MetaVue).

*Oxidative Fluorescence Microscopy*

In situ production of reactive oxygen species (ROS) was assessed by fluorescence microscopy using the fluorescent dye dihydroethidium (DHE; Molecular Probes). In the presence of superoxide, DHE is rapidly oxidized to ethidium bromide, which gets trapped by intercalation with DNA. Ethidium bromide is excited at 488 nm with an emission spectrum of 610 nm. Unfixed frozen tissue sections of the ascending and descending aorta (10 μm) were incubated with DHE (8 μM) in a humidified, light-protected chamber at 37°C for 1 h. In some studies, slides were preincubated with 200 U/ml polyethylene glycol-SOD (Sigma) to verify specificity of the fluorescent dye. Images were taken by use of a Spot RT digital camera (Diagnostic Instruments) that was connected to a fluorescence microscope (Nikon Eclipse 80i; Nikon). Identical image acquisition setup was used for all slides. Fluorescence signal intensities were analyzed using image analysis software (MetaVue). To account for autofluorescence, images were thresholded and fluorescent pixels were expressed relative to the pixel count of the entire vessel circumference.

*Real-Time RT-PCR Detection of mRNA*

In mice infused with PBS or 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, the RNA of renal cortical tissue was extracted with TriFast reagent (Peqlab) (6). First-strand cDNA was synthesized with TaqMan RT reagents (Applied Biosystems) using random hexamers. Polymerase chain reaction was performed with an ABI Prism 7000 sequence detector and SYBR green reagents (Applied Biosystems) according to the manufacturer's

instructions. All samples were run in duplicate. The amount of the specific mRNA of interest was normalized to 18S rRNA and expressed as relative copies. Dissociation curves were performed to confirm the specificity of the polymerase chain reaction. Primers were designed using Primer Express software (version 2.0), and selected sequences were subjected to the National Center for Biotechnical Information basic local alignment search tool (BLAST) search. Primer sequences for mouse DDAH1, DDAH2, PRMT1, PRMT3, and collagen I were as follows: DDAH1, forward, CATGTCTTGCTGCACCGAAC, and reverse, GACCTTTGCGCTTTCTGG; DDAH2, forward, GGTTGATGGAGTGCGTAAAGC, and reverse, TCCACAATTCGGAGTCCCAA; PRMT1, forward, AATGGGATGAGCCTCCAGC, and reverse, TGCTTGGCCACAGGAAACTT; PRMT3, forward, TTACCCTGAGAACCACAAAGACG, and reverse, AGTACCCAGCAACTGCCGTG; and collagen I, forward, TCACCTACAGCACCCTTGTGG, and reverse, CCCAAGTTCCGGTGTGACTC. The DDAH1 primers were designed to recognize the endogenous mouse DDAH1 sequence but not the human DDAH1 transgene.

### *Statistical Analysis*

Statistical analysis was performed by use of a SPSS software package (version 14.0). Figures and results are given as means ± SE; n equals the number of animals. For boxplot figures, the lower and upper bounds of the boxes indicate the 25th and 75th percentile values, respectively, and the horizontal lines indicate the 50% percentile (i.e., the median). Pearson correlation coefficients were calculated when indicated.

For statistical analysis, a general linear univariate model was applied using genotype (WT vs. TG) and treatment (PBS vs. 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II vs. 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II) as fixed factors. The Bonferroni post hoc test was used to account for multiple comparisons. Statistical significance was accepted at a treatment- or genotype-related $P$ value of $P < 0.05$.

## RESULTS

### *Animals*

Three animals treated with ANG II ($n = 1$ WT in the 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II group, and $n = 2$ TG in the 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II group) died following pump implantation. The WT animal died 2 wk after pump implantation, and necropsy revealed no major abnormalities, i.e., no signs of infection or infarction. The two TG mice died 4 and 6 days after the initiation of drug infusion, and necropsy revealed intrathoracic hemorrhage. In PBS-treated mice, a significant increase in body weight (corrected for osmotic minipump) was observed [WT, 2.4 ± 0.4 g, $P = 0.001$; and TG, 1.2 ± 0.4 g, $P = 0.024$; WT vs. TG, $P$ = not significant (NS)], whereas the infusion of ANG II was associated with weight loss (1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II WT, −1.9 ± 0.6 g, $P = 0.013$; TG, −0.6 ± 0.6 g, $P$ = NS; WT vs. TG, $P$ = NS; 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II WT, −2.4 ± 0.4 g, $P = 0.0003$; TG, −2.3 ± 0.6 g, $P = 0.005$; WT vs. TG, $P$ = NS).

### *Systemic Effects of ANG II Infusion*

*Blood pressure and heart rate.* Baseline tail-cuff SBP did not differ between WT and TG mice (119.6 ± 2.6 vs. 113.2 ± 1.9 mmHg, each $n = 28$, $P$ = NS). Infusion of ANG II at a dose of 1.0 or 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ caused a rapid, sustained increase of blood pressure to a similar extent in both WT and TG mice (Fig. 1). Interestingly, neither SBP before death ($r = 0.016$, $P$ = NS) nor the increase in SBP from baseline ($r = -0.17$, $P$ = NS) correlated with plasma ADMA levels.

Treatment with ANG II was associated with a decrease in heart rate, and this effect was significant after 2 wk of treatment (WT mice, PBS vs. 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II vs. 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, 646 ± 13 vs. 537 ± 15 vs. 530 ± 19 beats/min, $P < 0.001$; and TG mice, PBS vs. 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II vs. 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, 629 ± 15 vs. 574 ± 14 vs. 541 ± 31 beats/min, $P < 0.001$). All heart rate data obtained during the entire period of ANG II infusion were evaluated together in a repeated-measures analysis, and significant treatment ($P = 0.003$) and genotype ($P = 0.013$) effects were observed.

*Metabolic cages.* Baseline urine output (48 ± 3 $\mu l \cdot g^{-1} \cdot day^{-1}$, $n = 56$) and albuminuria (195 ± 13 mg/g

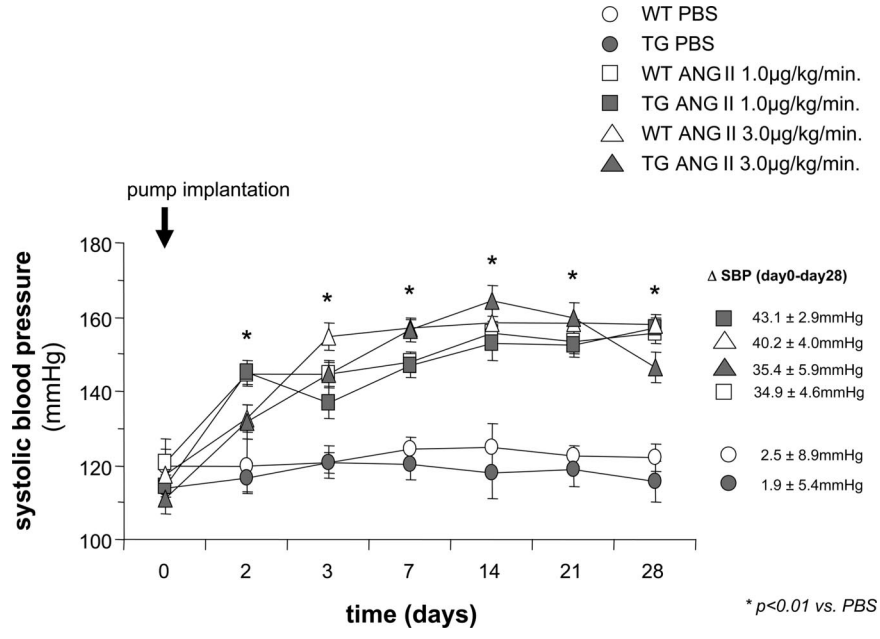


Fig. 1. Chronic infusion of ANG II and blood pressure in wild-type (WT) and human dimethylarginine dimethylaminohydrolase (DDAH)1 gene transgenic (TG) mice. SBP, systolic blood pressure.

creatinine, $n = 56$) did not differ between treatment groups and genotypes. The infusion of ANG II caused marked diuresis in both WT and TG mice (Table 1). Similarly, a significant increase in albuminuria was noted in mice treated with ANG II (Table 1). In PBS-treated animals, no significant change in urine output and urinary albumine excretion was observed.

*Heart weight and vascular hypertrophy.* The infusion of ANG II was also associated with myocardial and vascular hypertrophy as indicated by an increase in total wet heart weight and an increase in the wall-to-lumen ratio of the descending aorta (Table 1).

By use of immunofluorescence, scarce macrophage infiltration as indicated by MOMA-2 staining was noted, and the staining was restricted to the adventitia and the perivascular tissue, whereas the intima and media were largely devoid of macrophages (data not shown). Immunostaining for iNOS was barely detectable in PBS-treated mice, whereas a marked and dose-dependent expression was observed in ANG II-treated mice. The staining pattern and intensity did not differ between WT and TG mice (data not shown).

### *Plasma Levels of Aldosterone*

The infusion of ANG II at 1.0 or 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ caused a marked and dose-dependent increase in plasma aldosterone levels in WT and TG mice ($P < 0.001$; Table 1).

### *ANG II Infusion and Plasma ADMA Levels*

Despite profound hemodynamic effects and signs of target organ damage, the chronic infusion of ANG II had no impact on plasma levels of ADMA (Table 1). As expected, TG mice exhibited markedly lower ADMA levels (~37% lower than in WT mice) with virtually no overlap compared with WT animals (Table 1). Plasma aldosterone levels did not correlate with plasma ADMA levels ($r = -0.22$, $P$ = NS). The lack of effect of ANG II infusion on plasma ADMA levels was corroborated in mice infused with 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, in which ADMA levels were measured by ELISA and LC-MS both at baseline and after treatment (Fig. 2). There was an excellent correlation between ADMA levels obtained by ELISA versus LC-MS ($r = 0.95$, $P = 0.0006$; Fig. 3). In agreement with previous observations (22, 32), ELISA-derived ADMA levels were higher compared with those from LC-MS; the mean difference was 0.12 μM or 17% (0.685 vs. 0.569 μM, $P = 0.008$). However, a comparison of both methods in a Bland-Altman plot in which the mean of both methods ($x$-axis) was plotted against the difference of both methods ($y$-axis) demonstrated that values were within the limits of agreement (values are means ± 2 SD; data not shown). In addition, the concordance correlation coefficient (CCC), which evaluates the accuracy and precision between two measures based on the expected value of the squared distance function (20), suggested reasonably strong concordance (CCC = 0.680, and 95% lower confidence boundary = 0.514).

By means of the ELISA assay, a small increase of plasma ADMA levels was observed in WT mice. However, this finding could not be confirmed by LC-MS (Fig. 2). Baseline SDMA and L-arginine levels were similar in WT and TG mice (SDMA, 0.17 ± 0.02 vs. 0.17 ± 0.01 μM; and L-arginine, 65.6 ± 4.2 vs. 76.0 ± 7.4 μM, $P$ = NS) and remained unaltered by treatment (SDMA, 0.17 ± 0.02 vs. 0.17 ± 0.01 μM; L-arginine, 66.6 ± 8.7 vs. 60.7 ± 5.8 μM). The baseline and treatment-related L-arginine/ADMA ratio significantly differed between genotypes (baseline WT vs. TG, 98.7 ± 9.3 vs. 177.2 ± 26.5, $P = 0.01$; and treatment WT vs. TG, 95.2 ± 13.1 vs. 177.5 ± 22.6, $P = 0.006$) but did not change following ANG II infusion.

### *Renal Histomorphology and Immunohistochemistry*

The infusion of ANG II was associated with signs of glomerulosclerosis and interstitial fibrosis. Light microscopic evidence of glomerular damage was mild and similar between genotypes (Fig. 4*A*). Glomerular collagen IV staining was significantly enhanced in mice infused with 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, whereas the lower dose of ANG II had no effect on collagen IV content (WT mice, PBS vs. 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II vs. 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, 10.9 ± 0.8% vs. 11.2 ± 0.9% vs. 18.3 ± 0.8%; and TG mice, PBS vs. 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II vs. 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II, 9.6 ± 0.5% vs. 9.6 ± 0.7% vs. 18.6 ± 0.6%; treatment $P$ value, $P < 0.001$; and genotype $P$ value, $P$ = NS). ANG II caused marked renal interstitial fibrosis, which was significantly reduced in TG mice (Fig. 4*B*). Immunohistochemical findings of reduced renal interstitial fibrosis in TG mice were confirmed by RT-PCR of collagen I mRNA (Table 2). ANG II caused marked macrophage infiltration within the kidneys (Fig. 4*C*). F4/80 immunohistochemistry revealed a perivascular and periglomerular distribution pattern in ANG II-treated mice, and glomeruli were largely devoid of macrophages.

Table 1. *Target organ damage, hormone measurements, and plasma ADMA levels under chronic infusion of ANG II in WT and TG mice*

| | WT | | | TG | | | | |
|---|---|---|---|---|---|---|---|---|
| | PBS | ANG II, 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ | ANG II, 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ | PBS | ANG II, 1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ | ANG II, 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ | Treatment, $P$ Value | Genotype, $P$ Value |
| Diuresis, $\mu l \cdot g^{-1} \cdot day^{-1}$ | 29±6 | 110±11 | 132±23 | 32±5 | 86±9 | 118±15 | <0.001 | NS |
| Albuminuria, mg/g creatinine | 162±18 | 1,605±387 | 2,113±580 | 249±80 | 1,270±279 | 2,165±608 | <0.001 | NS |
| Heart weight, mg/g body wt | 4.81±0.11 | 5.63±0.21 | 5.78±0.15 | 4.76±0.21 | 5.69±0.24 | 6.11±0.19 | <0.001 | NS |
| Aortic wall-to-lumen ratio | 0.40±0.04 | 0.56±0.06 | 0.66±0.06 | 0.38±0.01 | 0.58±0.04 | 0.65±0.07 | <0.001 | NS |
| Plasma aldosterone, pg/ml | 257±19 | 1,871±346 | 5,355±606 | 240±67 | 2,161±333 | 5,835±350 | <0.001 | NS |
| Plasma ADMA, μM | 1.07±0.07 | 0.97±0.06 | 0.84±0.04 | 0.59±0.06 | 0.62±0.05 | 0.57±0.02 | NS | <0.001 |

Values are means ± SE. ADMA, asymmetric dimethylarginine; WT, wild-type; TG, human dimethylarginine dimethylaminohydrolase (DDAH)1 gene transgenic; NS, not significant.

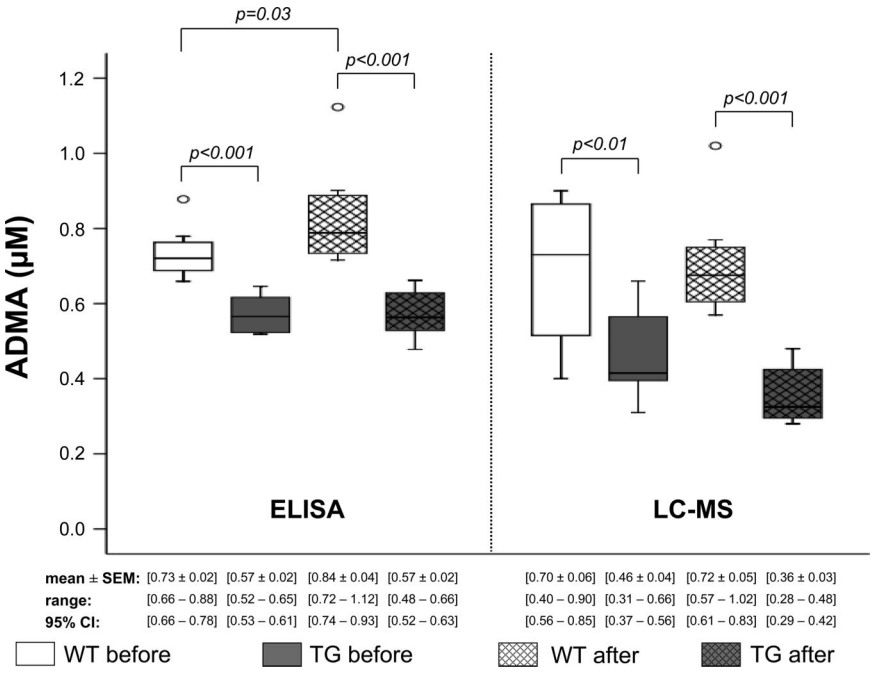


Fig. 2. ELISA- and liquid chromatography-tandem mass spectrometry (LC-MS)-derived asymmetric dimethylarginine (ADMA) levels in mice under high dose of ANG II (3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$).

*Oxidative Fluorescence Microscopy*

The infusion of ANG II dose dependently increased oxidative stress in WT mice as evidenced by DHE fluorescence in both the ascending and descending aorta (Fig. 5, *A* and *B*). In contrast, in TG mice only 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II was associated with enhanced DHE fluorescence. Overall, DHE fluorescence in mice treated with ANG II was significantly greater in WT as opposed to TG animals.

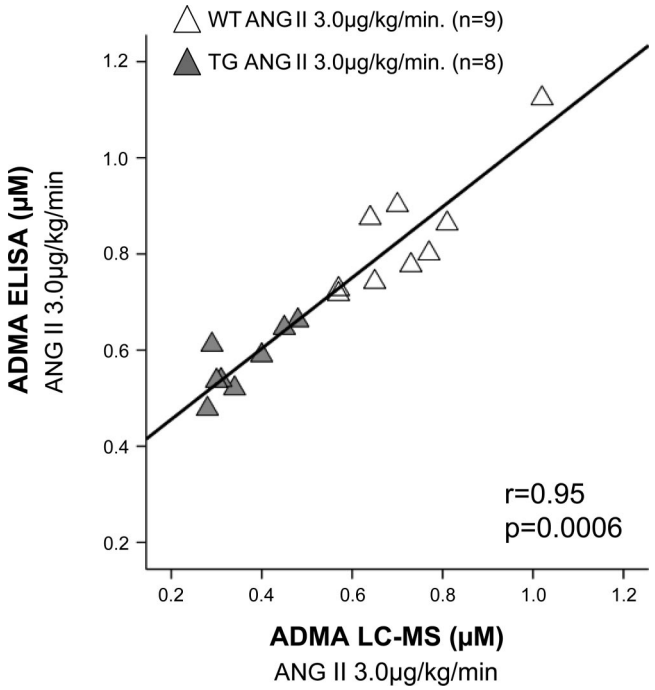


Fig. 3. Correlation of ELISA- and LC-MS-derived ADMA levels in mice following high-dose ANG II infusions (3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$). *n*, Number of animals.

*ANG II Infusion and Renal mRNA Expression of Collagen I and ADMA-Generating and -Degrading Enzymes*

The infusion of ANG II was associated with a significant increase in renal mRNA expression of PRMT1 and DDAH2, whereas PRMT3 and mouse DDAH1 expression remained unaltered (Table 2).

## DISCUSSION

The salient findings of the present study are that *1*) the chronic infusion of ANG II over 4 wk at a dose associated with marked hypertension and target organ damage has no net effect on plasma levels of ADMA and *2*) the overexpression of DDAH nevertheless protects against ANG II-induced vascular oxidative stress and renal interstitial fibrosis. The lack of effect of ANG II infusions on plasma ADMA levels observed in our studies stands in contrast to recent reports by others (13). In these experiments, Hasegawa and colleagues (13) studied the effect of ANG II infusions (1.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ delivered via osmotic minipumps over 2 wk) on plasma ADMA levels in both WT and DDAH2 TG mice. Although ADMA levels were twofold higher in ANG II- versus saline-treated WT mice (0.72 vs. 1.48 μM, $P < 0.01$), DDAH2 TG mice were, in part, resistant to ANG II-induced elevations of ADMA levels (0.52 vs. 0.80 μM, $P < 0.05$).

Notably, the results of Hasegawa et al. (13) agree with our data in several aspects. At a similar dose, these authors observed a comparable blood pressure increase regardless of whether the transgene was present or not. The reduced interstitial fibrosis in the kidney in our study is reminiscent of the effect of DDAH2 on the perivascular fibrosis reported by Hasegawa et al. (13). Similar to our study, these authors noted less vascular oxidative stress in TG versus WT mice following ANG II infusion (13). The effect of a DDAH2 transgene on vascular hypertrophy in the study by Hasegawa et al. (13) may be explained by the recent observation that the DDAH2 isoen-

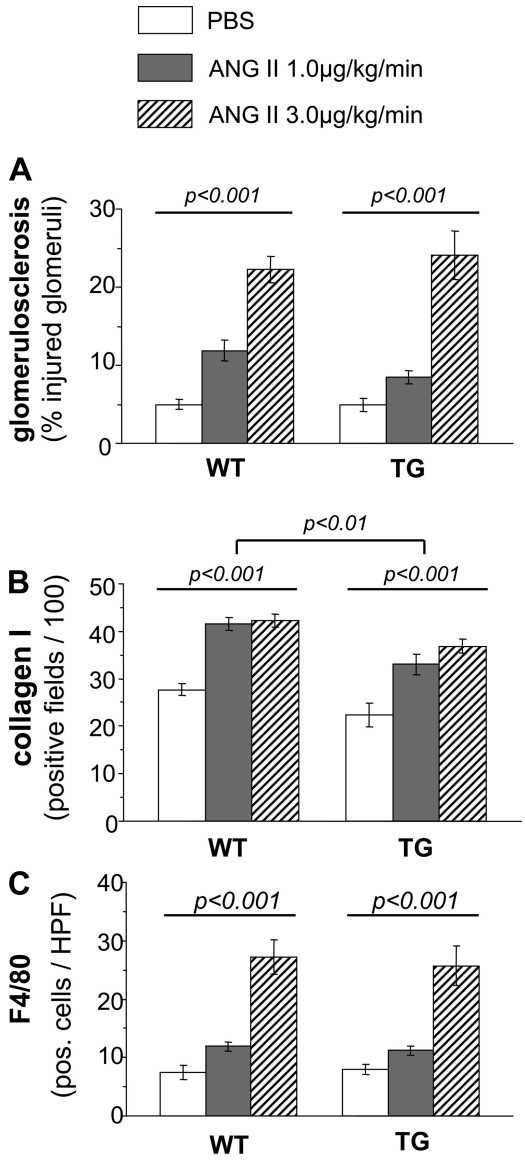


Fig. 4. Chronic infusion of ANG II and renal histomorphology. *A*: chronic infusion of ANG II and glomerulosclerosis in WT and TG mice. *B*: chronic infusion of ANG II and interstitial fibrosis (collagen I staining). *C*: chronic infusion of ANG II and renal macrophage infiltration (F4/80 staining). Pos cells, positive cells; HPF, high power field.

zyme plays a greater role for ADMA metabolism in vascular tissue, whereas DDAH1 has a more pronounced effect on serum ADMA levels (39). This may explain the more pronounced effect of DDAH1 (in our study) versus DDAH2 (13) overexpression on systemic ADMA levels compared with WT littermates (37% vs. 26% decrease). In that regard, the lack of tissue ADMA measurements must be considered a limitation of our study since tissue ADMA levels might be affected by ANG II, unlike plasma ADMA.

Given the similarities, the discrepant results on the effects of ANG II on ADMA levels are hard to explain. Due to our longer duration of ANG II treatment (4 vs. 2 wk), we may have missed a transient increase of ADMA after 2 wk. A further explanation for the discrepant findings may result from different detection methods for ADMA (HPLC vs. ELISA/LC-MS), the variation of which in the literature is substantial, especially for HPLC methods (24). We took several steps to exclude other possible explanations. First, we used an adequate sample size. Second, we confirmed our initial negative observation by also studying a higher dose of ANG II, namely 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$. Third, to corroborate our findings, ADMA levels were measured both by ELISA as well as LC-MS. Although we measured a small (~15%) increase of ADMA levels by ELISA in WT mice infused with 3.0 $\mu g \cdot kg^{-1} \cdot min^{-1}$ ANG II (and only compared with pretreatment levels), this increase was very minor and far from the 100% increase observed by Hasegawa et al. (13). In addition, we could not confirm this apparent increase with LC-MS measurements, despite good agreement between both methods in many other respects. As observed by others (22, 32), the ELISA yielded higher ADMA levels than LC-MS but showed an excellent discrimination between WT and TG mice with virtually no overlap of ADMA levels between genotypes.

Apart from the studies by Hasegawa and colleagues (13), little is known about how ANG II might affect ADMA metabolism. In vitro studies by Chen et al. (5) suggest that ANG II-induced elevations of ADMA levels are the consequence of an enhanced expression of PRMT as well as reduced activity of DDAH, but these authors used rather high doses of ANG II (1 μM) and ADMA (500 μM for DDAH activity assay). We observed a minor yet significant ANG II-induced upregulation of PRMT1 and DDAH2 gene expression in the kidney, whereas PRMT3 and endogenous mouse DDAH1 expression remained unaltered. The combined upregulation of ADMA-generating (PRMT1) and -degrading (DDAH2) enzymes may explain that no net effect of ANG II treatment on systemic ADMA levels was observed. Interestingly, enhanced mRNA expression of renal PRMT1 and DDAH2 during ANG II infusions mirrors the mRNA expression pattern observed during RAS blockade in a rat model of diabetes. Thus Onozato et al. (28) observed reduced expression of both enzymes during treatment with the ANG II receptor blocker telmisartan. Furthermore, our findings agree with earlier observations of Tojo et al. (37), who reported that DDAH protein expression was increased in rats by stimulating an endogenous ANG II production (by means of a low-salt diet) as well as by an infusion of exogenous ANG II.

Others have reported that ADMA levels may affect the expression of ACE (34) or the pressor response to ANG II (8). We did not observe any relationship between ANG II-induced hypertension and plasma ADMA levels. Evidence for an association between high blood pressure and elevated ADMA levels comes from small clinical studies (29, 35). Other reports, including several large-scale clinical studies, did not confirm such an association (25, 31). Our blood pressure results are limited because we only used a noninvasive tail-cuff method, a technique that has received criticism in the past. Although we may have missed subtle blood pressure differences between genotypes with this technique, the overall effect of ANG II treatment on blood pressure was clearly evident and within the range reported by others investigators with similar doses in which either the tail-cuff method or telemetric devices were being used (13, 41).

Despite the lack of ANG II to alter plasma levels of ADMA, we observed a functional relevance of the enzyme DDAH for ANG II-induced formation of ROS and renal interstitial fibro-

Table 2. *Renal mRNA expression of collagen I and ADMA generating and degrading enzymes*

| | WT | | TG | | | |
|---|---|---|---|---|---|---|
| | PBS | ANG II, 1.0 μg·kg$^{-1}$·min$^{-1}$ | PBS | ANG II, 1.0 μg·kg$^{-1}$·min$^{-1}$ | Treatment, *P* Value | Genotype, *P* Value |
| Mouse DDAH1, relative copies | 25,289±1,178 | 28,185±2,896 | 23,726±2,529 | 27,215±1,742 | NS | NS |
| DDAH2, relative copies | 602±92 | 820±77 | 533±78 | 716±47 | 0.013 | NS |
| PRMT1, relative copies | 319±59 | 543±67 | 374±61 | 580±77 | 0.011 | NS |
| PRMT3, relative copies | 258±47 | 237±34 | 246±35 | 197±40 | NS | NS |
| Collagen I, relative copies | 4,926±494 | 8,308±1,367 | 3,499±137 | 5,346±490 | 0.015 | 0.038 |

Values are means ± SE. PRMT, protein arginine methyltransferases.

sis. ANG II is known to stimulate the generation of oxygen free radicals ($O_2^-$) (30), which may in turn lead to endothelial NOS (eNOS) uncoupling via depletion of the cofactor tetrahydrobiopterin (3). In such a state, NOS itself becomes a major source for ROS generation. There is growing evidence that elevated ADMA levels may facilitate eNOS uncoupling (36). Such a mechanism may serve as an explanation of why TG mice were less susceptible to ANG II-induced vascular oxidative stress. In addition, the enzyme DDAH is redox sensitive, which results from a critical sulfhydryl group within the active catalytic site of DDAH (18). Enhanced expression of DDAH may, therefore, be vasoprotective. Finally, we speculate that ADMA may play a permissive role for target organ damage. Even apparently normal levels of factors, which can contribute to vascular damage, may become deleterious in the presence of comorbid conditions such as high ANG II levels and/or hypertension.

In addition to reduced vascular oxidative stress, TG mice developed less renal interstitial fibrosis. This finding is in line with recent observations by Matsumoto and colleagues (23) in which adenovirus-mediated overexpression of DDAH1 significantly reduced glomerular and interstitial fibrosis in subtotally nephrectomized rats. The infiltration of macrophages and T lymphocytes into the interstitium is a prominent finding and precedes interstitial fibrosis in ANG II-dependent forms of hypertension (17, 21). Several strategies aimed at reducing inflammation, including the blockade of tumor necrosis factor-α (TNF-α), have been shown to reduce ANG II-dependent kidney damage (26). The potential effect of ADMA on these mechanisms will require further studies.

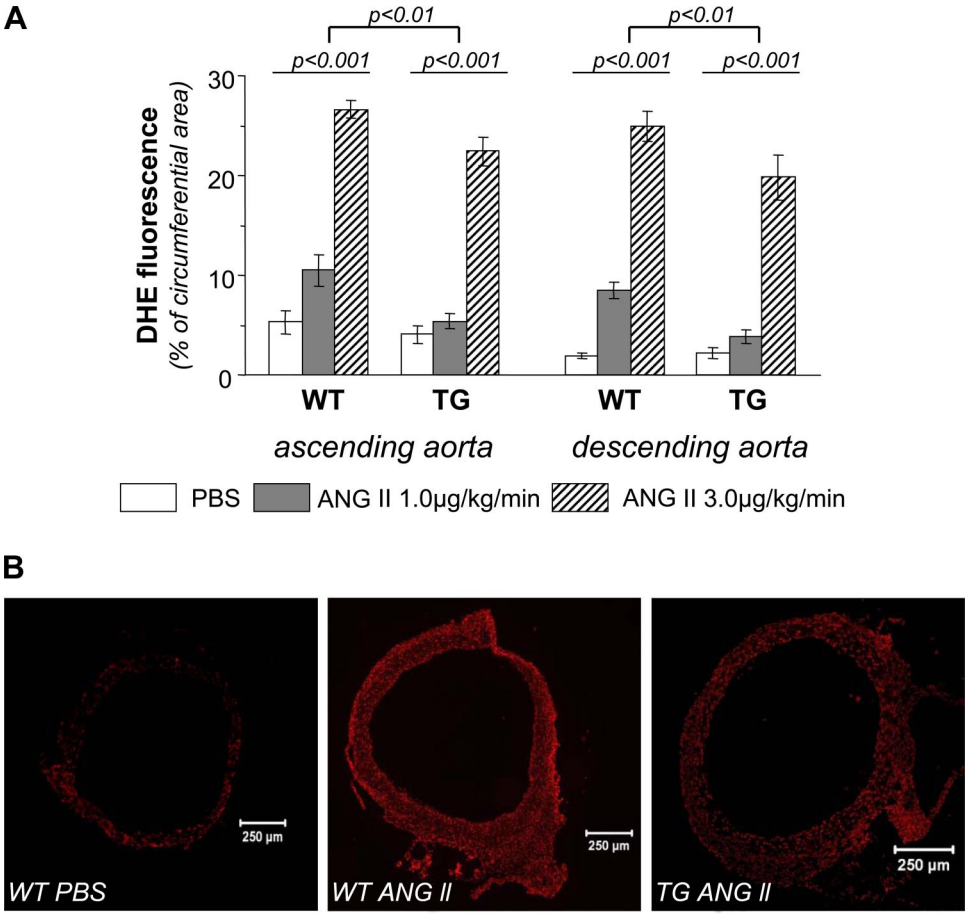


Fig. 5. Chronic infusion of ANG II and vascular oxidative stress. *A*: dihydroethidium (DHE) fluorescence within the ascending and descending aorta. *B*: DHE fluorescence in PBS, ANG II-treated (1.0 μg·kg$^{-1}$·min$^{-1}$) WT, and ANG II-treated (1.0 μg·kg$^{-1}$·min$^{-1}$) TG animal (ascending aorta, ×4 objective).

In conclusion, our study helps to delineate the relevance of ADMA in ANG II-induced hypertension and target organ damage in mice. Our data indicate that ADMA contributes to ANG II-induced oxidative stress and renal interstitial fibrosis. However, ANG II did not increase ADMA levels in our animals, in contrast to recent studies by others (13). The lack of an increase of ADMA may explain the unexpectedly modest role of this mediator.

## GRANTS

This work was supported by the Interdisciplinary Center for Clinical Research at the University Hospital of the University of Erlangen-Nuremberg, project B12, and by the Deutsche Forschungsgemeinschaft (KFO 106/2-1).

## REFERENCES


1. **Achan V, Broadhead M, Malaki M, Whitley G, Leiper J, MacAllister R, Vallance P.** Asymmetric dimethylarginine causes hypertension and cardiac dysfunction in humans and is actively metabolized by dimethylarginine dimethylaminohydrolase. *Arterioscler Thromb Vasc Biol* 23: 1455–1459, 2003.
2. **Aslam S, Santha T, Leone A, Wilcox C.** Effects of amlodipine and valsartan on oxidative stress and plasma methylarginines in end-stage renal disease patients on hemodialysis. *Kidney Int* 70: 2109–2115, 2006.
3. **Chalupsky K, Cai H.** Endothelial dihydrofolate reductase: critical for nitric oxide bioavailability and role in angiotensin II uncoupling of endothelial nitric oxide synthase. *Proc Natl Acad Sci USA* 102: 9056–9061, 2005.
4. **Chen JW, Hsu NW, Wu TC, Lin SJ, Chang MS.** Long-term angiotensin-converting enzyme inhibition reduces plasma asymmetric dimethylarginine and improves endothelial nitric oxide bioavailability and coronary microvascular function in patients with syndrome X. *Am J Cardiol* 90: 974–982, 2002.
5. **Chen MF, Xie XM, Yang TL, Wang YJ, Zhang XH, Luo BL, Li YJ.** Role of asymmetric dimethylarginine in inflammatory reactions by angiotensin II. *J Vasc Res* 44: 391–402, 2007.
6. **Chomczynski P.** A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 15: 532–534, 536–537, 1993.
7. **Dayoub H, Achan V, Adimoolam S, Jacobi J, Stuehlinger MC, Wang BY, Tsao PS, Kimoto M, Vallance P, Patterson AJ, Cooke JP.** Dimethylarginine dimethylaminohydrolase regulates nitric oxide synthesis: genetic and physiological evidence. *Circulation* 108: 3042–3047, 2003.
8. **De Gennaro Colonna V, Bonomo S, Ferrario P, Bianchi M, Berti M, Guazzi M, Manfredi B, Muller EE, Berti F, Rossoni G.** Asymmetric dimethylarginine (ADMA) induces vascular endothelium impairment and aggravates postischemic ventricular dysfunction in rats. *Eur J Pharmacol* 557: 178–185, 2007.
9. **Delles C, Schneider MP, John S, Gekle M, Schmieder RE.** Angiotensin converting enzyme inhibition and angiotensin II $AT_1$-receptor blockade reduce the levels of asymmetrical $N^G$, $N^G$-dimethylarginine in human essential hypertension. *Am J Hypertens* 15: 590–593, 2002.
10. **Fliser D, Wagner KK, Loos A, Tsikas D, Haller H.** Chronic angiotensin II receptor blockade reduces (intra)renal vascular resistance in patients with type 2 diabetes. *J Am Soc Nephrol* 16: 1135–1140, 2005.
11. **Handtrack C, Cordasic N, Klanke B, Veelken R, Hilgers KF.** Effect of the angiotensinogen genotype on experimental hypertension in mice. *J Mol Med* 85: 343–350, 2007.
12. **Hartner A, Cordasic N, Klanke B, Veelken R, Hilgers KF.** Strain differences in the development of hypertension and glomerular lesions induced by deoxycorticosterone acetate salt in mice. *Nephrol Dial Transplant* 18: 1999–2004, 2003.
13. **Hasegawa K, Wakino S, Tatematsu S, Yoshioka K, Homma K, Sugano N, Kimoto M, Hayashi K, Itoh H.** Role of asymmetric dimethylarginine in vascular injury in transgenic mice overexpressing dimethylarginie dimethylaminohydrolase 2. *Circ Res* 101: e2–e10, 2007.
14. **Hilgers KF, Langenfeld MR, Schlaich M, Veelken R, Schmieder RE.** 1166 A/C polymorphism of the angiotensin II type 1 receptor gene and the response to short-term infusion of angiotensin II. *Circulation* 100: 1394–1399, 1999.
15. **Ito A, Egashira K, Narishige T, Muramatsu K, Takeshita A.** Angiotensin-converting enzyme activity is involved in the mechanism of increased endogenous nitric oxide synthase inhibitor in patients with type 2 diabetes mellitus. *Circulation* 66: 811–815, 2002.
16. **Ito A, Egashira K, Narishige T, Muramatsu K, Takeshita A.** Renin-angiotensin system is involved in the mechanism of increased serum asymmetric dimethylarginine in essential hypertension. *Jpn Circ J* 65: 775–778, 2001.
17. **Johnson RJ, Alpers CE, Yoshimura A, Lombardi D, Pritzl P, Floege J, Schwartz SM.** Renal injury from angiotensin II-mediated hypertension. *Hypertension* 19: 464–474, 1992.
18. **Leiper J, Murray-Rust J, McDonald N, Vallance P.** S-nitrosylation of dimethylarginine dimethylaminohydrolase regulates enzyme activity: further interactions between nitric oxide synthase and dimethylarginine dimethylaminohydrolase. *Proc Natl Acad Sci USA* 99: 13527–13532, 2002.
19. **Leiper J, Nandi M, Torondel B, Murray-Rust J, Malaki M, O'Hara B, Rossiter S, Anthony S, Madhani M, Selwood D, Smith C, Wojciak-Stothard B, Rudiger A, Stidwill R, McDonald NQ, Vallance P.** Disruption of methylarginine metabolism impairs vascular homeostasis. *Nat Med* 13: 198–203, 2007.
20. **Lin LI.** A concordance correlation coefficient to evaluate reproducibility. *Biometrics* 45: 255–268, 1989.
21. **Mai M, Geiger H, Hilgers KF, Veelken R, Mann JF, Dammrich J, Luft FC.** Early interstitial changes in hypertension-induced renal injury. *Hypertension* 22: 754–765, 1993.
22. **Martens-Lobenhoffer J, Westphal S, Awiszus F, Bode-Boger SM, Luley C.** Determination of asymmetric dimethylarginine: liquid chromatography-mass spectrometry or ELISA? *Clin Chem* 51: 2188–2189, 2005.
23. **Matsumoto Y, Ueda S, Yamagishi S, Matsuguma K, Shibata R, Fukami K, Matsuoka H, Imaizumi T, Okuda S.** Dimethylarginine dimethylaminohydrolase prevents progression of renal dysfunction by inhibiting loss of peritubular capillaries and tubulointerstitial fibrosis in a rat model of chronic kidney disease. *J Am Soc Nephrol* 18: 1525–1533, 2007.
24. **Meinitzer A, Puchinger M, Winklhofer-Roob BM, Rock E, Ribalta J, Roob JM, Sundl I, Halwachs-Baumann G, Marz W.** Reference values for plasma concentrations of asymmetrical dimethylarginine (ADMA) and other arginine metabolites in men after validation of a chromatographic method. *Clin Chim Acta* 384: 141–148, 2007.
25. **Meinitzer A, Seelhorst U, Wellnitz B, Halwachs-Baumann G, Boehm BO, Winkelmann BR, Marz W.** Asymmetrical dimethylarginine independently predicts total and cardiovascular mortality in individuals with angiographic coronary artery disease (the ludwigshafen risk and cardiovascular health study). *Clin Chem* 53: 273–283, 2007.
26. **Muller DN, Shagdarsuren E, Park JK, Dechend R, Mervaala E, Hampich F, Fiebeler A, Ju X, Finckenberg P, Theuer J, Viedt C, Kreuzer J, Heidecke H, Haller H, Zenke M, Luft FC.** Immunosuppressive treatment protects against angiotensin II-induced renal damage. *Am J Pathol* 161: 1679–1693, 2002.
27. **Napoli C, Sica V, de Nigris F, Pignalosa O, Condorelli M, Ignarro LJ, Liguori A.** Sulfhydryl angiotensin-converting enzyme inhibition induces sustained reduction of systemic oxidative stress and improves the nitric oxide pathway in patients with essential hypertension. *Am Heart J* 148: e5, 2004.
28. **Onozato ML, Tojo A, Leiper J, Fujita T, Palm F, Wilcox CS.** Expression of DDAH and PRMT isoforms in the diabetic rat kidney; effects of angiotensin II receptor blocker. *Diabetes* 57: 172–180, 2008.
29. **Perticone F, Sciacqua A, Maio R, Perticone M, Maas R, Boger RH, Tripepi G, Sesti G, Zoccali C.** Asymmetric dimethylarginine, L-arginine, and endothelial dysfunction in essential hypertension. *J Am Coll Cardiol* 46: 518–523, 2005.
30. **Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK, Harrison DG.** Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. *J Clin Invest* 97: 1916–1923, 1996.
31. **Schnabel R, Blankenberg S, Lubos E, Lackner KJ, Rupprecht HJ, Espinola-Klein C, Jachmann N, Post F, Peetz D, Bickel C, Cambien F, Tiret L, Munzel T.** Asymmetric dimethylarginine and the risk of cardiovascular events and death in patients with coronary artery disease: results from the atherogene study. *Circ Res* 97: e53–e59, 2005.
32. **Schulze F, Wesemann R, Schwedhelm E, Sydow K, Albsmeier J, Cooke JP, Boger RH.** Determination of asymmetric dimethylarginine (ADMA) using a novel ELISA assay. *Clin Chem* 42: 1377–1383, 2004.

33. **Schwedhelm E, Maas R, Tan-Andresen J, Schulze F, Riederer U, Boger RH.** High-throughput liquid chromatographic-tandem mass spectrometric determination of arginine and dimethylated arginine derivatives in human and mouse plasma. *J Chromatogr A* 851: 211–219, 2007.
34. **Suda O, Tsutsui M, Morishita T, Tasaki H, Ueno S, Nakata S, Tsujimoto T, Toyohira Y, Hayashida Y, Sasaguri Y, Ueta Y, Nakashima Y, Yanagihara N.** Asymmetric dimethylarginine produces vascular lesions in endothelial nitric oxide synthase-deficient mice: involvement of renin-angiotensin system and oxidative stress. *Arterioscler Thromb Vasc Biol* 24: 1682–1688, 2004.
35. **Surdacki A, Nowicki M, Sandmann J, Tsikas D, Boeger RH, Bode-Boeger SM, Kruszelnicka-Kwiatkowska O, Kokot F, Dubiel JS, Froelich JC.** Reduced urinary excretion of nitric oxide metabolites and increased plasma levels of asymmetric dimethylarginine in men with essential hypertension. *J Cardiovasc Pharmacol* 33: 652–658, 1999.
36. **Sydow K, Munzel T.** ADMA and oxidative stress. *Atheroscler Suppl* 4: 41–51, 2003.
37. **Tojo A, Kimoto M, Wilcox CS.** Renal expression of constitutive NOS and DDAH: separate effects of salt intake and angiotensin. *Kidney Int* 58: 2075–2083, 2000.
38. **Vallance P, Leiper J.** Cardiovascular biology of the asymmetric dimethylarginine: dimethylarginine dimethylaminohydrolase pathway. *Arterioscler Thromb Vasc Biol* 24: 1023–1030, 2004.
39. **Wang D, Gill PS, Chabrashvili T, Onozato ML, Raggio J, Mendonca M, Dennehy K, Li M, Modlinger P, Leiper J, Vallance P, Adler O, Leone A, Tojo A, Welch WJ, Wilcox CS.** Isoform-specific regulation by $N^G$,$N^G$-dimethylarginine dimethylaminohydrolase of rat serum asymmetric dimethylarginine and vascular endothelium-derived relaxing factor/NO. *Circ Res* 101: 627–635, 2007.
40. **Warnholtz A, Ostad MA, Heitzer T, Thuneke F, Frohlich M, Tschentscher P, Schwedhelm E, Boger R, Meinertz T, Munzel T.** $AT_1$-receptor blockade with irbesartan improves peripheral but not coronary endothelial dysfunction in patients with stable coronary artery disease. *Atherosclerosis* 194: 439–445, 2007.
41. **Xue B, Pamidimukkala J, Hay M.** Sex differences in the development of angiotensin II-induced hypertension in conscious mice. *Am J Physiol Heart Circ Physiol* 288: H2177–H2184, 2005.
42. **Yilmaz MI, Saglam M, Sonmez A, Caglar K, Cakir E, Kurt Y, Eyileten T, Tasar M, Acikel C, Oguz Y, Vural A, Yenicesu M.** Improving proteinuria, endothelial functions and asymmetric dimethylarginine levels in chronic kidney disease: ramipril versus valsartan. *Blood Purif* 25: 327–335, 2007.