Elsevier Editorial System(tm) for Translational Research
Manuscript Draft

Manuscript Number: TR-11-111R1

Title: The combined ratios of L-arginine, asymmetric and symmetric dimethylarginine as biomarkers in spontaneously hypertensive rats

Article Type: Full Length Article

Keywords: arginine; asymmetric dimethylarginine; symmetric dimethylarginine; hypertension; nitric oxide; spontaneously hypertensive rat

Corresponding Author: Dr You-Lin Tain, M.D., Ph.D.

Corresponding Author's Institution: Chang Gung Memorial Hospital-Kaohsiung

First Author: You-Lin Tain, M.D., Ph.D.

Order of Authors: You-Lin Tain, M.D., Ph.D.; Chien-Ning Hsu, PhD; Li-Tung Huang, MD; Ying-Tung Lau, PhD; Ching-Yuang Lin, MD, PhD

Manuscript Region of Origin: TAIWAN

Abstract: Hypertension and hypertensive end-organ damage have been associated with decreased nitric oxide (NO) bioavailability. Asymmetric and symmetric dimethylarginine (ADMA and SDMA) can both inhibit NO availability by competition with L-arginine (L-Arg). However, whether combined analysis of these three parameters can serve as an ideal biomarker of hypertension remains unclear. We measured the plasma and renal levels of L-Arg, ADMA, and SDMA in spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) control rats at three stages: 4-wk-old (pre-hypertensive), 12-wk-old (hypertensive), and 24-wk-old (end-organ damage). The plasma and renal L-Arg/ADMA ratio (AAR) and the ADMA/SDMA ratio (ASR) were computed for all three age stages. Our results revealed an ADMA level increase and an AAR decrease in plasma and kidneys may develop early on, even before the onset of hypertension in 4-wk-old SHRs. The renal ADMA level and AAR were restored in SHRs at 24 wk of age, which might protect SHRs against kidney injury. We found that the plasma AAR is superior to the levels of L-Arg and ADMA in plasma, and predicted blood pressure and urinary NOx levels; and renal AAR is a strong independent marker of renal dimethylarginine dimethylaminohydrolase (DDAH) activity. The plasma ASR was strongly correlated to blood pressure. However, renal DDAH activity was related to the renal ASR, but not the plasma ASR. In conclusion, the AAR and ASR may serve as better markers for disease activity and progression than each individual parameter. Clinical use of these ratios to elucidate the role of ADMA in hypertension awaits further validation.

***Cover Letter**

Dear Editor,

Here we submit our revised article entitled “The combined ratios of L-arginine, asymmetric and symmetric dimethylarginine as biomarkers in spontaneously hypertensive rats” solely to Translational Research. This manuscript contains a concise text, three tables and four figures. The results presented in this paper have not been published previously in whole or part. All authors have contributed significantly and all are in agreement with the content of this manuscript. The authors also declare that there is no conflict of interest in this manuscript.

Thank you very much for your review and consideration of publication.

Sincerely yours,

You-Lin Tain M.D., Ph.D.
Department of Pediatrics,
Kaohsiung Chang Gung Memorial Hospital and
Chang Gung University College of Medicine, Kaohsiung, Taiwan
E-mail address: tainyl@hotmail.com
Tel: +886-7-7317123 ext 8723
Fax: +886-7-7338009

Dear Dr. Laurence,

Thank you very much for your letter regarding our article ID TR-11-111 entitled "The combined ratios of L-arginine, asymmetric and symmetric dimethylarginine as biomarkers in spontaneously hypertensive rats". We greatly appreciate your kindness in considering our article for publication.

Please find below point-by-point responses to each of the reviewers' comments:

RESPONSES TO REVIEWERS' COMMENTS

Reviewer: 1

1. This is a case-cohort analysis assessing biomarkers of L-Arg and NO metabolism in SHR rats vs. WKY rats. The rationale for the study is that SHR rats, despite end-organ hypertensive damage, appear to be protected against kidney injury. The abstract highlights key data. The introduction is well-written. The materials and methods section is straightforward.

RESPONSE: We thank reviewer #1 for his/her support.

2. A description of pathological and morphological measurements and statistical assessment would have been useful.

RESPONSE: Thanks for your valuable comments. We have added the morphological findings into the following sections:

a. In the Materials and Methods section (page 7, last line 4), we added "One kidney was removed, decapsulated, divided into the cortex and medulla, and snap-frozen for Western blot and HPLC. The other kidney was perfusion-fixed for histological analysis" and "Histological analysis was performed on 4-μm sections of formalin-fixed kidney blocked in paraffin wax and stained with periodic acid-Schiff (PAS). The level of renal injury was assessed on a blind basis by evaluating the glomerular injury. Glomerular injury (GI) scores representing sclerotic damage to glomeruli (N = 100) were calculated using the 0 to 4+ scale described previously[17]" (page 8, last line 2) into the text.

b. We added the following statements (page 11, line 15) "The glomerular injury (GI) score did not differ significantly between the SHRs and WKYs at 4 wk and 12 wk, but tended to be higher in the SHRs than in the WKYs at 24 wk of age." to the Results section.

c. In the Discussion section (page 15, line 12), we added "In addition, the kidneys of the WKY rats exhibited almost no glomerular injury at any of the ages studied. In SHR kidneys, only some glomeruli showed sclerosis at 24 wk of age. These pathological findings were consistent with a previous study showing that pathological changes in SHR kidneys do not progress rapidly until 45 wk of age.[19]" to the text.

3. The results suggest that ADMA increases in plasma and kidneys early though ADMA is lower at 12 and 24 weeks, AAR decreases in both and urinary NOx is lower in SHR animals with stable functional parameters. By 24 weeks, ADMA and AAR levels are comparable in SHR and WKY animals. If not changes in DDAH activity, what would be the likely mechanism of restoration of ADMA if it was protective? This is difficult to answer with the studies as noted with the only suggestion being changes in ADMA transport due to changes in CAT-1. This would benefit from additional studies

examining transport activity if possible.
RESPONSE: Here, unfortunately, we failed to meet the reviewer's request. We tried to approach this experiment using a radioisotope assay; however, the space and facilities required were not available at our hospital. We would have been unable to complete this experiment within 3 months if we had cooperated with an outside lab. Although decreased CAT activity in SHRs probably impact ADMA transport was not done in our hands, this point was supported by the following studies: First is observation that a decreased CAT activity can reduce cellular uptake of ADMA [Ref 21]. Second, Moss et al. showed that the CAT activity was lower in SHRs than in WKY rats in RBCs [Ref 22]. As an alternative, we have added the related reference and rephrased the following statement in the Discussion section (page 16, line 8): "As the kidney is a major organ in ADMA metabolism, as renal CAT-1 levels are decreased in SHR rats, as a decreased CAT activity can reduce cellular uptake of ADMA,[21] and as it was shown in a previous report that the CAT activity was lower in SHRs than in WKY rats in red blood cells,[22] another possibility is that the SHR kidney protects against injury by reducing the ADMA uptake from circulation into the kidneys."

4. Hepatic ADMA production should be examined given the likelihood that it is a major contributor to circulating ADMA levels. The AAR data could be summarized more succinctly given that it appears to be a finding without marked biological relevance in this study. The urinary Nox studies add little to the work, given the variety of parameters that might be influencing them.
RESPONSE: Unfortunately we did not harvest the livers in this study. As we mentioned in the Discussion, ADMA can be produced and/or metabolized in many organs (e.g., liver, lung, etc.), and elucidation of the active sites affecting ADMA homeostasis might provide a specific ADMA-lowering approach for future study. We consider this to be a very important issue and we will put a lot of effort into looking into this in future studies.

5. The AAR data could be summarized more succinctly given that it appears to be a finding without marked biological relevance in this study. The urinary Nox studies add little to the work, given the variety of parameters that might be influencing them.
RESPONSE: We thank the reviewer for this comment, and have taken care to soften our statement regarding the AAR. We agree with the reviewer that measurement of urinary NOx has limitations and adds little to the work. We have deleted the part of urinary NOx in the Discussion section.

6. With the noted finding of melatonin and the presence of ADMA inhibition, why not consider an intervention in the SHR group as well to determine how that intervention alters metabolism in this study and whether those changes, if they correlate with WKY findings, are validating.
RESPONSE: Thanks again for your valuable suggestions. So far, specific ADMA-lowering agents remain unavailable. We are working on some interventions to lower ADMA and restore the AAR in other projects. Therefore, we are greatly thankful for your review and consideration of publication of this fundamental paper.

7. Kidney histology should be examined. Damage may ensue in the presence of good function and this is important to note.

RESPONSE: Please see response to Q2.

8. The discussion is well-written and the tables and figures are helpful. A diagram outlining theoretical mechanisms based on the data would be a great supplement to these.
RESPONSE: Thanks for your suggestion. We have added a simplified scheme as Fig 4.

Reviewer: 2
1. The authors should clearly distinguish between a) biomarker use as an index of disease or injury, and 2) pathophysiologic mechanisms. By mixing these two distinct but related concepts, the discussion is sometimes confusing and misleading. In a related manner, much of the discussion should be re-written to focus on clear hypotheses; there are frequently so many ratios presented, and mixing plasma and kidney at the three ages, that the concepts being advanced are confusing or lost.
RESPONSE: We have re-written our discussion (page 17, line 3) and obtained help from an English-speaking copy-editor to improve the readability of the manuscript.

2. Also the authors in several instances use correlations to indicate mechanisms or predictive ability which have not been proved. Occasionally the authors compare SHR of one age to WKY of another age; those are inappropriate comparisons. The number of the correlations presented in the tables can be lowered to those that are particularly important to decrease the density of material.
RESPONSE: Thanks for your valuable comments. We have reanalyzed our data to avoid comparing SHRs of one age with WKY rats of another age. We have re-written the statement in the Results and re-labeled the significance in the tables and figures. We also took your advice and removed some less important correlations from Table 3.

3. Language is unclear and poorly constructed in certain aspects and needs to be scrutinized throughout. An example is the first sentence of the discussion.
RESPONSE: The manuscript has been edited by a professional native English copy-editor (OxBioSci) in order to improve the language.

We have made every editorial change and style revision recommended by the reviewers in the edited version. Thank you very much for your review and for considering our manuscript for publication in your journal.

You-Lin Tain, MD, PhD
Division of Pediatric Nephrology
Kaohsiung Chang Gung Memorial Hospital, Kaohsiung, Taiwan
E-mail: tainyl@hotmail.com or tainyl@adm.cgmh.com.tw
Office phone: +886-7-7317123 ext. 8723
Phone: +886-0975-056995
Fax: +886-7-7338009

***Brief Commentary**

Background: Nitric oxide (NO) deficiency contributes to hypertension. Asymmetric and symmetric dimethylarginine (ADMA and SDMA) both can inhibit NO availability by competition with L-arginine. However, whether combined analysis of these three parameters serve biomarkers of hypertension remains unclear.

Translational Significance: We found that plasma L-arginine-to-ADMA ratio (AAR) is superior to L-Arg and ADMA, to predict BP and NO levels. Renal DDAH activity was related to AAR and ADMA-to-SDMA ratio (ASR). Thus, the AAR and ASR may serve as markers for hypertension. These findings provide evidence for further clinical use of these ratios to elucidate the role of ADMA in hypertension.

The combined ratios of L-arginine, asymmetric and symmetric dimethylarginine as biomarkers in spontaneously hypertensive rats

Chien-Ning Hsu[a], Li-Tung Huang[b], Ying-Tung Lau[c], Ching-Yuang Lin[d],You-Lin Tain[b]

[a]Departments of Pharmacy and [b]Pediatrics, Kaohsiung Chang Gung Memorial Hospital, and Chang Gung University College of Medicine, Taiwan

[c]Department of Physiology and Pharmacology, College of Medicine, Chang Gung University, and Chang Gung Institute of Technology, Taoyuan, Taiwan

[d]College of Medicine, China Medical University, Clinical Immunological Center, and Division of Pediatric Nephrology, China Medical University Hospital, Taichung, Taiwan

Corresponding author:

You-Lin Tain, MD, PhD.

Department of Pediatrics, Chang Gung Memorial Hospital at Kaohsiung, 123 Dabi Rd., Niausung, Kaohsiung 833, Taiwan

E-mail address: tainyl@hotmail.com

Tel: +886-975056995, Fax: +886-7-7338009

Abstract

Hypertension and hypertensive end-organ damage have been associated with decreased nitric oxide (NO) bioavailability. Asymmetric and symmetric dimethylarginine (ADMA and SDMA) can both inhibit NO availability by competition with L-arginine (L-Arg). However, whether combined analysis of these three parameters can serve as an ideal biomarker of hypertension remains unclear. We measured the plasma and renal levels of L-Arg, ADMA, and SDMA in spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) control rats at three stages: 4-wk-old (pre-hypertensive), 12-wk-old (hypertensive), and 24-wk-old (end-organ damage). The plasma and renal L-Arg/ADMA ratio (AAR) and the ADMA/SDMA ratio (ASR) were computed for all three age stages. Our results revealed an ADMA level increase and an AAR decrease in plasma and kidneys may develop early on, even before the onset of hypertension in 4-wk-old SHRs. The renal ADMA level and AAR were restored in SHRs at 24 wk of age, which might protect SHRs against kidney injury. We found that the plasma AAR is superior to the levels of L-Arg and ADMA in plasma, and predicted blood pressure and urinary NOx levels; and renal AAR is a strong independent marker of renal dimethylarginine dimethylaminohydrolase (DDAH) activity. The plasma ASR was strongly correlated to blood pressure. However, renal DDAH activity was related to the renal ASR, but not the plasma ASR. In conclusion, the AAR and ASR may serve as better markers for disease activity and progression than each individual parameter. Clinical use of these ratios to elucidate the role of ADMA in hypertension awaits further validation.

Key words: asymmetric dimethylarginine, symmetric dimethylarginine, hypertension, nitric oxide, spontaneously hypertensive rat.

Running title: AAR and ASR in SHR

Abbreviation:

AAR= L-Arginine-to-ADMA ratio

ADMA= asymmetric dimethylarginine

ASR= ADMA-to-SDMA ratio

DDAH= dimethylarginine dimethylaminohydrolase

NOS= nitric oxide synthase

PRMT= protein arginine methyltransferase

SHR= spontaneously hypertensive rat

WKY**=** Wistar Kyoto

Introduction

Nitric oxide (NO) results in vasodilatation to regulate systemic blood pressure (BP) and local blood flow. There is a growing body of evidence demonstrating a close relationship between NO deficiency and the development of hypertension and hypertensive end-organ damage.[1-3] NO deficiency can occur for many reasons, one of which is increased asymmetric dimethylarginine (ADMA). ADMA acts as an inhibitor to compete with L-arginine (L-Arg) for nitric oxide synthase (NOS) to generate NO.[4,5] Therefore, the L-Arginine-to-ADMA ratio (AAR) has been proposed to represent NO bioavailability.[6] ADMA is mainly metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), and approximately 20% of ADMA is excreted by the kidneys into the urine. Unlike ADMA, another structural isomer, symmetric dimethylarginine (SDMA), is excreted by the kidneys only. SDMA may serve as a marker of renal function and indirectly inhibit NO availability by competition with L-Arg for transporter uptake.[7] Both ADMA and SDMA are formed by posttranslational methylation of L-Arg by protein arginine methyltransferase (PRMT).

Spontaneously hypertensive rats (SHRs), a commonly employed experimental model of hypertension, exhibited a rise in BP starting from 5–6 wk of age, a steep increase between 6 wk and 20 wk of age, and progressive development of many

features of hypertensive end-organ damage.[8] Unlike salt-sensitive hypertensive models, SHRs are resistant to kidney damage.[9,10] We recently found that melatonin concurrently prevented increases in plasma ADMA and blood pressure in young SHRs.[11] In addition, preservation of renal L-Arg availability and restoration of the plasma AAR were found to be associated with a reduction in blood pressure. These findings suggest that L-Arg and ADMA may serve as biomarkers to predict pathologic processes and responses to therapy.

Although there is ample evidence linking ADMA with vascular disease and adverse cardiovascular outcomes,[12,13] few studies have been performed to determine whether combined analysis of L-Arg and SDMA and their ratios with ADMA (AAR= L-Arginine-to-ADMA ratio; ASR= ADMA-to-SDMA ratio) in the plasma and kidneys may unravel their relative importance in the development of hypertension and end-organ damage.

The ASR may provide indirect information about the DDAH activity, because only ADMA is metabolized by the enzyme;[14] however, whether the renal ASR can be taken to represent the renal DDAH activity is unclear. In addition, whether tissue ADMA levels or the related ratios are superior to those in the plasma, predicting end-organ damage, remains unknown.

This study aimed to elucidate the roles of the levels of plasma and renal L-Arg,

ADMA, SDMA, AAR and ASR in hypertension and hypertensive end-organ damage, which might provide a potential therapeutic approach to prevent disease progression via restoration of the NO pathway.

Materials and Methods

Animal protocol

This experiment was approved and was performed in accordance with the Guidelines for Animal Experiments of Chang Gung Memorial Hospital and Chang Gung University. Male SHRs and normotensive Wistar-Kyoto (WKY) control rats at different ages were obtained and were maintained under standard conditions with free access to tap water and standard rat chow: 3 wk of age (N = 6), 11 wk of age (N = 6), and 23 wk of age (N = 6). Blood pressure was measured in conscious rats by an indirect tail-cuff method (BP-2000, Visitech Systems, Inc., Apex, NC, USA) after systematic training.[11] Three stable consecutive measures were taken and averaged. Twenty-four-hour urine collections were performed for the determination of total protein by the Bradford method and NOx (NO_2^-+NO_3^-) levels by the Greiss reaction.[15] All rats were sacrificed 1 wk later. Heparinized blood samples were collected. The kidneys and heart were harvested and stored at -80°C until analysis. One kidney was removed, decapsulated, divided into the cortex and medulla, and snap-frozen for Western blot and HPLC. The other kidney was perfusion-fixed for histological analysis.

Detection of L-Arg, ADMA, and SDMA by HPLC

The levels of L-Arg, ADMA, and SDMA in the plasma and kidney were measured using HPLC (HP series 1100, Agilent Technologies, Inc., Santa Clara, CA, USA) with the o-phthaldialdehyde 3-mercaptopropionic acid (OPA-3MPA) derivatization reagent, as described previously.[11] Standards contained concentrations of L-Arg, ADMA, and SDMA in the range of 1–100 μM, 0.5–5 μM, and 0.5–5 μM respectively. The recovery rate was approximately 90–105%. The tissue concentration was factored for the protein concentration, which was represented as μM/mg protein.

DDAH activity

Dimethylarginine dimethylaminohydrolase (DDAH) activity was measured by a colorimetric assay measuring the rate of citrulline production, as optimized by ourselves.[16] The kidney cortex was homogenized by sodium phosphate buffer. The tissue homogenate was pre-incubated with urease for 15 min, then 100 μl (2 mg) of homogenate were incubated with 1mM ADMA for 45 min at 37ºC. After deproteinization, the supernatant was incubated with color mixture at 60ºC for 110 min. Each sample was analyzed with a paired blank (which omitted ADMA) to prevent the citrulline interference effect. The absorbance was measured by spectrophotometry at 466 nm. The DDAH activity was represented as μM citrulline formation/g protein/min at 37ºC.

Histological study

Histological analysis was performed on 4-μm sections of formalin-fixed kidney

blocked in paraffin wax and stained with periodic acid-Schiff (PAS). The level of renal injury was assessed on a blind basis by evaluating the glomerular injury. Glomerular injury (GI) scores representing sclerotic damage to glomeruli (N = 100) were calculated using the 0 to 4+ scale described previously.[17]

Western blot

Western blot analysis was performed as described previously.[15,17] The following antibodies were used: for protein arginine methyltransferase-1 (PRMT-1), rabbit anti-human PRMT-1 (1:200; Millipore, Billerica, MA, USA); for dimethylarginine dimethylaminohydrolase (DDAH), goat anti-rat DDAH-1 (1:500, overnight incubation; Santa Cruz, Santa Cruz, CA, USA) and goat anti-rat DDAH-2 (1:100, overnight incubation; Santa Cruz), followed by donkey anti-goat secondary antibody; for cationic amino acid transporter-1 (CAT-1), rabbit anti-rat CAT-1 (1:250; Abcam, Cambridge, MA, USA). The bands of interest were visualized using ECL reagents (PerkinElmer, Waltham, MA, USA) and quantified by densitometry (Quantity One Analysis software; Bio-Rad, Hercules, CA, USA), calculated as the integrated optical density (IOD) minus the background value. The IOD was adjusted for Ponceau red staining (PonS) to correct for variations in the total protein loading; protein abundance was expressed as IOD/PonS.

Statistical analysis

Morphological and biochemical parameters are presented as the mean ± SEM. Differences between specific means were compared using one-way ANOVA with post-hoc Fisher's least significant difference (LSD) tests. Correlations between two variables were examined using Pearson tests. All analyses were performed using the

statistical package SPSS version 14. A value of $P < 0.05$ was accepted as indicating statistical significance.

Results

As shown in Table 1, the body weights (BWs) of the SHRs were slightly greater than those of the WKYs at 4 wk and 24 wk of age. The SHRs exhibited significantly elevated systolic and diastolic BP and mean arterial pressure (MAP) as compared with the WKYs at 12 wk of age, and the BP was persistently high at 24 wk of age. The SHRs had a greater heart weight (HW) and heart weight/BW ratio than the WKYs at both 12 wk and 24 wk of age, suggesting the early development of cardiac hypertrophy. Urine protein excretion was slightly higher in the SHRs than in the WKYs at 4 wk and 24 wk of age. In contrast, the left kidney weight/BW ratio and clearance of creatinine (CCr) did not differ between the SHRs and WKYs of all ages. In agreement with previous studies, we demonstrated three stages of hypertension in SHRs, at 4 wk (prehypertensive stage), 12 wk (hypertensive stage), and 24 wk of age (hypertensive end-organ damage stage). The urine NOx (NO_2^- + NO_3^-) levels did not differ between the SHRs and WKYs at 4 wk and 12 wk, but were significantly lower in the SHRs than in the WKYs at 24 wk of age. The glomerular injury (GI) score did not differ significantly between the SHRs and WKYs at 4 wk and 12 wk, but tended to be higher in the SHRs than in the WKYs at 24 wk of age.

Table 2 shows that there was no difference in the plasma L-Arg level between the SHRs and WKYs in the three age groups. However, the plasma L-Arg level was lower in the SHRs at 12 wk and 24 wk of age than at 4 wk of age. The plasma ADMA level was higher in the SHRs vs. the WKYs in all three age groups. The plasma SDMA level was slightly increased in the SHRs vs. the WKYs at 12 wk of age. In the kidney, the L-Arg, ADMA, and SDMA levels did not differ between the SHRs and the WKYs at all ages, with the exception that the SHRs had a higher renal

ADMA concentration than the WKYs at 4 wk of age. Further, the renal ADMA level was found to be significantly higher in 12- and 24-wk-old WKYs as compared with 4-wk-old WKYs. The renal SDMA level at 24 wk of age in both the SHRs and WKYs was higher than those in the respective strains at 4 wk of age.

The plasma AAR was lower in the SHRs than in the WKYs in the three age groups. The plasma AAR was significantly decreased in the SHRs at 12 wk and 24 wk of age vs. 4-wk-old SHRs (Fig 1A). The renal AAR was lower in 4-wk-old SHRs than in WKYs, but this was not the case at 12 and 24 wk of age (Fig 1B). At 12 wk of age, the renal AAR was significantly decreased in the WKYs but increased in the SHRs as compared with their respective strains at 4wk of age. Unlike the AAR, the plasma ASR was higher in 24-wk-old SHRs than in age-matched WKYs, but this was not the case at 4 and 12 wk of age (Fig 1C). In contrast to the renal AAR, the ASR in the kidney was higher in SHRs than in WKYs at 4 wk of age, but not at 12 and 24 wk (Fig 1D).

The results shown in Table 3 demonstrate the correlations between blood pressure and L-Arg/ADMA/SDMA profiles. Both SBP and DBP were negatively related to the plasma L-Arg level and AAR, but positively related to the plasma ADMA level and ASR. There was a moderate negative correlation between MAP and the plasma L-Arg level and AAR. Yet MAP was positively related to the plasma ADMA level and ASR. In addition, the plasma AAR (r = 0.403; P = 0.015) and ASR (r = -0.364; P = 0.029) showed weak correlations with the urine NOx level. The L-Arg/ADMA/SDMA profiles were not correlated with other biochemical

parameters, such as clearance of creatinine and proteinuria. On the other hand, the levels of L-Arg, ADMA, SDMA, and their combined ratios in the kidney were not significantly correlated with clinical and biochemical parameters.

A stepwise regression model was used to examine the association between SBP (dependent variable) and the levels of L-Arg, ADMA, SDMA in the plasma and kidney, and their combined ratios. Our data showed that the plasma AAR was the strongest independent predictor of SBP ($P < 0.001$; $F = 28.277$), followed by the plasma SDMA level ($P < 0.001$; $F = 21.140$). The strongest predictor of DBP was the plasma AAR ($P < 0.001$; $F = 33.028$), followed by the plasma ASR ($P < 0.001$; $F = 26.716$). Similarly, the stepwise regression model indicated that the plasma AAR was the strongest independent predictor of MAP ($P < 0.001$; $F = 34.965$), followed by the plasma ASR ($P < 0.001$; $F = 28.297$). The plasma AAR was also a significantly independent predictor of the urine NOx level ($P = 0.015$; $F = 6.606$).

Figure 2A shows that the renal DDAH activity was higher in 4-wk-old SHRs than in WKYs, but this was not the case at 12 and 24 wk of age. The results indicated that elevation of the renal ADMA level in SHRs is associated with increased renal DDAH activity (ADMA-metabolizing enzyme). The renal DDAH activity was related to the renal L-Arg level ($r = -0.438$, $P = 0.007$), AAR ($r = -0.681$, $P < 0.001$, Fig 2B), and ASR ($r = 0.396$, $P = 0.017$, Fig 2C). A stepwise regression model with renal DDAH activity as the dependent variable showed that the renal AAR was the strongest independent predictor of DDAH activity ($P < 0.001$; $F = 29.483$).

Next, we evaluated the expression proteins that regulate the ADMA pathway. As shown in Figure 3B, we observed that the protein levels of CAT-1—the

transporter for ADMA—in the kidney were lower in the SHRs than in the WKY rats at 12 and 24 wk of age. The levels of PRMT-1—the ADMA-synthesizing enzyme—in the kidney did not differ between the SHRs and WKYs (Fig. 3C). The protein levels of DDAH-1 and-2, ADMA-metabolizing enzymes, were significantly lower in 12-wk-old SHR kidneys than those in WKYs (Fig. 3D and E). However, the renal expression of DDAH-1 and -2 did not differ between the SHRs and WKYs at 24 wk of age.

Discussion

Increases in the levels of ADMA in the plasma and kidneys, reductions in the AAR in the plasma and kidneys, and an increase in the renal ASR, develop before the onset of hypertension in 4-wk-old SHRs. The urinary NOx (NO_2^- + NO_3^-) levels were lower in the SHRs than in the WKY rats at 24 wk of age. In addition, an elevated plasma ADMA level and a decreased plasma AAR were found in the SHRs at 24 wk of age. These findings supported an association between reduced NO bioavailability and the development of hypertension and end-organ damage, which was in agreement with other studies.[1-3,18]

In the line with previous studies,[9,10,18] our data showed that SHRs are resistant to kidney injury because the renal outcome parameters are similar between SHRs and WKY rats at 24 wk of age (hypertensive end-organ damage stage). In addition, the kidneys of the WKY rats exhibited almost no glomerular injury at any of the ages studied. In SHR kidneys, only some glomeruli showed sclerosis at 24 wk of age. These pathological findings were consistent with a previous study showing that pathological changes in SHR kidneys do not progress rapidly until 45 wk of age.[19] Interestingly, the renal ADMA level and AAR were restored in SHRs at 24 wk of age. It is worthy of note that the changes in ADMA in the plasma across the three age groups were not identical to those seen in the kidney. As SHRs are resistant to kidney damage, elevation of ADMA levels were observed in the plasma but not in the kidneys at 12 and 24 wk of ages, which implied that the ability to reduce renal ADMA concentrations might be a protective mechanism against kidney damage. This concept is supported by a recent report showing that overexpression of DDAH to reduce ADMA can prevent progression of renal dysfunction.[20]

How do SHRs maintain their renal ADMA levels? We found that the changes

in renal DDAH activity across the three age groups were similar to those seen in the renal ADMA levels. Four-wk-old SHRs displayed a significantly elevated ADMA concentration and DDAH activity concurrently in the kidney but only a slightly increased ADMA level in the plasma. These findings suggested that renal DDAH activity might be upregulated to metabolize the excessive ADMA in the plasma and kidney to maintain a normal plasma ADMA level. However, this compensatory mechanism fails, and therefore the plasma ADMA level was increased by approximately 50% in the SHRs at 12 and 24 wk of age. As the kidney is a major organ in ADMA metabolism, as renal CAT-1 levels are decreased in SHR rats, as a decreased CAT activity can reduce cellular uptake of ADMA,[21] and as it was shown in a previous report that the CAT activity was lower in SHRs than in WKY rats in red blood cells,[22] another possibility is that the SHR kidney protects against injury by reducing the ADMA uptake from circulation into the kidneys. Intracellular ADMA is regulated by complex mechanisms, as reviewed by us and others,[4,5] further study is required in order to clarify whether restoration of renal ADMA homeostasis can protect kidney against hypertensive end-organ damage. Moreover, because ADMA can be produced in many organs, elucidation of the active sites causing excessive circulating ADMA might provide a specific ADMA-lowering approach for future study.

Importantly, we found a better correlation between renal DDAH activity and renal AAR than between renal DDAH activity and either L-Arg or ADMA. Although the plasma AAR has been considered as a marker of NO bioavailability,[7] little is known about this ratio in the kidney. We showed for the first time that the renal AAR is a strong independent predictor of renal DDAH activity, although the renal AAR was not highly-correlated with other clinical parameters. In contrast, the

plasma AAR was superior to the levels of L-Arg and ADMA in the plasma, and was related to a number of outcome factors, such as systolic and diastolic blood pressure, mean arterial pressure, and urinary NOx levels. Indeed, the plasma AAR was lowest in SHRs at 24 wk of age, which exhibited the poorest outcome.

Next, our previous study showed that restoration of reduced plasma AAR by melatonin is associated with a reduction in blood pressure in young SHRs.[11] We also observed that a decreased plasma AAR contributes to the developmental programming of adult hypertension, which can both be prevented by maternal supplementation with L-citrulline.[17] Accordingly, combined analysis of L-Arg and ADMA as represented by the AAR in plasma may serve as an ideal maker of hypertension to monitor prognosis and therapeutic response.

Although SDMA is used as a renal function marker,[6,13] we found that the plasma Cr level is not correlated with the SDMA level. As SHRs are resistant to kidney injury, therefore, the SDMA level might not be a good marker in SHRs with mild kidney disease. However, ASR, the combined ratio of ADMA and SDMA in plasma, was significantly correlated with SBP, DBP, and MBP. Given the fact that ADMA is mainly metabolized by DDAH and SDMA is only excreted by the kidney, the plasma ASR may indirectly represent DDAH activity.[6] We found that renal DDAH activity is related to the renal ASR (r = 0.396; P = 0.017), but not the plasma ASR (r = 0.116; P = 0.501). This finding suggested that the ASRs in the plasma and kidney are different, and these ratios should be interpreted cautiously with regards to representing DDAH activity in future studies.

In summary, the development of increases in ADMA in the plasma and kidneys may have begun in young SHRs preceding hypertension. In addition, the SHR kidney might be protected against injury via a reduction in the renal ADMA

concentration. The L-Arg and SDMA and subsequent ratios with ADMA—the AAR and ASR— may serve as better markers than each individual parameter for disease activity and disease progression. As the renal AAR and ASR are related to renal DDAH activity, both may aid in unraveling the importance of ADMA in hypertension and end-organ damage in future translational studies. Clinical validation will be required to compare panels of markers and achieve a consensus on which combination offers the most valuable clinical information.

Acknowledgements

This work was supported by grant NHRI-EX100-9826SC from the National Health Research Institutes, Taiwan and grant NSC 98-2314-B-182A-006-MY3 from the National Science Council, Taiwan. All authors have read the journal's policy on conflicts of interest and have none to declare.

References

1. Wilcox CS. Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? Am J Physiol Regul Integr Comp Physiol 2005;89:R913–R935.
2. Török J. Participation of nitric oxide in different models of experimental hypertension. Physiol Res 2008;57:813–825.
3. Baylis C. Arginine, arginine analogs and nitric oxide production in chronic kidney disease. Nat Clin Pract Nephrol 2006;2:209–220.
4. Teerlink T, Luo Z, Palm F, et al. Cellular ADMA: Regulation and action. Pharmacol Res 2009;60:448–460.
5. Tain YL, Huang LT, Asymmetric dimethylarginine: Clinical application in pediatric medicine. J Formos Med Assoc 2011;110:70–77.
6. Kielstein JT, Salpeter SR, Bode-Boeger SM, et al. Symmetric dimethylarginine (SDMA) as endogenous marker of renal function—a meta-analysis. Nephrol Dial Transplant 2006;21:2446–2451.
7. Bode-Boger SM, Scalera F, Ignarro LJ. The l-arginine paradox: Importance of the l-arginine/asymmetrical dimethylarginine ratio. Pharmacol Ther 2007;114:295–306.
8. Pinto YM, Paul M, Ganten D. Lessons from rat models of hypertension: from Goldblatt to genetic engineering. Cardiovasc Res 1998;39:77–88.
9. Uchino K, Frohlich ED, Nishikimi T, et al. Spontaneously hypertensive rats demonstrate increased renal vascular alpha 1-adrenergic receptor responsiveness. Am J Physiol 1991;260:R889–R893.
10. Numabe A, Komatsu K, Frohlich ED. Effects of ANG-converting enzyme and alpha1-adrenoceptor inhibition on intrarenal hemodynamics in SHR. Am J

Physiol 1994;266:R1437–R1442.

11. Tain YL, Huang LT, Lin IC, et al. Melatonin prevents hypertension and increased asymmetric dimethylarginine in young spontaneous hypertensive rats. J Pineal Res 2010;49:390–398.
12. Böger RH. The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor. Cardiovasc Res 2003;59:824–833.
13. Jacobi J, Tsao PS. Asymmetrical dimethylarginine in renal disease: limits of variation or variation limits? A systematic review. Am J Nephrol 2008;28:224–237.
14. Iapichino G, Umbrello M, Albicini M, et al. Time course of endogenous nitric oxide inhibitors in severe sepsis in humans. Minerva Anestesiol 2010;76:325–333.
15. Tain YL, Freshour G, Dikalova A, et al. Vitamin E reduces glomerulosclerosis, restores renal neuronal NOS, and suppresses oxidative stress in the 5/6 nephrectomized rat. Am J Physiol Renal Physiol 2007;292:F1404–F1410.
16. Tain YL, Baylis C. Determination of dimethylarginine dimethylaminohydrolase activity in the kidney. Kidney Int 2007;72:886–889.
17. Tain YL, Hsieh CS, Lin IC, et al. Effects of maternal L-citrulline supplementation on renal function and blood pressure in offspring exposed to maternal caloric restriction: the impact of nitric oxide pathway. Nitric Oxide 2010;23:34–41.
18. Verhagen AM, Koomans HA, Joles JA. Predisposition of spontaneously hypertensive rats to develop renal injury during nitric oxide synthase inhibition. Eur J Pharmacol 2001;411:175–180.

19. Feld LG, Van Liew JB, Galaske RG, et al. Selectivity of renal injury and proteinuria in the spontaneously hypertensive rat. Kidney Int 1977;12:332–343.
20. Matsumoto Y, Ueda S, Yamagishi S, et al. 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 2007;18:1525–1533.
21. Luo Z, Teerlink T, Griendling K, et al. Angiotensin II and NADPH oxidase increase ADMA in vascular smooth muscle cells. Hypertension 2010;56:498–504.
22. Moss MB, Brunini TM, Soares De Moura R, et al. Diminished L-arginine bioavailability in hypertension. Clin Sci (Lond) 2004;107:391–397.

Legends

Figure 1. (A) Plasma and (B) renal L-arginine to ADMA ratio (AAR), and (C) plasma and (D) renal ADMA to SDMA ratio (ASR) in SHRs and WKY rats at different ages. N = 6/group; *$P < 0.05$ vs. 4-wk-old respective strains; $^{\#}P < 0.05$ vs. respective age-matched WKYs.

Figure 2. (A) In vitro DDAH activity in the kidney in SHRs and WKY rats at different ages. (B) The correlation between renal DDAH activity and renal L-arginine and the ADMA ratio (AAR). (C) The correlation between renal DDAH activity and renal ADMA and the SDMA ratio (ASR). N = 6/group; *$P < 0.05$ vs. 4-wk-old respective strains; $^{\#}P < 0.05$ vs. respective age-matched WKYs.

Figure 3. Representative western blots (A) showing CAT-1 (~68 kDa), PRMT-1 (~42kDa), DDAH-1 (~34kDa), and DDAH-2 (~30kDa) bands in WKY rats and SHRs at 12 and 24 wk of age. Relative abundance of renal cortical (B) CAT-1, (C) PRMT-1, (D) DDAH-1, and (E) DDAH-2. N = 6 per group; $^{*}P < 0.05$ 12 wk vs. 24 wk; $^{\#}P < 0.05$ vs. respective age-matched WKYs.

Figure 4. A simplified scheme of L-Arginine (Arg), ADMA, SDMA, and ratios with ADMA (AAR= L-Arginine-to-ADMA ratio; ASR= ADMA-to-SDMA ratio) in the plasma and kidneys of 4- and 24-wk-old SHRs. Distinct changes in the plasma and renal levels of ADMA may occur upon synthesis by PRMT, degradation by DDAH, or transport by CAT. See text for a detailed description. CAT = cationic amino acid transporter; DDAH= dimethylarginine dimethylaminohydrolase; PRMT= protein

arginine methyltransferases.

The combined ratios of L-arginine, asymmetric and symmetric dimethylarginine as biomarkers in spontaneously hypertensive rats

Chien-Ning Hsu[a], Li-Tung Huang[b], Ying-Tung Lau[c], Ching-Yuang Lin[d],You-Lin Tain[b]

[a]Departments of Pharmacy and [b]Pediatrics, Kaohsiung Chang Gung Memorial Hospital, and Chang Gung University College of Medicine, Taiwan

[c]Department of Physiology and Pharmacology, College of Medicine, Chang Gung University, and Chang Gung Institute of Technology, Taoyuan, Taiwan

[d]College of Medicine, China Medical University, Clinical Immunological Center, and Division of Pediatric Nephrology, China Medical University Hospital, Taichung, Taiwan

Corresponding author:

You-Lin Tain, MD, PhD.

Department of Pediatrics, Chang Gung Memorial Hospital at Kaohsiung, 123 Dabi Rd., Niausung, Kaohsiung 833, Taiwan

E-mail address: tainyl@hotmail.com

Tel: +886-975056995, Fax: +886-7-7338009

Abstract

Hypertension and hypertensive end-organ damage have been associated with decreased nitric oxide (NO) bioavailability. Asymmetric and symmetric dimethylarginine (ADMA and SDMA) can both inhibit NO availability by competition with L-arginine (L-Arg). However, whether combined analysis of these three parameters can serve as an ideal biomarker of hypertension remains unclear. We measured the plasma and renal levels of L-Arg, ADMA, and SDMA in spontaneously hypertensive rats (SHRs) and Wistar-Kyoto (WKY) control rats at three stages: 4-wk-old (pre-hypertensive), 12-wk-old (hypertensive), and 24-wk-old (end-organ damage). The plasma and renal L-Arg/ADMA ratio (AAR) and the ADMA/SDMA ratio (ASR) were computed for all three age stages. Our results revealed an ADMA level increase and an AAR decrease in plasma and kidneys may develop early on, even before the onset of hypertension in 4-wk-old SHRs. The renal ADMA level and AAR were restored in SHRs at 24 wk of age, which might protect SHRs against kidney injury. We found that the plasma AAR is superior to the levels of L-Arg and ADMA in plasma, and predicted blood pressure and urinary NOx levels; and renal AAR is a strong independent marker of renal dimethylarginine dimethylaminohydrolase (DDAH) activity. The plasma ASR was strongly correlated to blood pressure. However, renal DDAH activity was related to the renal ASR, but not the plasma ASR. In conclusion, the AAR and ASR may serve as better markers for disease activity and progression than each individual parameter. Clinical use of these ratios to elucidate the role of ADMA in hypertension awaits further validation.

Key words: asymmetric dimethylarginine, symmetric dimethylarginine, hypertension, nitric oxide, spontaneously hypertensive rat.

Running title: AAR and ASR in SHR

Abbreviation:

AAR= L-Arginine-to-ADMA ratio

ADMA= asymmetric dimethylarginine

ASR= ADMA-to-SDMA ratio

DDAH= dimethylarginine dimethylaminohydrolase

NOS= nitric oxide synthase

PRMT= protein arginine methyltransferase

SHR= spontaneously hypertensive rat

WKY**=** Wistar Kyoto

Introduction

Nitric oxide (NO) results in vasodilatation to regulate systemic blood pressure (BP) and local blood flow. There is a growing body of evidence demonstrating a close relationship between NO deficiency and the development of hypertension and hypertensive end-organ damage.[1-3] NO deficiency can occur for many reasons, one of which is increased asymmetric dimethylarginine (ADMA). ADMA acts as an inhibitor to compete with L-arginine (L-Arg) for nitric oxide synthase (NOS) to generate NO.[4,5] Therefore, the L-Arginine-to-ADMA ratio (AAR) has been proposed to represent NO bioavailability.[6] ADMA is mainly metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH), and approximately 20% of ADMA is excreted by the kidneys into the urine. Unlike ADMA, another structural isomer, symmetric dimethylarginine (SDMA), is excreted by the kidneys only. SDMA may serve as a marker of renal function and indirectly inhibit NO availability by competition with L-Arg for transporter uptake.[7] Both ADMA and SDMA are formed by posttranslational methylation of L-Arg by protein arginine methyltransferase (PRMT).

Spontaneously hypertensive rats (SHRs), a commonly employed experimental model of hypertension, exhibited a rise in BP starting from 5–6 wk of age, a steep increase between 6 wk and 20 wk of age, and progressive development of many

features of hypertensive end-organ damage.[8] Unlike salt-sensitive hypertensive models, SHRs are resistant to kidney damage.[9,10] We recently found that melatonin concurrently prevented increases in plasma ADMA and blood pressure in young SHRs.[11] In addition, preservation of renal L-Arg availability and restoration of the plasma AAR were found to be associated with a reduction in blood pressure. These findings suggest that L-Arg and ADMA may serve as biomarkers to predict pathologic processes and responses to therapy.

Although there is ample evidence linking ADMA with vascular disease and adverse cardiovascular outcomes,[12,13] few studies have been performed to determine whether combined analysis of L-Arg and SDMA and their ratios with ADMA (AAR= L-Arginine-to-ADMA ratio; ASR= ADMA-to-SDMA ratio) in the plasma and kidneys may unravel their relative importance in the development of hypertension and end-organ damage.

The ASR may provide indirect information about the DDAH activity, because only ADMA is metabolized by the enzyme;[14] however, whether the renal ASR can be taken to represent the renal DDAH activity is unclear. In addition, whether tissue ADMA levels or the related ratios are superior to those in the plasma, predicting end-organ damage, remains unknown.

This study aimed to elucidate the roles of the levels of plasma and renal L-Arg,

ADMA, SDMA, AAR and ASR in hypertension and hypertensive end-organ damage, which might provide a potential therapeutic approach to prevent disease progression via restoration of the NO pathway.

Materials and Methods

Animal protocol

This experiment was approved and was performed in accordance with the Guidelines for Animal Experiments of Chang Gung Memorial Hospital and Chang Gung University. Male SHRs and normotensive Wistar-Kyoto (WKY) control rats at different ages were obtained and were maintained under standard conditions with free access to tap water and standard rat chow: 3 wk of age (N = 6), 11 wk of age (N = 6), and 23 wk of age (N = 6). Blood pressure was measured in conscious rats by an indirect tail-cuff method (BP-2000, Visitech Systems, Inc., Apex, NC, USA) after systematic training.[11] Three stable consecutive measures were taken and averaged. Twenty-four-hour urine collections were performed for the determination of total protein by the Bradford method and NOx (NO_2^-+NO_3^-) levels by the Greiss reaction.[15] All rats were sacrificed 1 wk later. Heparinized blood samples were collected. The kidneys and heart were harvested and stored at -80°C until analysis. One kidney was removed, decapsulated, divided into the cortex and medulla, and snap-frozen for Western blot and HPLC. The other kidney was perfusion-fixed for histological analysis.

Detection of L-Arg, ADMA, and SDMA by HPLC

The levels of L-Arg, ADMA, and SDMA in the plasma and kidney were measured using HPLC (HP series 1100, Agilent Technologies, Inc., Santa Clara, CA, USA) with the o-phthaldialdehyde 3-mercaptopropionic acid (OPA-3MPA) derivatization reagent, as described previously.[11] Standards contained concentrations of L-Arg, ADMA, and SDMA in the range of 1–100 μM, 0.5–5 μM, and 0.5–5 μM respectively. The recovery rate was approximately 90–105%. The tissue concentration was factored for the protein concentration, which was represented as μM/mg protein.

DDAH activity

Dimethylarginine dimethylaminohydrolase (DDAH) activity was measured by a colorimetric assay measuring the rate of citrulline production, as optimized by ourselves.[16] The kidney cortex was homogenized by sodium phosphate buffer. The tissue homogenate was pre-incubated with urease for 15 min, then 100 μl (2 mg) of homogenate were incubated with 1mM ADMA for 45 min at 37ºC. After deproteinization, the supernatant was incubated with color mixture at 60ºC for 110 min. Each sample was analyzed with a paired blank (which omitted ADMA) to prevent the citrulline interference effect. The absorbance was measured by spectrophotometry at 466 nm. The DDAH activity was represented as μM citrulline formation/g protein/min at 37ºC.

Histological study

Histological analysis was performed on 4-μm sections of formalin-fixed kidney

blocked in paraffin wax and stained with periodic acid-Schiff (PAS). The level of renal injury was assessed on a blind basis by evaluating the glomerular injury. Glomerular injury (GI) scores representing sclerotic damage to glomeruli (N = 100) were calculated using the 0 to 4+ scale described previously.[17]

Western blot

Western blot analysis was performed as described previously.[15,17] The following antibodies were used: for protein arginine methyltransferase-1 (PRMT-1), rabbit anti-human PRMT-1 (1:200; Millipore, Billerica, MA, USA); for dimethylarginine dimethylaminohydrolase (DDAH), goat anti-rat DDAH-1 (1:500, overnight incubation; Santa Cruz, Santa Cruz, CA, USA) and goat anti-rat DDAH-2 (1:100, overnight incubation; Santa Cruz), followed by donkey anti-goat secondary antibody; for cationic amino acid transporter-1 (CAT-1), rabbit anti-rat CAT-1 (1:250; Abcam, Cambridge, MA, USA). The bands of interest were visualized using ECL reagents (PerkinElmer, Waltham, MA, USA) and quantified by densitometry (Quantity One Analysis software; Bio-Rad, Hercules, CA, USA), calculated as the integrated optical density (IOD) minus the background value. The IOD was adjusted for Ponceau red staining (PonS) to correct for variations in the total protein loading; protein abundance was expressed as IOD/PonS.

Statistical analysis

Morphological and biochemical parameters are presented as the mean ± SEM. Differences between specific means were compared using one-way ANOVA with post-hoc Fisher's least significant difference (LSD) tests. Correlations between two variables were examined using Pearson tests. All analyses were performed using the

statistical package SPSS version 14. A value of $P < 0.05$ was accepted as indicating statistical significance.

Results

As shown in Table 1, the body weights (BWs) of the SHRs were slightly greater than those of the WKYs at 4 wk and 24 wk of age. The SHRs exhibited significantly elevated systolic and diastolic BP and mean arterial pressure (MAP) as compared with the WKYs at 12 wk of age, and the BP was persistently high at 24 wk of age. The SHRs had a greater heart weight (HW) and heart weight/BW ratio than the WKYs at both 12 wk and 24 wk of age, suggesting the early development of cardiac hypertrophy. Urine protein excretion was slightly higher in the SHRs than in the WKYs at 4 wk and 24 wk of age. In contrast, the left kidney weight/BW ratio and clearance of creatinine (CCr) did not differ between the SHRs and WKYs of all ages. In agreement with previous studies, we demonstrated three stages of hypertension in SHRs, at 4 wk (prehypertensive stage), 12 wk (hypertensive stage), and 24 wk of age (hypertensive end-organ damage stage). The urine NOx (NO_2^- + NO_3^-) levels did not differ between the SHRs and WKYs at 4 wk and 12 wk, but were significantly lower in the SHRs than in the WKYs at 24 wk of age. The glomerular injury (GI) score did not differ significantly between the SHRs and WKYs at 4 wk and 12 wk, but tended to be higher in the SHRs than in the WKYs at 24 wk of age.

Table 2 shows that there was no difference in the plasma L-Arg level between the SHRs and WKYs in the three age groups. However, the plasma L-Arg level was lower in the SHRs at 12 wk and 24 wk of age than at 4 wk of age. The plasma ADMA level was higher in the SHRs vs. the WKYs in all three age groups. The plasma SDMA level was slightly increased in the SHRs vs. the WKYs at 12 wk of age. In the kidney, the L-Arg, ADMA, and SDMA levels did not differ between the SHRs and the WKYs at all ages, with the exception that the SHRs had a higher renal

ADMA concentration than the WKYs at 4 wk of age. Further, the renal ADMA level was found to be significantly higher in 12- and 24-wk-old WKYs as compared with 4-wk-old WKYs. The renal SDMA level at 24 wk of age in both the SHRs and WKYs was higher than those in the respective strains at 4 wk of age.

The plasma AAR was lower in the SHRs than in the WKYs in the three age groups. The plasma AAR was significantly decreased in the SHRs at 12 wk and 24 wk of age vs. 4-wk-old SHRs (Fig 1A). The renal AAR was lower in 4-wk-old SHRs than in WKYs, but this was not the case at 12 and 24 wk of age (Fig 1B). At 12 wk of age, the renal AAR was significantly decreased in the WKYs but increased in the SHRs as compared with their respective strains at 4wk of age. Unlike the AAR, the plasma ASR was higher in 24-wk-old SHRs than in age-matched WKYs, but this was not the case at 4 and 12 wk of age (Fig 1C). In contrast to the renal AAR, the ASR in the kidney was higher in SHRs than in WKYs at 4 wk of age, but not at 12 and 24 wk (Fig 1D).

The results shown in Table 3 demonstrate the correlations between blood pressure and L-Arg/ADMA/SDMA profiles. Both SBP and DBP were negatively related to the plasma L-Arg level and AAR, but positively related to the plasma ADMA level and ASR. There was a moderate negative correlation between MAP and the plasma L-Arg level and AAR. Yet MAP was positively related to the plasma ADMA level and ASR. In addition, the plasma AAR (r = 0.403; P = 0.015) and ASR (r = -0.364; P = 0.029) showed weak correlations with the urine NOx level. The L-Arg/ADMA/SDMA profiles were not correlated with other biochemical

parameters, such as clearance of creatinine and proteinuria. On the other hand, the levels of L-Arg, ADMA, SDMA, and their combined ratios in the kidney were not significantly correlated with clinical and biochemical parameters.

A stepwise regression model was used to examine the association between SBP (dependent variable) and the levels of L-Arg, ADMA, SDMA in the plasma and kidney, and their combined ratios. Our data showed that the plasma AAR was the strongest independent predictor of SBP ($P < 0.001$; $F = 28.277$), followed by the plasma SDMA level ($P < 0.001$; $F = 21.140$). The strongest predictor of DBP was the plasma AAR ($P < 0.001$; $F = 33.028$), followed by the plasma ASR ($P < 0.001$; $F = 26.716$). Similarly, the stepwise regression model indicated that the plasma AAR was the strongest independent predictor of MAP ($P < 0.001$; $F = 34.965$), followed by the plasma ASR ($P < 0.001$; $F = 28.297$). The plasma AAR was also a significantly independent predictor of the urine NOx level ($P = 0.015$; $F = 6.606$).

Figure 2A shows that the renal DDAH activity was higher in 4-wk-old SHRs than in WKYs, but this was not the case at 12 and 24 wk of age. The results indicated that elevation of the renal ADMA level in SHRs is associated with increased renal DDAH activity (ADMA-metabolizing enzyme). The renal DDAH activity was related to the renal L-Arg level ($r = -0.438$, $P = 0.007$), AAR ($r = -0.681$, $P < 0.001$, Fig 2B), and ASR ($r = 0.396$, $P = 0.017$, Fig 2C). A stepwise regression model with renal DDAH activity as the dependent variable showed that the renal AAR was the strongest independent predictor of DDAH activity ($P < 0.001$; $F = 29.483$).

Next, we evaluated the expression proteins that regulate the ADMA pathway. As shown in Figure 3B, we observed that the protein levels of CAT-1—the

transporter for ADMA—in the kidney were lower in the SHRs than in the WKY rats at 12 and 24 wk of age. The levels of PRMT-1—the ADMA-synthesizing enzyme—in the kidney did not differ between the SHRs and WKYs (Fig. 3C). The protein levels of DDAH-1 and-2, ADMA-metabolizing enzymes, were significantly lower in 12-wk-old SHR kidneys than those in WKYs (Fig. 3D and E). However, the renal expression of DDAH-1 and -2 did not differ between the SHRs and WKYs at 24 wk of age.

Discussion

Increases in the levels of ADMA in the plasma and kidneys, reductions in the AAR in the plasma and kidneys, and an increase in the renal ASR, develop before the onset of hypertension in 4-wk-old SHRs. The urinary NOx (NO_2^- + NO_3^-) levels were lower in the SHRs than in the WKY rats at 24 wk of age. In addition, an elevated plasma ADMA level and a decreased plasma AAR were found in the SHRs at 24 wk of age. These findings supported an association between reduced NO bioavailability and the development of hypertension and end-organ damage, which was in agreement with other studies.[1-3,18]

In the line with previous studies,[9,10,18] our data showed that SHRs are resistant to kidney injury because the renal outcome parameters are similar between SHRs and WKY rats at 24 wk of age (hypertensive end-organ damage stage). In addition, the kidneys of the WKY rats exhibited almost no glomerular injury at any of the ages studied. In SHR kidneys, only some glomeruli showed sclerosis at 24 wk of age. These pathological findings were consistent with a previous study showing that pathological changes in SHR kidneys do not progress rapidly until 45 wk of age.[19] Interestingly, the renal ADMA level and AAR were restored in SHRs at 24 wk of age. It is worthy of note that the changes in ADMA in the plasma across the three age groups were not identical to those seen in the kidney. As SHRs are resistant to kidney damage, elevation of ADMA levels were observed in the plasma but not in the kidneys at 12 and 24 wk of ages, which implied that the ability to reduce renal ADMA concentrations might be a protective mechanism against kidney damage. This concept is supported by a recent report showing that overexpression of DDAH to reduce ADMA can prevent progression of renal dysfunction.[20]

How do SHRs maintain their renal ADMA levels? We found that the changes

in renal DDAH activity across the three age groups were similar to those seen in the renal ADMA levels. Four-wk-old SHRs displayed a significantly elevated ADMA concentration and DDAH activity concurrently in the kidney but only a slightly increased ADMA level in the plasma. These findings suggested that renal DDAH activity might be upregulated to metabolize the excessive ADMA in the plasma and kidney to maintain a normal plasma ADMA level. However, this compensatory mechanism fails, and therefore the plasma ADMA level was increased by approximately 50% in the SHRs at 12 and 24 wk of age. As the kidney is a major organ in ADMA metabolism, as renal CAT-1 levels are decreased in SHR rats, as a decreased CAT activity can reduce cellular uptake of ADMA,[21] and as it was shown in a previous report that the CAT activity was lower in SHRs than in WKY rats in red blood cells,[22] another possibility is that the SHR kidney protects against injury by reducing the ADMA uptake from circulation into the kidneys. Intracellular ADMA is regulated by complex mechanisms, as reviewed by us and others,[4,5] further study is required in order to clarify whether restoration of renal ADMA homeostasis can protect kidney against hypertensive end-organ damage. Moreover, because ADMA can be produced in many organs, elucidation of the active sites causing excessive circulating ADMA might provide a specific ADMA-lowering approach for future study.

Importantly, we found a better correlation between renal DDAH activity and renal AAR than between renal DDAH activity and either L-Arg or ADMA. Although the plasma AAR has been considered as a marker of NO bioavailability,[7] little is known about this ratio in the kidney. We showed for the first time that the renal AAR is a strong independent predictor of renal DDAH activity, although the renal AAR was not highly-correlated with other clinical parameters. In contrast, the

plasma AAR was superior to the levels of L-Arg and ADMA in the plasma, and was related to a number of outcome factors, such as systolic and diastolic blood pressure, mean arterial pressure, and urinary NOx levels. Indeed, the plasma AAR was lowest in SHRs at 24 wk of age, which exhibited the poorest outcome.

Next, our previous study showed that restoration of reduced plasma AAR by melatonin is associated with a reduction in blood pressure in young SHRs.[11] We also observed that a decreased plasma AAR contributes to the developmental programming of adult hypertension, which can both be prevented by maternal supplementation with L-citrulline.[17] Accordingly, combined analysis of L-Arg and ADMA as represented by the AAR in plasma may serve as an ideal maker of hypertension to monitor prognosis and therapeutic response.

Although SDMA is used as a renal function marker,[6,13] we found that the plasma Cr level is not correlated with the SDMA level. As SHRs are resistant to kidney injury, therefore, the SDMA level might not be a good marker in SHRs with mild kidney disease. However, ASR, the combined ratio of ADMA and SDMA in plasma, was significantly correlated with SBP, DBP, and MBP. Given the fact that ADMA is mainly metabolized by DDAH and SDMA is only excreted by the kidney, the plasma ASR may indirectly represent DDAH activity.[6] We found that renal DDAH activity is related to the renal ASR (r = 0.396; P = 0.017), but not the plasma ASR (r = 0.116; P = 0.501). This finding suggested that the ASRs in the plasma and kidney are different, and these ratios should be interpreted cautiously with regards to representing DDAH activity in future studies.

In summary, the development of increases in ADMA in the plasma and kidneys may have begun in young SHRs preceding hypertension. In addition, the SHR kidney might be protected against injury via a reduction in the renal ADMA

concentration. The L-Arg and SDMA and subsequent ratios with ADMA—the AAR and ASR— may serve as better markers than each individual parameter for disease activity and disease progression. As the renal AAR and ASR are related to renal DDAH activity, both may aid in unraveling the importance of ADMA in hypertension and end-organ damage in future translational studies. Clinical validation will be required to compare panels of markers and achieve a consensus on which combination offers the most valuable clinical information.

Acknowledgements

This work was supported by grant NHRI-EX100-9826SC from the National Health Research Institutes, Taiwan and grant NSC 98-2314-B-182A-006-MY3 from the National Science Council, Taiwan. All authors have read the journal's policy on conflicts of interest and have none to declare.

References

1. Wilcox CS. Oxidative stress and nitric oxide deficiency in the kidney: a critical link to hypertension? Am J Physiol Regul Integr Comp Physiol 2005;89:R913–R935.
2. Török J. Participation of nitric oxide in different models of experimental hypertension. Physiol Res 2008;57:813–825.
3. Baylis C. Arginine, arginine analogs and nitric oxide production in chronic kidney disease. Nat Clin Pract Nephrol 2006;2:209–220.
4. Teerlink T, Luo Z, Palm F, et al. Cellular ADMA: Regulation and action. Pharmacol Res 2009;60:448–460.
5. Tain YL, Huang LT, Asymmetric dimethylarginine: Clinical application in pediatric medicine. J Formos Med Assoc 2011;110:70–77.
6. Kielstein JT, Salpeter SR, Bode-Boeger SM, et al. Symmetric dimethylarginine (SDMA) as endogenous marker of renal function—a meta-analysis. Nephrol Dial Transplant 2006;21:2446–2451.
7. Bode-Boger SM, Scalera F, Ignarro LJ. The l-arginine paradox: Importance of the l-arginine/asymmetrical dimethylarginine ratio. Pharmacol Ther 2007;114:295–306.
8. Pinto YM, Paul M, Ganten D. Lessons from rat models of hypertension: from Goldblatt to genetic engineering. Cardiovasc Res 1998;39:77–88.
9. Uchino K, Frohlich ED, Nishikimi T, et al. Spontaneously hypertensive rats demonstrate increased renal vascular alpha 1-adrenergic receptor responsiveness. Am J Physiol 1991;260:R889–R893.
10. Numabe A, Komatsu K, Frohlich ED. Effects of ANG-converting enzyme and alpha1-adrenoceptor inhibition on intrarenal hemodynamics in SHR. Am J

Physiol 1994;266:R1437–R1442.

11. Tain YL, Huang LT, Lin IC, et al. Melatonin prevents hypertension and increased asymmetric dimethylarginine in young spontaneous hypertensive rats. J Pineal Res 2010;49:390–398.
12. Böger RH. The emerging role of asymmetric dimethylarginine as a novel cardiovascular risk factor. Cardiovasc Res 2003;59:824–833.
13. Jacobi J, Tsao PS. Asymmetrical dimethylarginine in renal disease: limits of variation or variation limits? A systematic review. Am J Nephrol 2008;28:224–237.
14. Iapichino G, Umbrello M, Albicini M, et al. Time course of endogenous nitric oxide inhibitors in severe sepsis in humans. Minerva Anestesiol 2010;76:325–333.
15. Tain YL, Freshour G, Dikalova A, et al. Vitamin E reduces glomerulosclerosis, restores renal neuronal NOS, and suppresses oxidative stress in the 5/6 nephrectomized rat. Am J Physiol Renal Physiol 2007;292:F1404–F1410.
16. Tain YL, Baylis C. Determination of dimethylarginine dimethylaminohydrolase activity in the kidney. Kidney Int 2007;72:886–889.
17. Tain YL, Hsieh CS, Lin IC, et al. Effects of maternal L-citrulline supplementation on renal function and blood pressure in offspring exposed to maternal caloric restriction: the impact of nitric oxide pathway. Nitric Oxide 2010;23:34–41.
18. Verhagen AM, Koomans HA, Joles JA. Predisposition of spontaneously hypertensive rats to develop renal injury during nitric oxide synthase inhibition. Eur J Pharmacol 2001;411:175–180.

19. Feld LG, Van Liew JB, Galaske RG, et al. Selectivity of renal injury and proteinuria in the spontaneously hypertensive rat. Kidney Int 1977;12:332–343.
20. Matsumoto Y, Ueda S, Yamagishi S, et al. 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 2007;18:1525–1533.
21. Luo Z, Teerlink T, Griendling K, et al. Angiotensin II and NADPH oxidase increase ADMA in vascular smooth muscle cells. Hypertension 2010;56:498–504.
22. Moss MB, Brunini TM, Soares De Moura R, et al. Diminished L-arginine bioavailability in hypertension. Clin Sci (Lond) 2004;107:391–397.

Legends

Figure 1. (A) Plasma and (B) renal L-arginine to ADMA ratio (AAR), and (C) plasma and (D) renal ADMA to SDMA ratio (ASR) in SHRs and WKY rats at different ages. N = 6/group; *$P < 0.05$ vs. 4-wk-old respective strains; $^{\#}P < 0.05$ vs. respective age-matched WKYs.

Figure 2. (A) In vitro DDAH activity in the kidney in SHRs and WKY rats at different ages. (B) The correlation between renal DDAH activity and renal L-arginine and the ADMA ratio (AAR). (C) The correlation between renal DDAH activity and renal ADMA and the SDMA ratio (ASR). N = 6/group; *$P < 0.05$ vs. 4-wk-old respective strains; $^{\#}P < 0.05$ vs. respective age-matched WKYs.

Figure 3. Representative western blots (A) showing CAT-1 (~68 kDa), PRMT-1 (~42kDa), DDAH-1 (~34kDa), and DDAH-2 (~30kDa) bands in WKY rats and SHRs at 12 and 24 wk of age. Relative abundance of renal cortical (B) CAT-1, (C) PRMT-1, (D) DDAH-1, and (E) DDAH-2. N = 6 per group; $^{*}P < 0.05$ 12 wk vs. 24 wk; $^{\#}P < 0.05$ vs. respective age-matched WKYs.

Figure 4. A simplified scheme of L-Arginine (Arg), ADMA, SDMA, and ratios with ADMA (AAR= L-Arginine-to-ADMA ratio; ASR= ADMA-to-SDMA ratio) in the plasma and kidneys of 4- and 24-wk-old SHRs. Distinct changes in the plasma and renal levels of ADMA may occur upon synthesis by PRMT, degradation by DDAH, or transport by CAT. See text for a detailed description. CAT = cationic amino acid transporter; DDAH= dimethylarginine dimethylaminohydrolase; PRMT= protein

arginine methyltransferases.

Figure1

Click here to download high resolution image

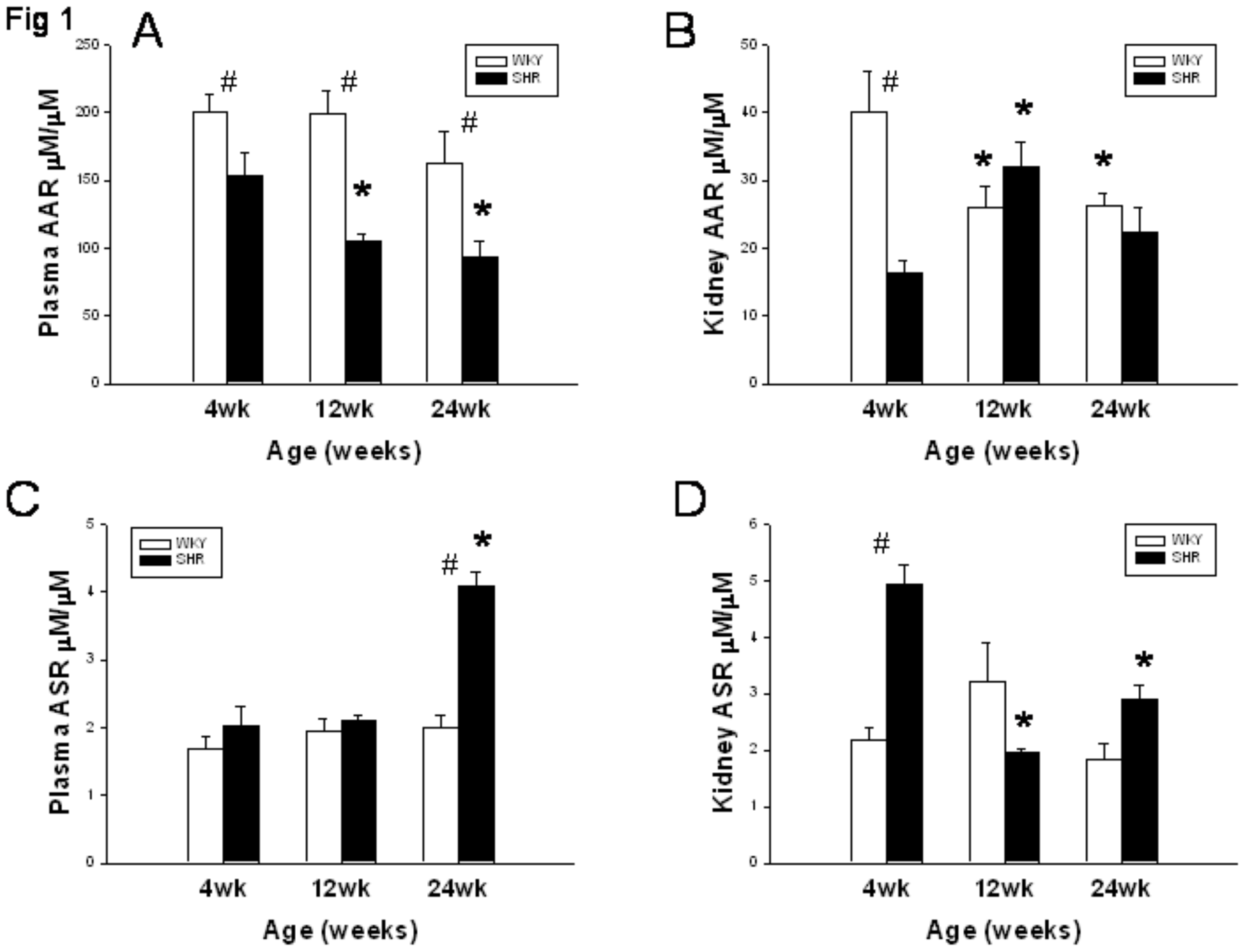


Figure2a

Click here to download high resolution image

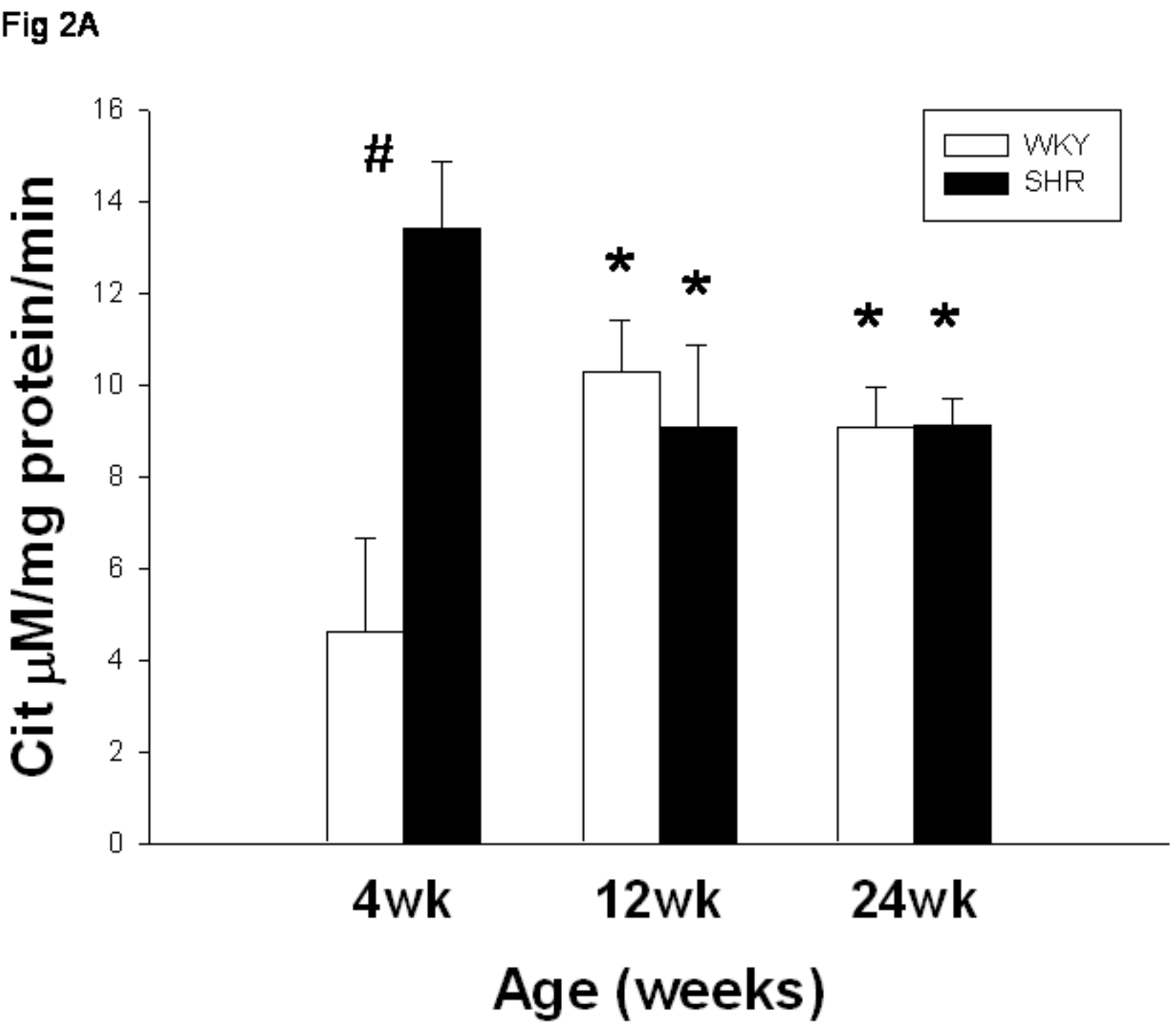


Figure2b

Click here to download high resolution image

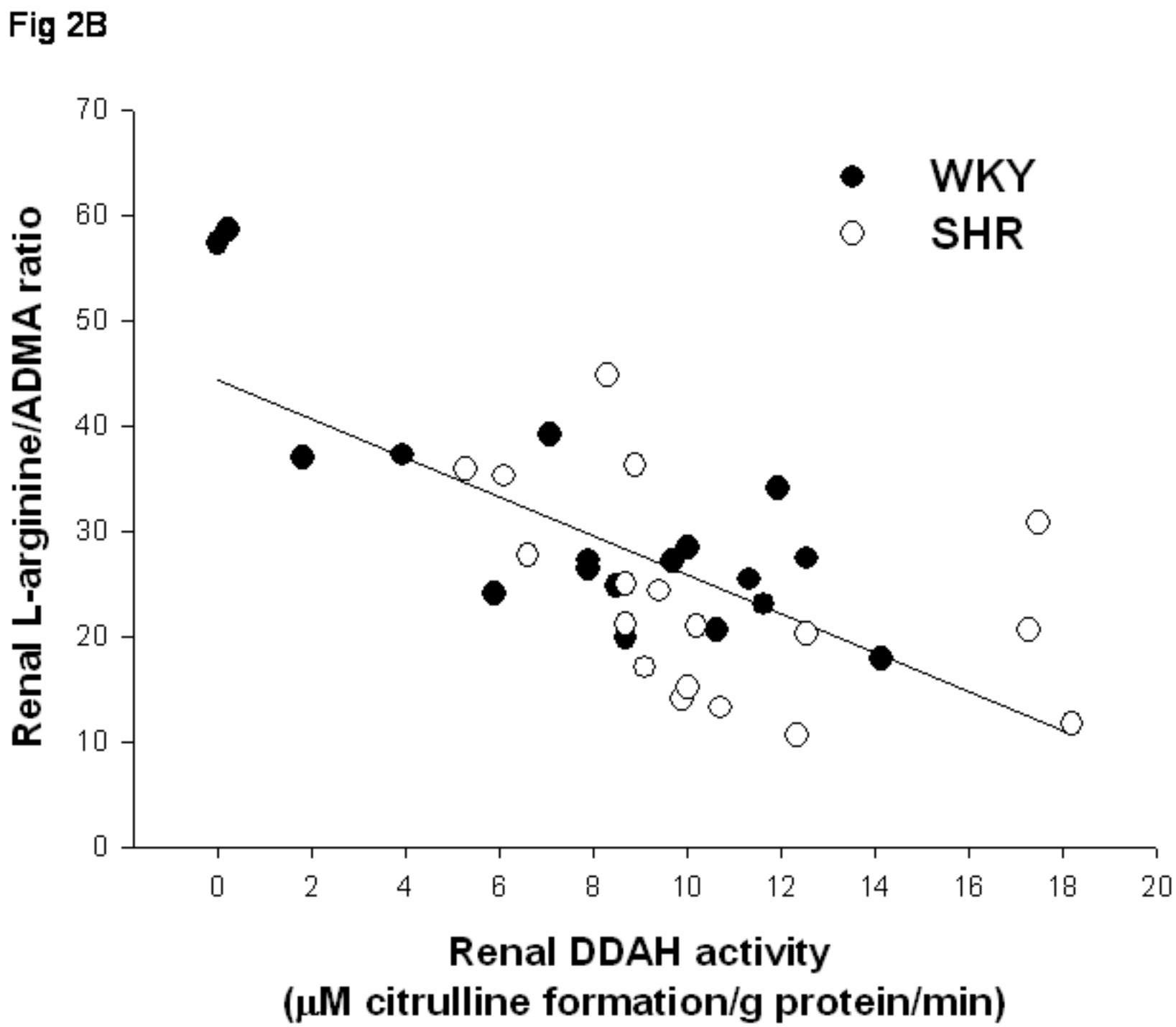


Figure2c

Click here to download high resolution image

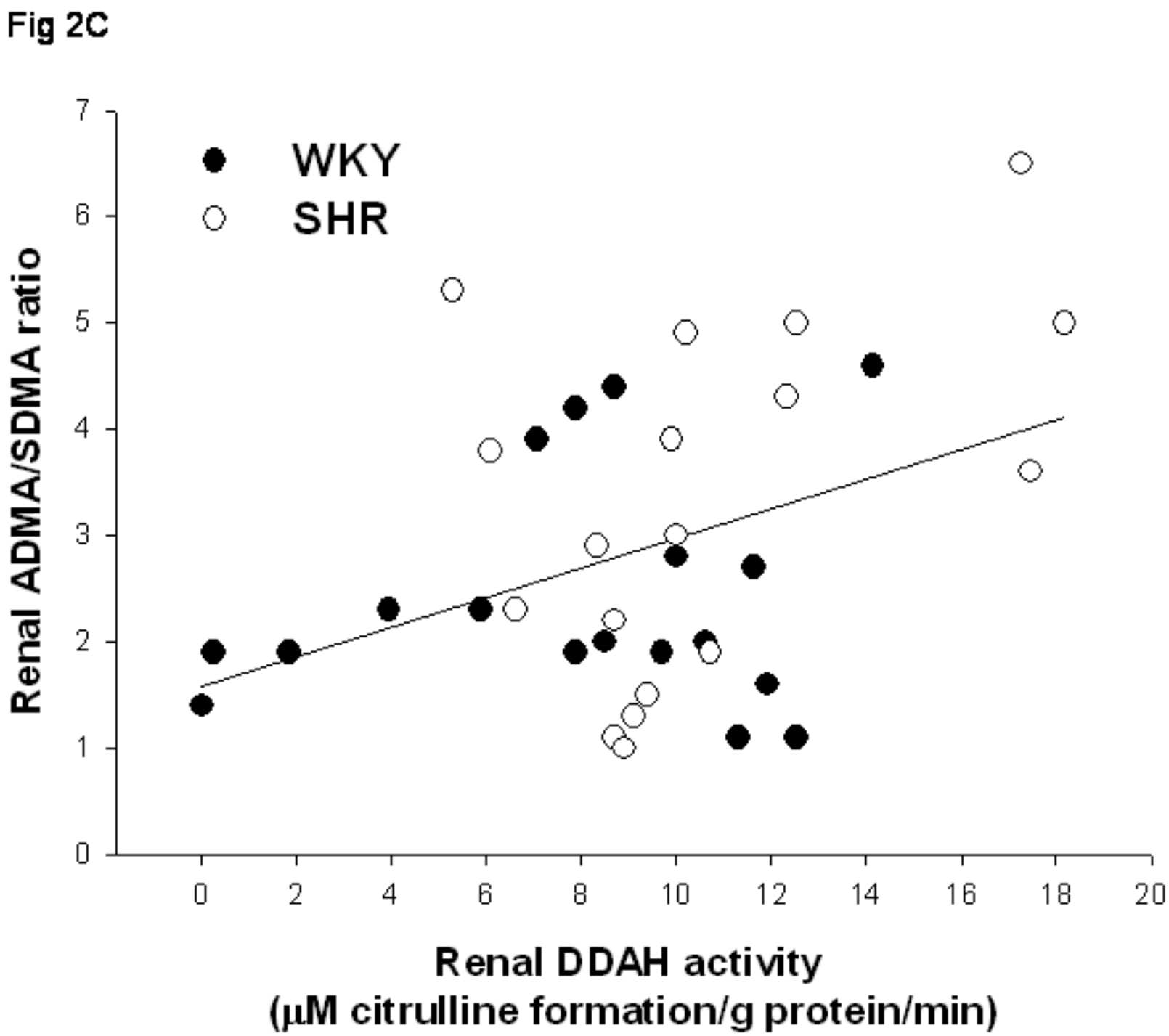


Figure3

Click here to download high resolution image

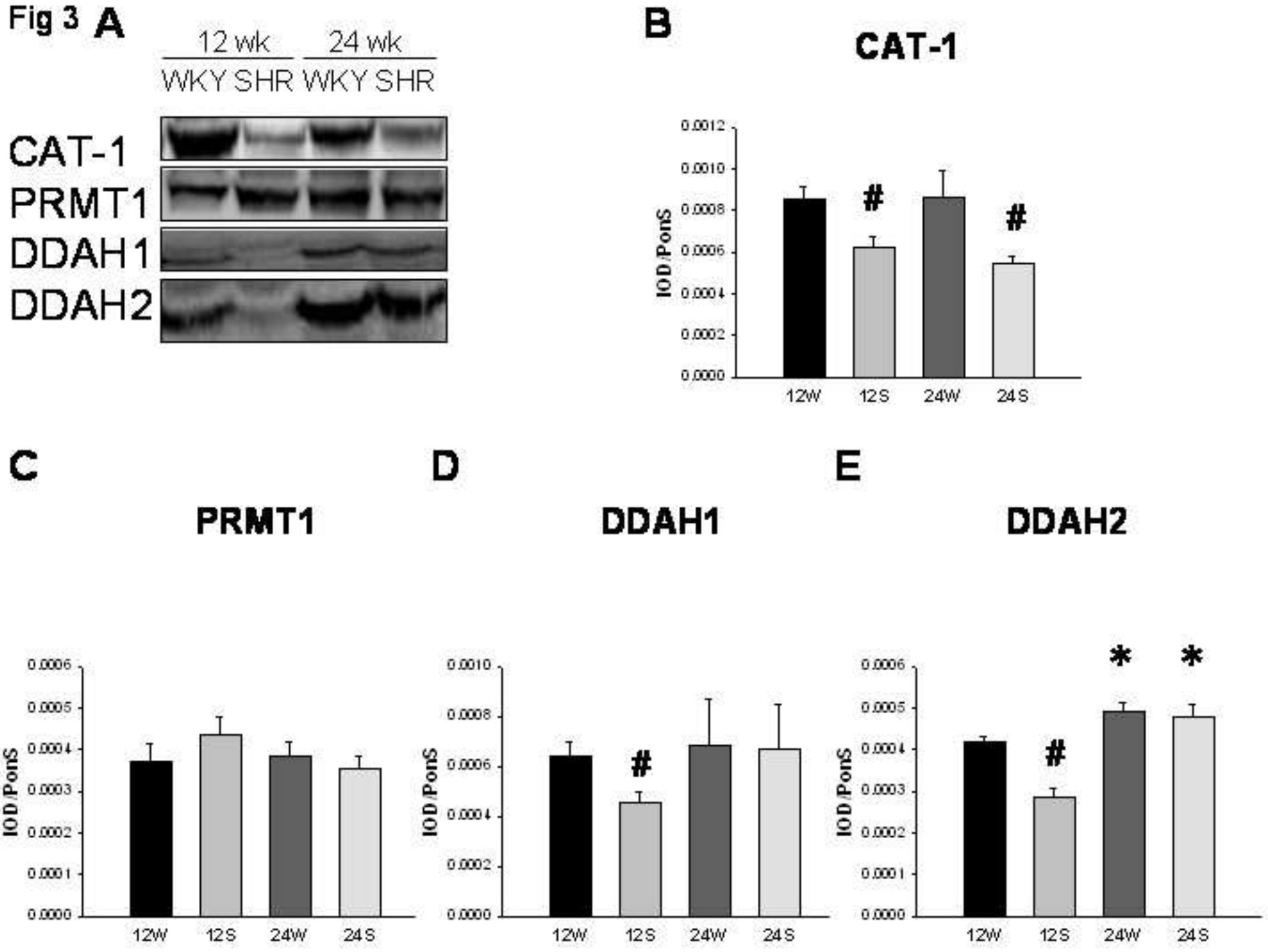
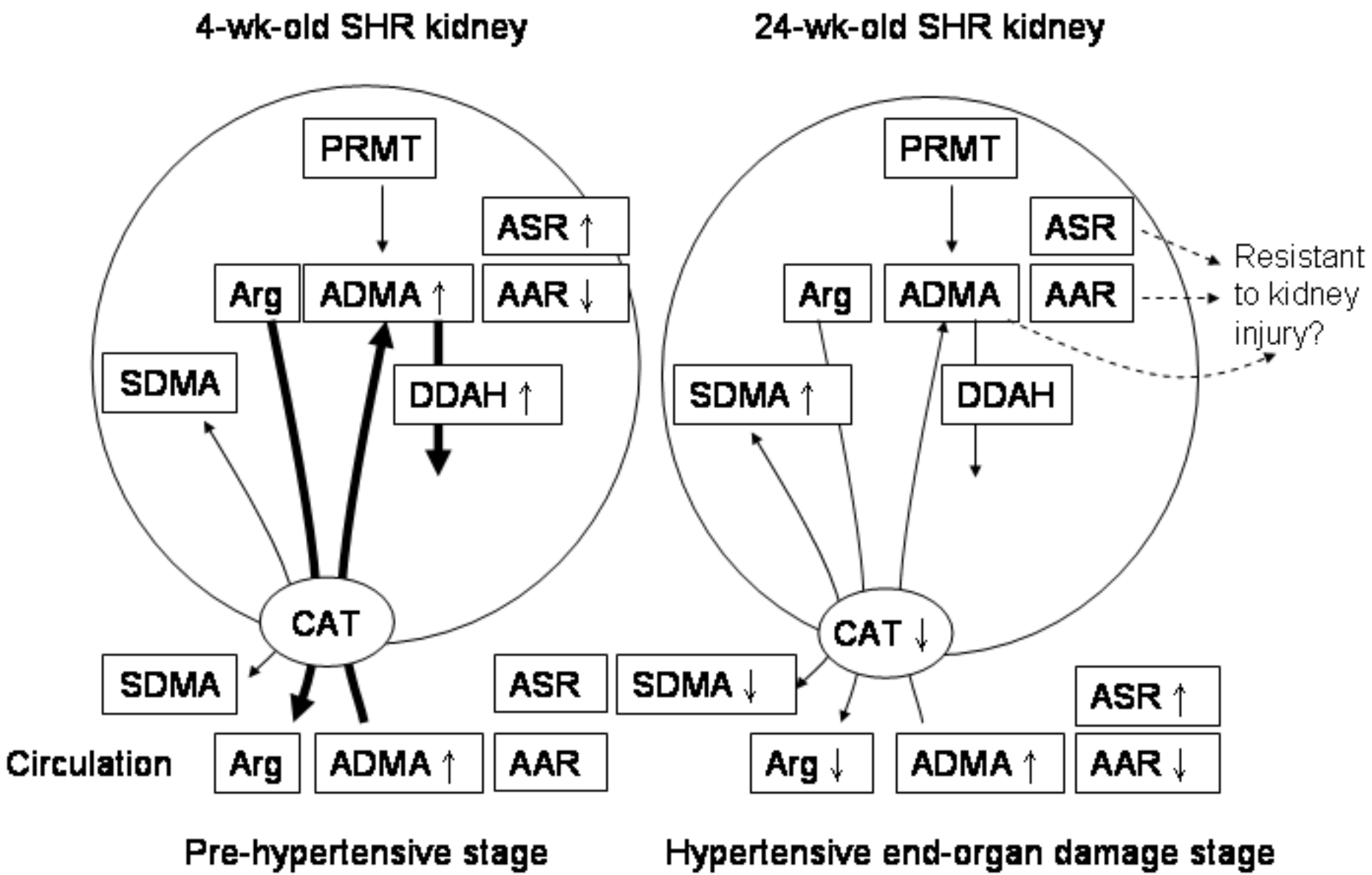


Figure4
Click here to download high resolution image

Fig 4



Table(s)

Table 1 Morphological and biochemical values in the different experimental groups.

	4 weeks		12 weeks		24 weeks	
	WKY	SHR	WKY	SHR	WKY	SHR
Body weight (g)	118 ± 4	144 ± 2#	274 ± 3*	288 ± 2*	318 ± 12*	352 ± 7*#
Systolic BP	112 ± 3	123 ± 3	134 ± 2*	181 ± 7*#	158 ± 6*	193 ± 5*#
Diastolic BP	75 ± 5	83 ± 6	78 ± 8	141 ± 8*#	119 ± 6*	164 ± 7*#
MAP	87 ± 3	97 ± 5	97 ± 6	155 ± 6*#	132 ± 6*	174 ± 6*#
Heart weight (g)	0.61 ± 0.05	0.72 ± 0.01	0.90 ± 0.06*	1.16 ± 0.03*#	1.25 ± 0.03*	1.62 ± 0.03*#
HW / 100g BW	0.51 ± 0.03	0.50 ± 0.00	0.33 ± 0.02	0.40 ± 0.01#	0.40 ± 0.01	0.46 ± 0.01#
Left kidney weight (g)	0.64 ± 0.03	0.83 ± 0.02#	1.22 ± 0.02*	1.22 ± 0.02*	1.34 ± 0.04*	1.47 ± 0.06*
Left kidney weight/100g BW	0.54 ± 0.01	0.58 ± 0.01	0.45 ± 0.01*	0.42 ± 0.01*	0.42 ± 0.01*	0.42 ± 0.01*
Urine protein excretion (mg/24hr)	6.78 ± 0.15	9.79 ± 1.37#	9.91 ± 1.00*	8.70 ± 0.32	5.77 ± 0.45	8.03 ± 0.49#
CCr (ml/min/kg BW)	3.86 ± 0.71	3.61 ± 0.63	5.82 ± 0.65*	5.9 ± 1.11*	6.9 ± 0.78*	6.75 ± 0.73*
UNOxV (μM/24hr/100g BW)	4.50 ± 0.59	3.06 ± 0.61	4.57 ± 1.09	4.57 ± 0.96	4.27 ± 0.78	1.85 ± 0.72*#
Glomerular injury score	0.33 ± 0.21	0.33 ± 0.21	0.5 ± 0.34	0.5 ± 0.34	0.33 ± 0.21	1.33 ± 0.71

MAP = mean arterial pressure; CCr = Clearance of creatinine; UNOxV = Total urinary NOx (NO_3^- + NO_2^-) excretion; N = 6/group; *$P < 0.05$ vs. 4-wk-old respective strains; #$P < 0.05$ vs. respective age-matched WKYs.

Table 2 L-Arginine, ADMA, and SDMA levels in the plasma and kidney.

	4 weeks		12 weeks		24 weeks	
	WKY	SHR	WKY	SHR	WKY	SHR
Plasma (μM)						
L-Arg	132.0 ± 8.2	130.4 ± 7.1	122.0 ± 7.1	103.9 ± 5.7*	100.3 ± 15.4*	83.9 ± 10.0*
ADMA	0.66 ± 0.01	0.88 ± 0.06#	0.63 ± 0.05	1.00 ± 0.08#	0.61 ± 0.03	0.90 ± 0.06#
SDMA	0.39 ± 0.04	0.46 ± 0.04	0.34 ± 0.04	0.49 ± 0.05#	0.32 ± 0.04	0.22 ± 0.01*
Kidney (μM/mg protein)						
L-Arg	36.5 ± 6.6	23.1 ± 3.5	40.4 ± 6.9	39.5 ± 2.6	42.5 ± 3.5	31.4 ± 4.5
ADMA	0.89 ± 0.06	1.41 ± 0.11#	1.55 ± 0.15*	1.28 ± 0.11	1.61 ± 0.06*	1.48 ± 0.21
SDMA	0.42 ± 0.08	0.29 ± 0.04	0.66 ± 0.21	0.55 ± 0.19	1.16 ± 0.07*	0.88 ± 0.13*

N = 6/group; *$P < 0.05$ vs. 4-wk-old respective strains; #$P < 0.05$ vs. respective age-matched WKYs.

Table 3 Correlations between plasma L-Arginine, ADMA, SDMA, their combined ratios and blood pressures.

Factor		L-arginine	ADMA	AAR	SDMA	ASR
SBP	*r* value	-0.634	0.358	-0.674	-0.314	0.622
	P value	<0.001	0.032	<0.001	0.062	<0.001
DBP	*r* value	-0.589	0.429	-0.702	-0.286	0.665
	P value	<0.001	0.009	<0.001	0.09	<0.001
MAP	*r* value	-0.618	0.419	-0.712	-0.301	0.669
	P value	<0.001	0.011	<0.001	0.074	<0.001

SBP = systolic blood pressure; DBP = diastolic blood pressure; MAP = mean arterial pressure.