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ORIGINAL ARTICLE

Effects of Arginine Supplementation on Exogenous Advanced Glycation End Product-induced Renal Inflammatory Mediator Expression in Rats

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Background: This study investigated the effects of dietary Arg supplementation on renal inflammatory mediator expression and oxidative damage in rats with exogenous advanced glycation end product (AGE) administration.
Methods: There were two groups of rats: the CA group was fed a common diet and given a tail vein injection of AGE–bovine serum albumin (BSA); and the AA group was fed the Arg-supplemented diet and injected with AGE–BSA. Arg provided 2% of the total energy. The tail vein injection and diets for the respective groups were given for 10 days. After that, all rats were sacrificed, and blood and kidneys were harvested for further analysis.
Results: Blood adhesion molecule expression and NO levels were higher in the AA group. Also, the kidney nitrotyrosine concentration, phospho-nuclear factor-κB p65 and inducible NO synthase protein expression was higher in the AA group than in the CA group. The finding of immunohistochemical staining was consistent with the results that the AA group had higher receptor of AGE expression in the kidneys.
Conclusion: Supplemental dietary Arg may have adverse effect in AGE-induced kidney inflammatory response and oxidative damage in rats.

1. Introduction

Due to the increasing prevalence of diabetes, disabilities associated with its complications are constantly growing. Diabetic complications are considered to be multifactorial in origin, with increasing evidence that one of the major pathways is the biochemical process of advanced glycation.[1] Advanced glycation involves the generation of a heterologous group of compounds known as advanced glycated end products (AGEs). AGEs can exert their biological effects through receptor-mediated mechanisms, the most important of which is the receptor of AGE (RAGE).[2] Interactions of AGEs with the RAGE induce activation of nuclear factor (NF)-κB, result in oxidative stress, and elicit inflammation that is implicated in the development of diabetic complications, especially diabetic nephropathy.[3–5]

Arginine (Arg) is a nonessential amino acid for healthy adults. It has been shown to possess numerous useful physiological properties. Arg is the substrate of NO synthase (NOS) and a precursor of NO. Recently, Arg has been added to enteral formulas at pharmacological levels in an attempt to boost immune function and improve clinical outcomes of patients.[6,7] Although a meta-analysis of several studies focusing on immunonutrition has indicated that Arg supplementation has no effect on infectious complications and may increase mortality in critically ill patients,[8] many studies have demonstrated the benefits of Arg supplementation on various diseases including diabetic nephropathy.[9–12] A study by Reyes et al[11] has reported that administration of Arg prevents the development of hyperfiltration and ameliorates proteinuria in diabetic rats. Also, a previous study carried out by our laboratory has found that supplemental dietary Arg decreases renal RAGE expression and oxidative damage in rats with type 2 diabetes.[12] However, the studies mentioned above concerned with the influence of Arg focused on the condition of diabetes. Studies investigating the effects of Arg on AGE-induced inflammation have been rare. Diabetes is a disease with complicated metabolic alterations. It has been difficult to separate the effects of various metabolic disorders from those of AGEs in diabetic animal models. In diabetes, AGEs accumulation results from chronic hyperglycemia concomitant with impaired renal function, because the kidneys are the major site of AGE clearance.[13] To investigate specifically Arg intervention in AGE-induced renal damage, we administered

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exogenous AGE-modified proteins to normal rats. A previous study has reported that short-term administration of exogenous AGE to rats leads to the covalent attachment of AGEs within the extracellular matrix in several tissues, and the abnormalities in tissues are similar to those observed in diabetes.[14] We hypothesized that dietary Arg supplementation reduces AGE-induced inflammatory mediator expression and attenuates oxidative damage in kidneys.

2. Materials and methods

2.1. Preparation of AGE protein

AGE-modified bovine serum albumin (AGE–BSA) was prepared as described previously.[15] Briefly, BSA (2 mg/mL) was incubated under sterile conditions with 33 mmol/L glycoaldehyde (GA; Sigma, St. Louis, MO, USA) at 37 °C for 7 days in phosphate-buffered saline (PBS) (pH 7.4). Then, GA that had not been incorporated into the BSA was removed by dialysis against PBS. All reagents were passed through a 0.2-μm positive charged Nylon membrane (Pall Life Sciences, Ann Arbor, MI, USA) to remove the endotoxin and were confirmed by the limulus amebocyte assay QCL-1000 kit (Cambrex, Walkersville, MD, USA). The extent of chemical modification was determined by reaction with 2,4,6- trinitrobenzenesulfonic acid (Sigma) using unmodified BSA as the calibration standard.[16] The extent of lysine modification by GA on BSA in the preparation of GA–BSA was approximately 70%. The percentage of lysine modification was similar to that previously reported.[15]

2.2. Animals

Twenty male Wistar rats, aged 5 weeks and weighing about 170–210 g, were purchased from the National Laboratory Animal Center (Taipei, Taiwan). All rats were maintained in a temperature- and humidity-controlled room with a 12:12-h light–dark cycle and were fed standard chow diet for 1 week before the study. Care of the laboratory animals was in full compliance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council, 1996), and protocols were approved by the institutional Animal Care and Use Committee at Taipei Medical University.

2.3. Study protocols

After adaptation for 1 week, rats were randomly divided into two groups: in the CA group, rats were fed a common semipurified diet and given a tail vein injection of 2 mg AGE–BSA; and in the AA group, rats were fed the Arg-supplemented diet and injected with 2 mg AGE–BSA. The experimental diets were isonitrogenous and identical in energy and nutrient distributions except for the difference in amino acid contents (Table 1). The Arg-supplemented diet replaced part of the casein by Arg, which provided 2% of the total energy. This amount of Arg was used because it has been proven to have regulatory effects on immune responses.[17] The tail vein injection and the diets for the respective group were given daily for a duration of 10 days. After 10 days, all rats were anesthetized and sacrificed by drawing arterial blood from the aorta of the abdomen. Blood samples were collected in tubes containing heparin, part of the kidney tissue was harvested for immunocytochemical analysis, and the remaining tissues were stored at −80 °C for further assays.

2.4. Analysis of CD11a/CD18 and CD11b/CD18 expression by monocytes

One hundred microliters of fresh blood was double-stained with 10 μL fluorescein-isothiocyanate-conjugated mouse monoclonal anti-rat CD11a or mouse anti-rat CD11b and phycoerythrin-conjugated mouse anti-rat CD18 (Serotec, Oxford, UK) for 15 min at 4 °C. Then, red blood cells were lysed with lysing reagents (Beckman Coulter, Fullerton, CA, USA). Proportions of CD11a/CD18 and CD11b/CD18 expression by monocytes were analyzed by flow cytometry (BD FACS CantoII; BD Biosciences, San Diego, CA, USA). Fluorescence data were collected, and the results are presented as a percentage of CD11a- or CD11b-presenting cells in 10^5 monocytes. Monocytes were gated according to forward and side scatter, and were analyzed for expression of CD11a/CD18 and CD11b/CD18.

Table 1 Composition of experimental diets

Component (g/kg)	CA group	AA group
Soybean oil	100	100
Casein	200	158
Arg	0	20.9
Salt mixture*	35	35
Vitamin mixture†	10	10
Methyl cellulose	31	31
Choline chloride	1	1
Methionine	3	3
Corn starch	620	641.1

AA = Arg-supplemented diet and advanced glycation end product–bovine serum albumin; CA = common diet and advanced glycation end product–bovine serum albumin.

* Contained the following (mg/g): calcium phosphate dibasic, 500; sodium chloride, 74; potassium sulfate, 52; potassium citrate monohydrate, 20; magnesium oxide, 24; manganese carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.6; curpric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; and chromium potassium sulfate, 0.55; † Contained the following (mg/g): thiamin hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3; calcium pantothenate, 1.6; D-biotin, 0.05; cyanocobalamin, 0.001; retinyl palmitate, 1.6; DL-α-tocopherol acetate, 20; cholecalciferol, 0.25; and menaquinone, 0.005.

2.5. Measurements of plasma soluble intercellular adhesion molecule, monocyte chemotactic protein and NO concentrations

Concentrations of soluble intercellular adhesion molecule (sICAM)-1 and monocyte chemotactic protein (MCP)-1 were measured using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA). NO is highly unstable in solution and cannot readily be assayed. However, NO is converted to stable nitrite and nitrate ions in aqueous solution. After conversion of nitrate to nitrite using nitrate reductase, nitrite concentrations were measured using the Griess reagent. Plasma concentrations of nitrite/nitrate were determined with a commercial kit (R&D Systems). All measurements were performed according to the manufacturer's instructions.

2.6. Analysis of nitrotyrosine in kidney homogenates

Kidney tissues were homogenized (1:3, w/v) in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The homogenates were centrifuged at 15,000 rpm for 20 min, and the cell debris was discarded. Supernatants were used for the analysis of nitrotyrosine. Nitrotyrosine concentrations were measured using a commercial ELISA kit (Millipore, Bedford, MA, USA). Nitro-BSA was coated onto the wells of microtiter strips, and nitrotyrosines were quantitated using an anti-nitrotyrosine antibody. Competition was accomplished by adding 50 μL supernatant and 50 μL of the primary antibody to the wells. Each competed with the coated nitrated proteins for antibody binding. The amount of antibody that bound to the coated nitro-BSA was inversely proportional to the amount of nitrotyrosine present in the samples added to the wells of the plate. Procedures followed the manufacturer's instructions.

3. Preparation of whole-cell extracts and western blotting for phospho-NF-κB p65 (serine 536), IκB-α, and inducible NOS in the kidneys

Kidney homogenate (1:3, w/v) was prepared in ice-cold 1% sodium dodecyl sulfate (Sigma) lysis buffer containing 50 mM Tris−HCl (pH 8), 10 mM EDTA (Sigma), 1 mM sodium pyrophosphate (Sigma), 10 mM NaF (Sigma), 1 mM Na_3VO_4 (Sigma), 1 mM β-glycerophosphate (Sigma), and a protease inhibitor cocktail (Roche Diagnostics). The homogenates were centrifuged at 15,000 rpm for 30 min, and the supernatants were used for western blotting as whole-cell extracts. Protein concentrations of the supernatant were determined using a Bradford Protein Assay Reagent kit (Bio-Rad, Richmond, CA, USA). Twenty micrograms of protein was loaded and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane in a wet-transfer apparatus. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 30 min and then incubated with a rabbit anti-phospho-NF-κB p65 (Ser 536) antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-IκB-α antibody (1:200; Santa Cruz Biotechnology), and rabbit polyclonal anti-inducible NOS (iNOS) (1:200; Santa Cruz Biotechnology) overnight, or a mouse anti-actin antibody (1:10,000; Sigma) for 1 hour. After the membranes were washed three times (for 10 minutes each) in TBS-T, a goat anti-rabbit immunoglobulin G (IgG) or anti-mouse IgG−horseradish peroxidase conjugate (Millipore, Bedford, MA, USA) was applied at a 1:10,000 dilution for 1 hour. Afterwards, membranes were washed three times with TBS-T for 30 minutes, and blots were developed with high-sensitivity chemiluminescence substrate Western Lighting Ultra (PerkinElmer Life Sciences, Waltham, MA, USA) and exposed to X-ray films. The relative intensity was measured to quantify the protein level. All blots were normalized against actin to adjust for the protein loaded.

3.1. RAGE immunohistochemistry

Kidneys were excised and immersed in 4% paraformaldehyde. The fixed kidneys were dehydrated through a graded series of different percentages of ethanol, embedded in paraffin, sectioned at 4-μm thickness, and placed on glass slides. For immunohistochemical demonstration of RAGE, a rabbit polyclonal anti-rat RAGE antibody (Thermo Fisher Scientific, Pittsburgh, PA, USA) was used. Sections were deparaffinized in xylene, rehydrated through a graded ethanol series to water, and antigen-retrieval pretreated by steaming in citrate buffer (BioGenex, San Ramon, CA, USA). After all tissue sections had been preincubated in a blocking solution containing 10% normal goat serum and 0.3% H_2O_2 in 0.1 M phosphate buffer for 1 hour to block endogenous peroxidase activity and nonspecific binding of antibodies, the slides were incubated overnight with the primary antibody (diluted 1:300) at 4°C. For peroxidase activity detection, tissue sections were incubated in biotinylated goat anti-rabbit IgG (diluted 1:300; Chemicon, Temecula, CA, USA) for 1 hour at room temperature. After reacting with the peroxidase-linked avidin−biotin complex (Vector, Burlingame, CA, USA) for 1 hour at room temperature, a diaminobenzidine solution kit (Vector) was used to detect RAGE immunoreactivity. Hematoxylin (Sigma) nuclear staining was also applied to contrast the cell nucleus and cytoplasm. All tissue sections were covered with a coverslip by Permount (Fisher Scientific, Loughborough, Leicestershire, UK), and measured using a digital image analysis system (Image Pro Plus 5.1; Media Cybernetics, Silver Spring, MD, USA) after the images were captured on a Zeiss Axiphot light microscope (Carl Zeiss, Jena, Germany) equipped with a 20× objective lens and a Nikon D1X digital camera (Tokyo, Japan). We used the "count/size" and "area" commands to determine the intensity of RAGE immunoreactivity. Automatic object counting and measuring processes were used to quantify the immunoreactive areas in the sections. Values are expressed as μm^2. At least 10 microscopic fields per section and three independent samples for each group were analyzed, and average areas were obtained for each group.

3.2. Statistical analysis

All data are expressed as the mean ± standard error of the mean. Differences among groups were analyzed by unpaired *t* test. A *p* value <0.05 was considered statistically significant.

4. Results

4.1. Body weight

There were no differences in the initial body weight or weight after feeding the diets for 10 days (224 ± 28 g in CA group vs. 244 ± 25 g in the AA group; $p > 0.05$) between the two groups.

4.2. Blood inflammatory mediators

Plasma sICAM-1 and MCP-1 levels and monocyte CD11b/CD18 expression was significantly higher in the AA group than the CA group. Also, plasma NO concentrations were higher in the Arg-supplemented group than in those without Arg (Table 2).

4.3. Nitrotyrosine levels in kidney homogenates

Kidney nitrotyrosine concentrations were significantly higher in the AA group than in the CA group (11.13 ± 0.3 μmol/L in the CA group vs. 13.39 ± 0.8 μmol/L in the AA group; $p < 0.05$).

4.4. Western blotting for IκB-α, phospho-NF-κB and iNOS in the kidneys

IκB-α expression in whole-cell extracts was significantly lower (Figure 1A) and phospho-NF-κB p65 protein expression was higher (Figure 1B) in the AA group than the CA group. The AA group had higher iNOS expression than the CA group had (Figure 2).

4.5. RAGE expression in kidneys

Immunohistochemical findings of RAGE-immunoreactive cells with brown cytoplasm and hematoxylin-stained nuclei are shown in Figure 3. The immunoreactive intensities were higher in the AA

Table 2 Plasma concentrations of sICAM-1, MCP-1, monocyte CD11a/CD18 and CD11b/CD18, and NO expression in the various groups at the end of the experiment

	CA	AA
sICAM-1 (ng/mL)	15.4 ± 0.4	17.9 ± 0.5*
MCP-1 (ng/mL)	481.4 ± 54.0	635.4 ± 29.9*
CD11a (%)	4.9 ± 0.9	5.2 ± 1.1
CD11b (%)	4.4 ± 0.9	10.2 ± 2.0*
NO (μmol/L)	3.8 ± 0.5	5.5 ± 0.5*

Data are presented as the mean ± standard error of the mean. Different treatments were analyzed by unpaired *t* test. * Significantly different from the CA group ($p < 0.05$).
AA = Arg-supplemented diet and advanced glycation end product−bovine serum albumin; CA = common diet and advanced glycation end product−bovine serum albumin; MCP-1 = monocyte chemotactic protein-1; sICAM-1 = soluble intracellular adhesion molecule-1.

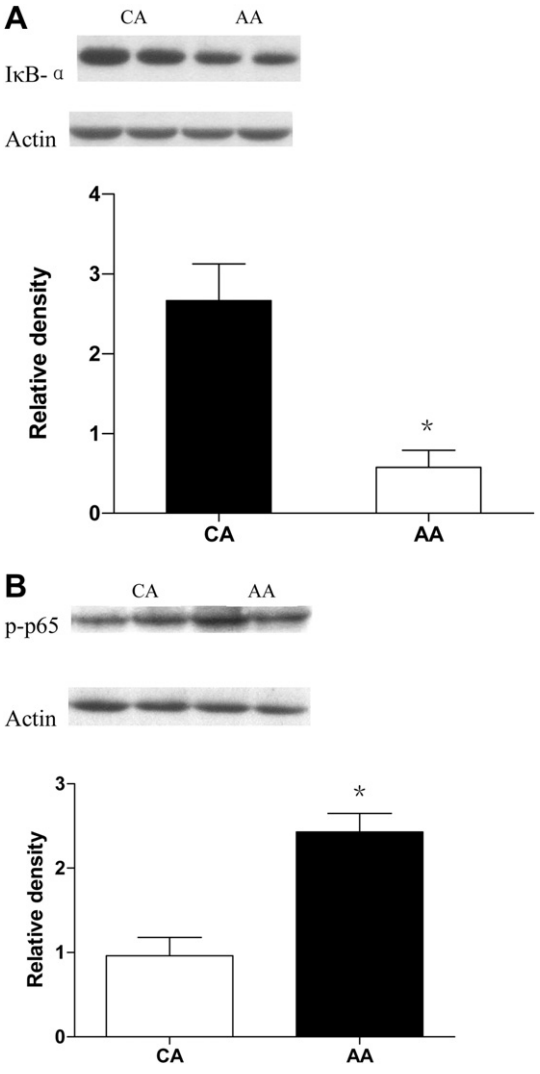

Figure 1 Western blot analysis of (A) phospho-p65 (NF-κB subunit) and (B) IκB-α proteins in kidneys at the end of the experiment. The upper pictures are western blot photographs. Equal loading of proteins is illustrated by β-actin bands. The lower scales are the mean ± standard error of the mean values of the densitometric analysis for six different observations. *Significantly differs from the CA group ($p < 0.05$). CA = common diet and advanced glycation end product–bovine serum albumin; NF = nuclear factor.

group (Figure 3B and 3D) than in the CA group (Figure 3A and 3C) in both the tubular and glomerular areas. Quantification of RAGE-immunoreactive areas of the groups is shown in Figure 3E. The immunoreactive areas of the AA group were significantly larger than those of the CA group.

5. Discussion

GA is a short-chain aldehyde, and reacts with amino groups to form a Schiff base, which rearranges to more-stable Amadori products that lead to AGE formation.[18] We used GA to prepare AGE-modified proteins in this study, because a previous study found that GA–BSA activated monocytes via RAGE, leading to the upregulation of adhesion molecule expression and cytokine production.[19] Also, GA-derived AGEs have been found to have diverse biological activities in various cells including mesangial cells.[20] A previous study has found that intravenously administered GA promotes intrarenal oxidative stress and might be an important factor in the development of diabetic nephropathy.[18]

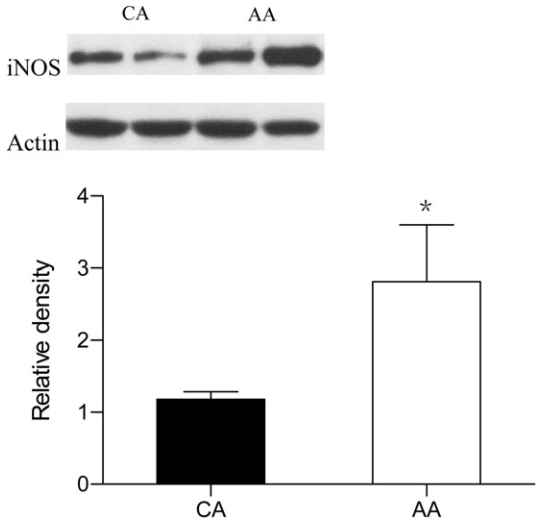

Figure 2 Western blot analysis of iNOS protein in the kidneys. Equal loading of proteins is illustrated by β-actin bands. iNOS expression in the kidneys was lower in the CA group than in the AA group. Data are presented as the mean ± standard deviation. Differences between groups were analyzed by t test ($n = 6$). *$p < 0.05$ compared to the CA group. AA = Arg-supplemented diet and advanced glycation end product–bovine serum albumin; CA = common diet and advanced glycation end product–bovine serum albumin; iNOS = inducible nitric oxide synthase.

AGEs are known to be pro-oxidant and proinflammatory compounds. A previous study has shown that AGEs lead to induction of reactive oxygen species and promote endothelial expression of inflammatory proteins.[21] Adhesion molecules play central roles in cell–cell and cell–matrix adhesion and participate in leukocyte binding, and transendothelial and interstitial migration. Excessive expression of adhesion molecules may induce an inflammatory response and tissue injury.[22] CD11a/CD18 and CD11b/CD18 are members of the leukocyte adhesion molecule-β_2 intergins (CD18). CD11a/CD18 and CD11b/CD18 are the most studied leukocyte adhesion molecules mediating tight binding to endothelial cells and migration through the vascular wall.[23] Integrins are ligands of ICAM-1 expressed on the vascular endothelium. In this study, we analyzed monocyte CD11a and CD11b expression, because monocyte adhesion to the endothelium is a key early event in endothelial dysfunction. The reported evidence also shows that monocyte CD11b expression increases in diabetes mellitus patients.[23] MCP-1 is a potent chemotactic factor, which is involved in recruiting peripheral leukocytes. MCP-1 is a contributor to the inflammatory process associated with diabetes.[24] In this study, we found higher adhesion molecule expression and MCP-1 levels in the Arg-supplemented group, indicating that Arg upregulated circulating monocytes to adhere to the endothelium as part of a generalized inflammatory response when an AGE was administered.

RAGE is a multiligand member of the immunoglobulin superfamily of cell surface receptors. It is the most extensively studied AGE-binding protein. RAGE is present on the surface of a variety of cells including endothelial cells, mononuclear phagocytes, hepatocytes and mesangial cells.[25] RAGE initiates the intracellular signaling that disrupts cellular function through its recognition and binding of AGEs, which in turn upregulates the expression of NF-κB. Sustained activation of NF-κB results in further amplification of the AGE–RAGE signal[26] and is thus implicated in the pathogenesis of several diseases including diabetes and renal failure.[27] The immunohistochemical findings in kidney RAGE expression observed in the AA group were consistent with the measurements of western blotting for IκB-α and phospho-NF-κB p65 in kidney homogenates. NF-κB is a widely expressed inducible transcription factor that regulates the expression of inflammatory proteins. In resting cells,

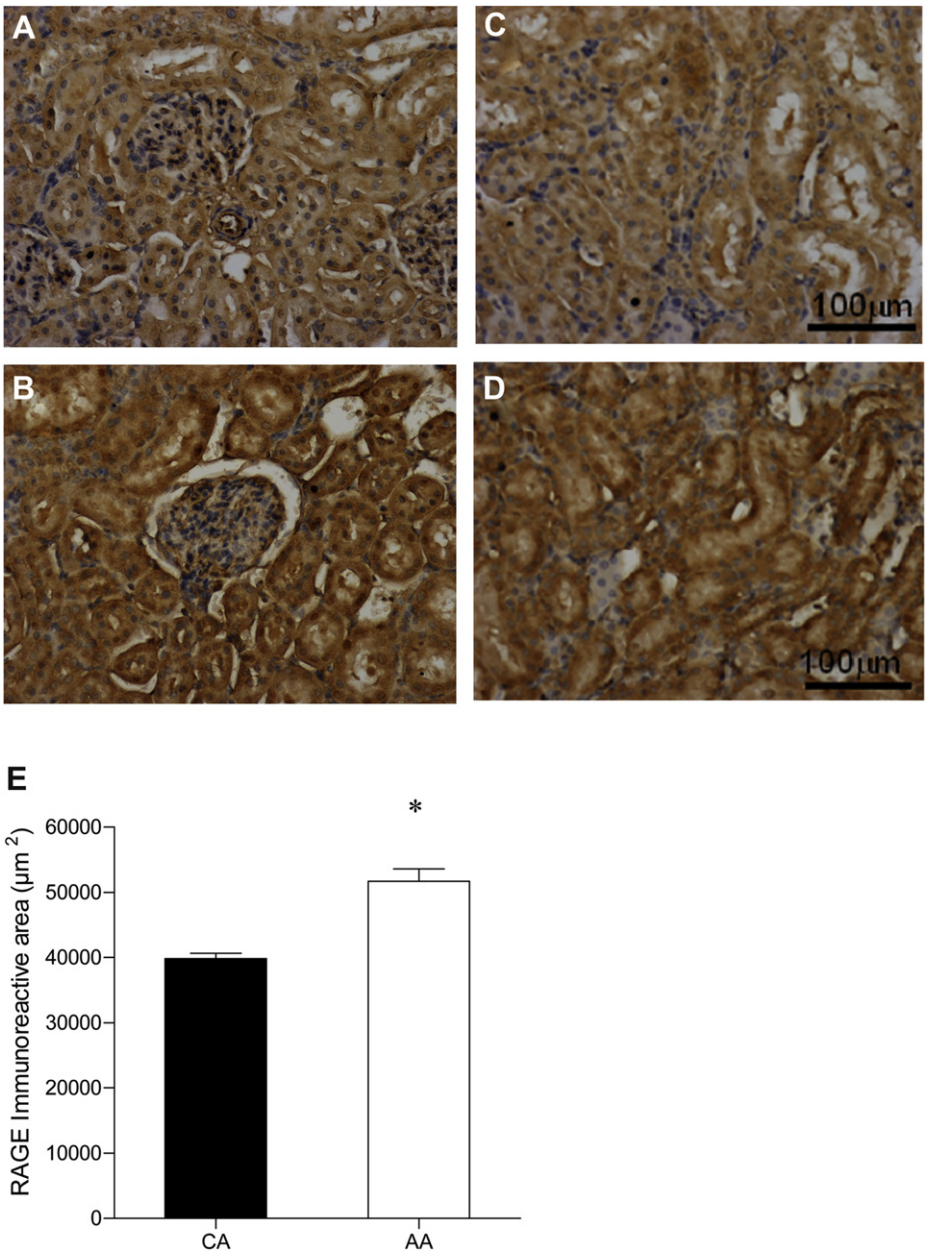

Figure 3 RAGE expression in kidneys of the CA (A, C) and AA (B, D) groups. RAGE-positive cells with brown cytoplasm and hematoxylin-stained nuclei were randomly distributed throughout the tissues, mostly in the renal tubules (A, B) rather than in the glomeruli (C, D). Expression of RAGE exhibited higher immunoreactive intensity in the AA group (B, D) than the CA group (A, C). Scale bars = 100 μm. (E) Quantification of RAGE immunoreactive areas among groups ($n = 3$). *$p < 0.05$ compared to the CA group. AA = Arg-supplemented diet and advanced glycation end product–bovine serum albumin; CA = common diet and advanced glycation end product–bovine serum albumin; RAGE = receptor for advanced glycation end product.

NF-κB resides in the cytoplasm in its inactive form bound to its inhibitor protein, IκB. Upon activation, IκB-α is phosphorylated and degraded, resulting in release and translocation of NF-κB to the nucleus.[28] Phosphorylation of the p65 subunit of NF-κB may contribute to complete p65 activation and subsequent DNA binding, which activates the transcription of target genes, such as adhesion molecules, cytokines, iNOS and RAGE.[28] We observed that, in the Arg-supplemented group, the whole-cell IκB-α was lower and phospho-p65 subunit was higher, indicating higher p65 nuclear translocation and activation, which may consequently lead to inflammatory mediator expression. Concomitant with increased renal NF-κB, higher iNOS expression and nitrotyrosine levels in the AA group were also observed. NO is a small molecule derived from Arg that is catalyzed by the enzyme, NOS. iNOS is an inducible form of NOS. An *in vitro* study has shown that AGE potently enhances activation of the iNOS–NO pathway in chondrocytes.[29] Nitrotyrosine is a marker of protein nitrosylation associated with oxidative stress.[30] When superoxide and NO exist in close proximity, they spontaneously form a powerful oxidant, peroxynitrite. Nitration in the 3-position of tyrosine is the major product of the attack of peroxynitrite on proteins.[30] The higher renal nitrotyrosine in the AA group indicated the greater extent of NO oxidation. These results contradicted our hypothesis that Arg supplementation has an adverse effect on renal inflammatory response. However, our results were similar to a previous study in which a high dose of L-Arg interacted with a precursor of AGE, methylglyoxal, to generate harmful superoxide anions in diabetes.[31] NO has several roles in modulating the immune response under various

physiological conditions. Some studies have shown that NO plays beneficial roles in maintaining homeostasis and protecting against organ damage. However, NO has also been implicated as a deleterious agent in some pathophysiological conditions.[32,33] Arg is the precursor of NO, therefore, it is possible that Arg administration enhanced excessive iNOS expression, which may consequently have aggravated AGE-induced oxidative stress and promoted NF-κB expression under the present experimental conditions.

In summary, this study showed that, compared with the group without Arg, dietary Arg supplementation resulted in higher plasma adhesion molecule levels and increased renal nitrotyrosine concentrations. Also, renal phospho-NF-κB p65 and iNOS protein expression was higher when exogenous AGE was administered. The results of immunohistochemical staining consistently showed that the number of immunoreactive RAGE cells in the kidneys was significantly greater in the Arg-supplemented group. These results suggest that supplemental dietary Arg may have adverse effects in AGE-induced renal inflammatory response and oxidative damage in rats.

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