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Original Article

Supplemental dietary arginine reduces renal RAGE expression and oxidative damage in rats with streptozotocin-induced type 2 diabetes

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SUMMARY

Background & aims: Arginine (Arg) was shown to have immunomodulatory effect and inhibits advanced glycation end product (AGE) formation in *in vitro* studies. This study investigated the effects of dietary Arg supplementation on renal receptor of AGE (RAGE) expressions and oxidative damage in diabetic rats.
Methods: There were 1 normal control (NC) group and 2 diabetic groups in this study. Rats in the NC group were fed with a chow diet. One diabetic group (DM) was fed a common semipurified diet, while the other diabetic group received a diet in which part of the casein was replaced by Arg (DM-Arg) for 8 wk. Diabetes was induced by an intraperitoneal injection of nicotinamide followed by streptozotocin. Rats with blood glucose levels exceeding 180 mg/dL were considered diabetic. Blood samples were collected at the baseline and 8 wk. Kidneys of the animals were harvested at the end of the study for further analysis.
Results: Plasma fructosamine contents were significantly higher in the diabetic groups than in the NC group. The DM group had higher fructosamine than the DM-Arg group. Kidney nitrotyrosine concentrations and nuclear factor-κB p65 protein expressions were significantly lower in the DM-Arg group than in the DM group. The result of immunohistochemical staining also showed that the expressions of RAGE in the kidneys were significantly lower in the DM-Arg group than in the DM group.
Conclusions: These results suggest that dietary Arg supplementation may decrease renal RAGE expressions and oxidative damage in rats with type 2 diabetes.

1. Introduction

Diabetes mellitus (DM) was the 5th leading cause of death in Taiwan in 2008.[1] It is a metabolic disorder characterized by hyperglycemia and both microvascular and macrovascular diseases. Diabetic complications are considered to be multifactorial in origin. The biochemical process of advanced glycation, which is accelerated in diabetes, was postulated to play a central role in these disorders.[2] Advanced glycation involves the generation of a heterogeneous group of compounds known as advanced glycated end products (AGEs). Increasing evidence showed that AGEs act directly to induce cross-linking of long-lived proteins, thus altering vascular structure and function. Also, AGEs can exert their biological effects through receptor-mediated mechanisms, the most important of which is the receptor of AGE (RAGE). RAGE is a signal transduction receptor which belongs to the immunoglobin superfamily. Binding of AGEs to the RAGE activates a number of pathways implicated in the development of diabetic complications, especially diabetic renal disease.[3]

Arginine (Arg) is a nonessential amino acid for healthy adults. It was shown to possess numerous useful physiological properties. Arg is often used in immunonutrition regimens and has proven to be an important immunomodulator.[4–6] Although a meta-analysis of several studies focusing on immunonutrition indicated that Arg supplementation has no effect on infectious complications and may increase mortality in critically ill patients, immune-enhancing diets containing Arg are associated with a reduced infection rate and shortened hospital stay in elective surgical patients.[7] On the other hand, several reports showed that Arg inhibits in vitro non-enzymatic glycation and AGE formation in serum and tissue

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proteins in humans and animals.[8,9] A study by Lucotti et al.[10] also found beneficial effects of oral Arg supplementation in reducing plasma AGE levels in type 2 diabetic patients. Because AGE-RAGE interactions in diabetes initiate signal transduction cascades of the inflammatory responses, we hypothesized that Arg supplementation reduces AGE production and consequently decreases RAGE expression and AGE-RAGE-induced organ injury.

With the global epidemic of type 2 DM, diabetes has become the leading cause of end-stage renal disease. Approximately 20%–30% of all diabetic subjects will develop evidence of diabetic nephropathy.[3] To the present, no study has investigated the effects of Arg on RAGE expressions in a diabetic condition. Therefore, we designed this study to investigate the effects of dietary Arg supplementation on renal RAGE expressions and oxidative damage in type 2 diabetic rats.

2. Materials and methods

2.1. Animals

Male 6-week-old Wistar rats weighing 180–220 g were purchased from the National Laboratory Animal Center (Taipei, Taiwan). The rats were maintained in a temperature- and humidity-controlled room with a 12-h light–dark cycle. 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 Animal Care Committee. All rats were allowed free access to a sterilized standard chow diet for 1 week before the study.

2.2. Study protocols

There were 1 normal control (NC, $n = 6$) group and 2 diabetic groups ($n = 13$ for each group) in this study. Rats were lightly anesthetized with ether. Diabetes was induced by an intraperitoneal injection of 150 mg/kg nicotinamide (Sigma Chemical Co, St Louis, MO) followed by streptozotocin (STZ; Sigma) at a dose of 65 mg/kg after 15 min.The induction procedure was repeated 1 d later. Nicotinamide was dissolved in 0.9% saline, and STZ was dissolved immediately before use in 0.05 mol/L sodium citrate (*p*H4.5). This model was designed to mimic the picture of type 2 diabetes in human, because nicotinamide protects beta cells against STZ toxicity established a relative insulin deficiency in rats.[11] Animals were allowed to eat laboratory chow ad libitum for 3d. Rats with fasting blood glucose levels exceeding 180 mg/dL were considered diabetic.Diabetic rats were divided into 2 groups according to the weight and blood glucose of each animal to make average weights and blood glucose levels between groups as similar as possible. Rats in the NC group were fed a chow diet. One diabetic group (DM) was fed a common semipurified diet, while the other diabetic group (DM-Arg) was supplied an identical diet except that part of the casein was replaced by Arg, which provided 2% of the total energy intake. This amount of Arg was found to have regulatory effects on immune responses.[12,13] The experimental diets were isonitrogenous and identical in energy and nutrient distributions (Table 1). Fasting blood samples were collected from the tail vein of each rat at the beginning of the experiment. After feeding the respective diets for 8 wk, all rats were anesthetized and sacrificed by drawing arterial blood from the aorta of the abdomen. Blood samples were collected in tubes containing heparin and were stored at −80 °C until the assay. Kidneys were rapidly harvested for further analysis.

2.3. Measurement of plasma glucose and fructosamine levels

Glucose levels were determined by colorimetric methods after an enzymatic reaction with peroxidase (Randox Co., Antrim, Ireland). Procedures followed the manufacturer's instructions. Fructosamine assays were performed according to the method of Chung et al.[14] The fructosamine concentration was measured as a function of the rate of reduction of nitroblue tetrazolium (NBT) in an alkaline solution. An NBT (Sigma) stock solution was prepared as 1 mmol/L NBT in 0.1 mol/L carbonate buffer (pH 10.35). Forty microliters of plasma samples were mixed with 1 mL 0.25 mM NBT reagent at 37 °C. The absorbance was read at 530 nm. Changes in absorbance (ΔA) were calculated over a 3-min interval, and rate readings were corrected by subtraction of 0.25 mM NBT as the reagent blank. All samples were run in duplicate.

2.4. Analysis of nitrotyrosine in kidney homogenates

A 25% kidney homogenate was prepared in ice-cold lysis buffer (10% sodium dodecylsulphate and 10 mM Tris base; pH7.5) containing a protease inhibitor cocktail (Complete, Roche Diagnostics Ltd, Germany), by using a homogenizer. The homogenates were centrifuged at 15,000 rpm for 20 min to discard the cell debris. Supernatants were used for the analysis of nitrotyrosine. Nitrotyrosine concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Millipore, Bedford, MA). Nitro-bovine serum albumin (BSA) was coated onto the wells of the microtiter strips and nitrotyrosines were quantitated using anti-nitrotyrosine antibodies. Competition was accomplished by adding 50 ul kidney supernatant samples and 50 μl primary antibodies to the wells. Each competes with the coated nitrated proteins for antibody binding. The amount of antibody that binds to the coated nitro-BSA is inversely proportional to the amount of nitrotyrosine present in the samples added to the well of the plate. Procedures followed the manufacturer's instructions.

2.5. Western blotting for nuclear factor (NF)-κB, IκB, and inducible nitric oxide synthase (iNOS) in the kidneys

Frozen kidney tissues were homogenized in ice-cold buffer as mentioned above and the supernatants were used for Western blotting. Protein concentrations of the supernatant were determined using a Bradford Protein Assay Reagent kit (Bio-Rad, Richmond, CA). Fifty microgram of protein was loaded and separated on 12% SDS -polyacrylamide gel electrophoresis (PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane in a wet-transfer apparatus. Membranes were blocked with 5% nonfat milk in phosphate-buffered saline (PBS) containing 0.1% Tween-20 (PBS-T) for 1 h and then incubated with rabbit anti-NFκB p65 antibodies (1:1000; Millipore, Billerica, MA), anti-IκB antibody (1:1000; Millipore) for 2 h, rabbit polyclonal anti-iNOS (1:200, Abcam, Cambridge, MA) overnight, or mouse anti-actin antibodies (1:5000; Sigma) for 1.5 h. After washing 4 times (for 5 min each) in PBS-T, goat anti-rabbit immunoglobin G (IgG) or anti-mouse IgG-horseradish peroxidase (HRP) conjugates (Millipore) were applied at 1:10,000 dilutions for 1 h. Membranes were washed 3 times with PBS-T over 30 min and blots were developed with enhanced chemiluminescence reagents (PerkinElmer Life Sciences, Waltham, MA) and exposed to x-ray films. The relative intensity was measured to quantify the protein level. All blots were normalized against actin to adjust the proteins loaded.

2.6. RAGE immunocytochemistry

To demonstrate RAGE immunoreactivity, consecutive frozen sections of the kidney (at a thickness of 10 μm) were obtained using a Cryostat (Bright, Huntingdon, UK) at −20 °C and mounted on silane-coated slides. All tissue sections were pre-incubated in