

Effects of dietary arginine on inflammatory mediator and receptor of advanced glycation endproducts (RAGE) expression in rats with streptozotocin-induced type 2 diabetes

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Arginine (Arg) is known to possess numerous useful physiological properties and have immunomodulatory effects. *In vitro* studies reported that Arg inhibits advanced glycation endproduct (AGE) formation; however, the effects of Arg on the receptor of AGE (RAGE) expression in inflammatory conditions are not clear. The present study investigated the effects of dietary Arg supplementation on inflammatory mediator production and RAGE expression in type 2 diabetic rats. There were one normal control (NC) group and two diabetic groups in the present study. Rats in the NC group were fed with a regular chow diet. One diabetic group (DM) was fed a common semi-purified diet while the other diabetic group received a diet in which part of the casein was replaced by Arg (DM-Arg) for 8 weeks. Diabetes was induced by an intraperitoneal injection of nicotinamide followed by streptozotocin. Rats with blood glucose levels exceeding 1800 mg/l were considered diabetic. Blood samples and the liver and lungs of the animals were collected at the end of the study for further analysis. Results showed that plasma glucose and fructosamine contents were significantly higher in the diabetic groups than those in the NC group. The DM group had higher fructosamine and C-reactive protein than the DM-Arg group. Immunohistochemical staining showed that the expressions of RAGE in liver and lung tissues were significantly lower in the DM-Arg group than in the DM group. These results suggest that supplemental dietary Arg can decrease AGE–RAGE interactions and consequently reduce tissue damage in rats with type 2 diabetes.

Arginine: Type 2 diabetes: Fructosamine: C-reactive protein: Receptor of advanced glycation endproducts (RAGE) expression

Diabetes mellitus was the fifth leading cause of death in Taiwan in 2008⁽¹⁾. Among all diabetic subjects, type 2 diabetes may account for about 90–95% of the diagnosed patients. Diabetes mellitus is a metabolic disorder characterised by chronic hyperglycaemia and the development of vascular diseases. Diabetic complications appear to be multifactorial in origin. Advanced glycated endproducts, which elicit a wide range of cell-mediated response, are believed to play a central role in these disorders⁽²⁾. Advanced glycation endproducts are a complex group of compounds formed via non-enzymic reactions between reducing sugars and amine residues on protein, lipids and nucleic acids. Advanced glycation endproduct formation proceeds slowly under normal ambient sugar concentrations, but is accelerated in the presence of hyperglycaemia^(2,3). Advanced glycation endproducts can exert their biological effects through receptor-mediated mechanisms, the most important of which is the receptor of advanced glycation endproducts (RAGE). RAGE is a multi-ligand receptor of cell surface molecules which acts as a counter-receptor for diverse molecules⁽⁴⁾. Binding of advanced glycation endproducts to

the RAGE activates a number of pathways implicated in chronic inflammation and the development of diabetic complications⁽⁴⁾.

Arginine (Arg) is a non-essential amino acid for healthy adults. It has been shown to possess numerous physiological properties. Previous studies have demonstrated that Arg supplementation decreases inflammation⁽⁵⁾ and improves immune function^(6–8). Currently, Arg is added to enteral formulas in an attempt to modulate immune function and improve clinical outcomes of critically ill patients⁽⁹⁾. A recent study by Martina *et al.*⁽¹⁰⁾ showed that Arg plus *N*-acetylcysteine (a substance that increases NO availability) administration improves endothelial function and reduces inflammatory markers in hypertensive patients with type 2 diabetes. A study by Lucotti *et al.*⁽¹¹⁾ also found beneficial effects of oral Arg supplementation in reducing plasma advanced glycation endproduct levels in type 2 diabetic patients. However, the effects of Arg on advanced glycation endproduct–RAGE interactions and subsequent inflammatory reactions in diabetes are not clear. Therefore, we designed the present study to investigate the effects of supplemental

Abbreviations: Arg, arginine; CRP, C-reactive protein; DM, diabetes without arginine; DM-Arg, diabetes with arginine; MCP-1, monocyte chemoattractant protein-1; NBT, nitroblue tetrazolium; NC, normal control; RAGE, receptor of advanced glycation endproducts.

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dietary Arg on inflammatory mediator production and organ RAGE expressions in a model of type 2 diabetes.

Materials and methods

Animals

Male Wistar rats, aged 6 weeks and weighing about 180 to 220 g at the beginning of the experiment, were used in the present study. All rats were housed in a temperature- and humidity-controlled room and were allowed free access to a 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.

Study protocols

Rats were assigned to one normal control (NC; six rats) group and two diabetic groups (thirteen rats in each group) in the present study. Diabetes was induced by an intraperitoneal injection of nicotinamide at 150 mg/kg (Sigma Chemical Co., St Louis, MO, USA) followed by streptozotocin (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 streptozotocin was dissolved immediately before use in 0.05 M-sodium citrate (pH 4.5). This is a well-established animal model to imitate type 2 diabetes⁽¹²⁾. Nicotinamide was used to protect pancreatic β cells against streptozotocin toxicity in order to build up a relative insulin deficiency in rodents as type 2 diabetes mellitus⁽¹³⁾. Animals were allowed to eat laboratory chow *ad libitum* for 3 d. Rats with fasting blood glucose levels exceeding 1800 mg/l were considered diabetic. Diabetic rats were divided into two groups according to the weight and blood glucose level 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 regular chow diet. One diabetic group (DM) was fed a common semi-purified diet. The other diabetic group (DM-Arg) was provided an identical diet except that part of the casein was replaced by Arg, which provided 2% of the total energy intake. The amount of Arg administered was approximately 1.5 g Arg/kg body weight per d. This amount of Arg was found to have regulatory effects on the immune response^(14,15). The experimental diets were isonitrogenous and identical in energy and nutrient distributions (Table 1). Insulin was not given to the diabetic rats during the experimental period. Fasting blood samples were collected from a tail vein of each rat at the beginning of the experiment. After feeding the respective diets for 8 weeks, all rats were anaesthetised and killed 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. Organs were rapidly harvested for further analysis.

Measurements and analytical procedures

Fructosamine assays were performed according to the method of Chung *et al.*⁽¹⁶⁾. The fructosamine concentration was

Table 1. Composition of the experimental diets (g/kg)

Component	Group	
	DM	DM-Arg
Soyabean oil	100	100
Casein	200	158
Arginine	0	20.9
Salt mixture*	35	35
Vitamin mixture†	10	10
Methyl cellulose	31	31
Choline chloride	1	1
Methionine	3	3
Maize starch	620	641.1

DM, diabetes without arginine; DM-Arg, diabetes with arginine.

* The salt mixture contained the following (mg/g): calcium phosphate diabolic, 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; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; chromium potassium sulfate, 0.55.

† The vitamin mixture 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 -tocopheryl acetate, 20; cholecalciferol, 0.25; menaquinone, 0.005.

measured as a function of the rate of reduction of nitroblue tetrazolium (NBT) in an alkaline solution. The NBT (Sigma) stock solution was prepared as 1 mM-NBT in 0.1 M-carbonate buffer (pH 10.35). Plasma samples (40 μl) were mixed with 1 ml 0.25 mM-NBT reagent at 37°C . The absorbance was read at 530 nm. Changes in absorbance 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. Plasma glucose, TAG, total cholesterol and HDL-cholesterol levels were determined by colorimetric methods after an enzymic reaction with peroxidase (Randox Co., Crumlin, County Antrim, UK). LDL-cholesterol was estimated by the Friedewald formula which is reliable when TAG levels are < 4000 mg/l. Concentrations of intercellular adhesion molecule-1, IL-6, PGE₂, monocyte chemotactic protein-1 (MCP-1) and C-reactive protein (CRP) were measured by using a commercially available sandwich ELISA kit (R&D Systems, Minneapolis, MN, USA). Procedures followed the manufacturer's instructions.

Advanced glycated endproducts receptor immunocytochemistry

To demonstrate RAGE immunoreactivity, consecutive frozen sections of the liver and lungs (at a thickness of 10 μm) were obtained using a cryostat (Bright, Huntingdon, Cambs, UK) at -20°C and mounted on silane-coated slides. All tissue sections were preincubated in a blocking solution containing 10% normal goat serum and 0.3% H₂O₂ in 0.1 M-phosphate buffer for 1 h to block endogenous peroxidase activity and the non-specific binding of antibodies. Sections were then incubated with a rabbit polyclonal primary antibody against RAGE (Thermo Fisher Scientific Inc., Pittsburg, PA, USA) and diluted 1:300 in 0.1 M-phosphate buffer overnight at 4°C . Immunostaining was visualised by detection of peroxidase activity. For peroxidase activity detection, tissue sections were incubated in biotinylated goat anti-rabbit IgG

Table 2. Plasma glucose, TAG, total cholesterol, HDL-cholesterol and LDL-cholesterol concentrations and fructosamine content of the groups at the end of the experiment*

(Mean values and standard deviations)

Group...	NC		DM		DM-Arg	
	Mean	SD	Mean	SD	Mean	SD
Glucose (mg/l)	1235 ^a	81	3047 ^b	1236	2630 ^b	831
Fructosamine (change in absorbance/min)	0.11 ^a	0.01	0.19 ^b	0.02	0.16 ^c	0.01
TAG (mg/l)	520 ^a	150	659 ^b	167	513 ^a	173
Total cholesterol (mg/l)	608 ^a	81	874 ^b	130	782 ^b	143
HDL-cholesterol (mg/l)	124 ^a	31	209 ^b	35	200 ^b	47
LDL-cholesterol (mg/l)	392 ^a	84	536 ^b	106	506 ^b	119

NC, normal control; DM, diabetes without arginine; DM-Arg, diabetes with arginine.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* Different treatments were analysed by one-way ANOVA using Duncan's test.

(diluted 1:300; Chemicon, Temecula, CA, USA) for 1 h at room temperature. After reacting with the peroxidase-linked avidin-biotin complex (Vector, Burlingame, CA, USA) for 1 h at room temperature, a diaminobenzidine solution kit (Vector) was used to detect RAGE immunoreactivity. Haematoxylin (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), and measured by 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 the RAGE immunoreactivity. The automatic object counting and measuring processes were used to quantify the immunoreactive areas in the sections. Values were expressed as μm^2 . At least ten microscopic fields per section and three independent samples for each group were analysed and the averaged areas were obtained for each group.

Statistical analysis

All data are expressed as the mean values and standard deviations. Differences among groups were analysed by ANOVA using Duncan's test. $P < 0.05$ was considered statistically significant.

Results

There were no differences in the initial body weights, food intake or weight after feeding the diets for 8 weeks between the two diabetic groups (data not shown).

Plasma glucose and lipids

Baseline plasma glucose levels were significantly higher in the diabetic groups than those in the NC group. There were no differences in glucose levels between the DM and DM-Arg groups (DM group 2072 (SD 448) mg/l; DM-Arg group 2120 (SD 500) mg/l v. NC group 1007 (SD 78) mg/l; $P < 0.05$). The plasma glucose, total cholesterol, HDL-cholesterol and LDL-cholesterol levels in the diabetic groups were significantly higher than those in the NC group. There were no differences in these parameters between the two diabetic groups at the end of the study (Table 2).

Plasma inflammatory mediators

Plasma CRP, MCP-1 and PGE₂ levels were significantly higher in the two diabetic groups compared with the NC group. There were no differences in MCP-1 or PGE₂ between the two diabetic groups. However, the CRP level in the DM-Arg group was significantly lower than that in the DM group (Table 3).

Table 3. Plasma concentrations of intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), C-reactive protein (CRP), IL-6 and PGE₂ in the various groups at the end of the experiment*

(Mean values and standard deviations)

Group...	NC		DM		DM-Arg	
	Mean	SD	Mean	SD	Mean	SD
ICAM-1 (ng/ml)	25.9	3.3	30.9	7.1	31.9	8.7
MCP-1 (ng/ml)	143.8 ^a	11.7	320.3 ^b	129.6	286.3 ^b	123.7
CRP (mg/l)	223 ^a	21	376 ^b	41	332 ^c	46
IL-6 (pg/ml)	124.4	10.6	113.9	9.3	115.4	8.3
PGE ₂ (ng/ml)	2.36 ^a	0.85	4.86 ^b	1.80	5.01 ^b	1.66

NC, normal control; DM, diabetes without arginine; DM-Arg, diabetes with arginine.

^{a,b,c} Mean values within a row with unlike superscript letters were significantly different ($P < 0.05$).

* Different treatments were analysed by one-way ANOVA using Duncan's test.

Advanced glycated endproducts receptor expressions in the liver and lungs

Immunocytochemical findings of RAGE-immunoreactive cells with brown cytoplasm and haematoxylin-stained nuclei were seen in liver (Fig. 1(A)–(C)) and lung (Fig. 1(E)–(G)) tissues. In the liver, the immunohistochemical distribution showed that the RAGE-positive cells were frequently observed in the hepatic plates, but not in the sinusoids. It was obvious that the RAGE-positive cells were mostly hepatocytes. In the lungs, the RAGE-positive cells were mostly observed in the

wall of alveoli. Immunohistochemical expressions in both the liver and lung tissues showed that the immunoreactive intensities of RAGE were lowest in the NC group (Fig. 1(A) and (E)). The intensities were lower in the DM-Arg group (Fig. 1(C) and (G)) than in the DM group (Fig. 1(B) and (F)). Quantification results of RAGE-immunoreactive areas among groups are shown in Fig. 1(D) and (H). The immunoreactive areas of the diabetic groups were significantly larger than those of the NC group. Compared with the DM group, the DM-Arg group had smaller RAGE-immunoreactive areas.

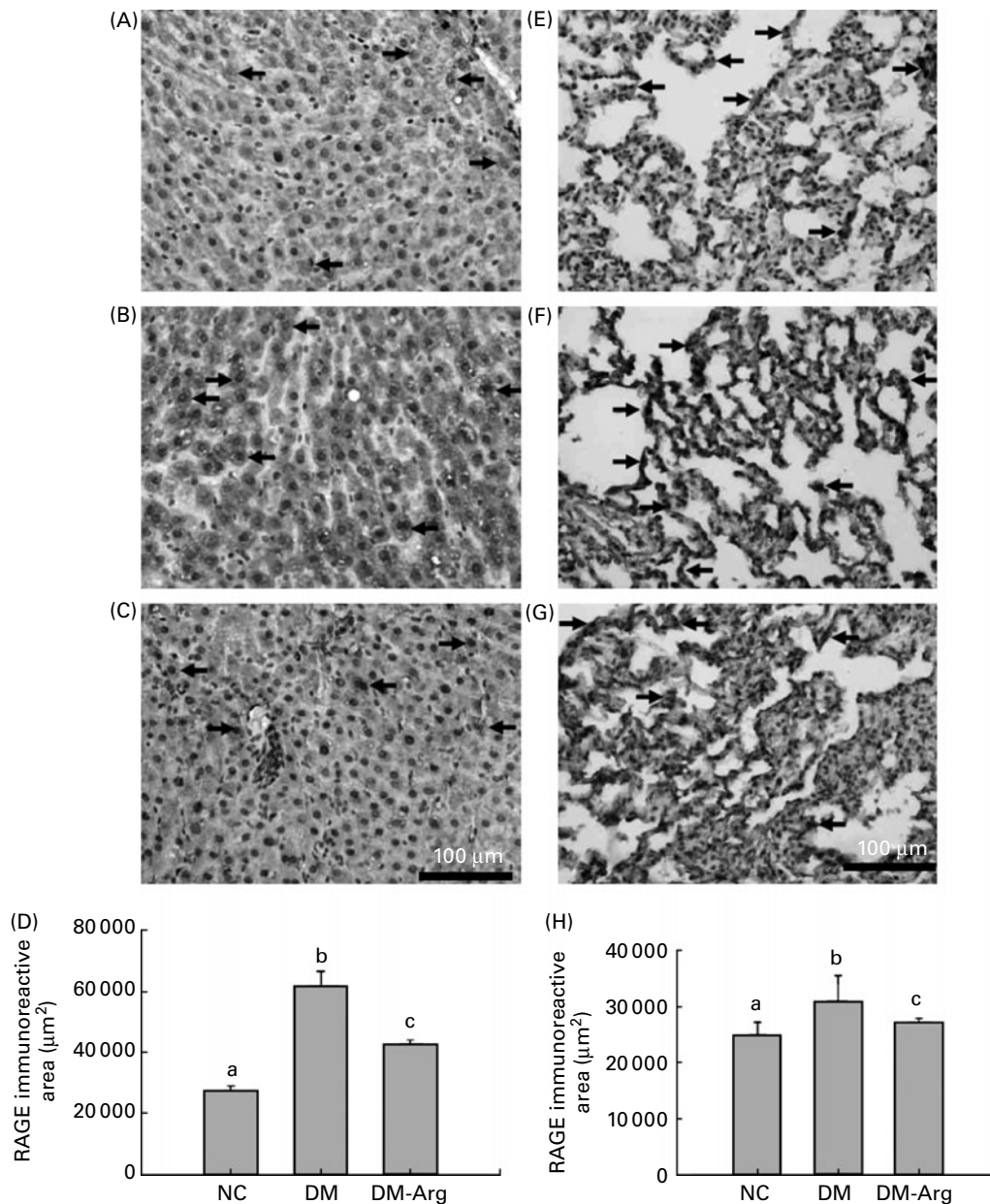


Fig. 1. Receptor of advanced glycation endproducts (RAGE) expression in the liver (A, B, C) and lungs (E, F, G) of the normal control (NC) (A, E), diabetic (DM) (B, F) and diabetic supplemented with arginine (DM-Arg) (C, G) groups. The RAGE-positive cells with brown cytoplasm and haematoxylin-stained nuclei were distributed randomly throughout the tissues, mostly in hepatocytes (A, B, C) and in the alveoli (E, F, G). The RAGE expressions in both of the liver and lung tissues exhibited higher immunoreactive intensities in the DM group (B, F) than in the DM-Arg group (C, G). Scale bars = 100 μm . Quantification of RAGE immunoreactive areas among groups ($n=3$) in the liver and lungs is shown in (D) and (H), respectively. Areas were assessed with Image-Pro Plus 5.1 (Media Cybernetics, Silver Spring, MD, USA) and were calculated as described in Materials and methods. Values are means, with standard deviations represented by vertical bars. ^{a,b,c}Mean values with unlike letters were significantly different ($P < 0.05$).

Discussion

In the present study, we used nicotinamide and streptozotocin to achieve a relative deficiency to imitate type 2 diabetes mellitus. Although this is a well-established model of type 2 diabetes mellitus in rodents, the features of insulin resistance and subclinical inflammation secondary to adiposity are not achieved with this method. Results of the present study demonstrated that almost all diabetic rats had hyperglycaemia and hypercholesterolaemia. Arg had no effect on lowering plasma glucose or cholesterol levels. However, TAG and fructosamine contents were reduced when Arg was administered. Accumulating data indicate that there is a link between dyslipidaemia and inflammation; lower plasma lipids may have a favourable effect on attenuating the inflammatory reaction⁽¹⁷⁾. Fructosamines are early glycation adducts which are formed through the condensation of glucose with primary amines. The fructosamine content can be used to quantify advanced glycation endproducts⁽¹⁶⁾. The findings of the present study were consistent with an animal study performed by Liu *et al.*⁽¹⁸⁾, in which they also found that supplemental dietary Arg decreased fructosamine without influencing fasting glucose levels in diabetic rats. Several reports showed that Arg inhibits *in vitro* non-enzymic glycation and advanced glycation endproduct formation in serum and tissue proteins from human subjects and animals^(19,20). The proposed mechanism was that the amino group of Arg can block an early stage of the Maillard reaction to form non-reactive substituted Amadori products, and with its guanidinium group, Arg may react with dicarbonyls (Amadori breakdown products) which results in fewer advanced glycation endproducts⁽¹⁹⁾. A previous report showed that oral Arg supplementation did not change plasma Arg levels in either control or diabetic rats when compared with their respective groups without Arg⁽²¹⁾. The authors postulated that the protective effect of organ function seen in diabetic rats given Arg was related to less formation of glycosylated products⁽²¹⁾.

Advanced glycation endproducts are known to be pro-inflammatory and pro-oxidant compounds. A previous report revealed that advanced glycation endproducts lead to an induction of reactive oxygen species and promote endothelial expression of IL-6, vascular cell adhesion molecule-1 and MCP-1⁽²²⁾. Prospective epidemiological studies have found that diabetic patients have increased leucocyte counts, pro-inflammatory cytokines such as IL-6 and TNF- α , and acute-phase reactants such as CRP^(23,24). Also, soluble adhesion molecule (intercellular adhesion molecule-1, vascular cell adhesion molecule-1) levels in diabetic patients were significantly higher than those in healthy controls⁽²⁵⁾. Although α 2-macroglobulin is an important acute-phase protein in response to inflammation⁽²⁶⁾, CRP is considered a potent modulator of inflammatory reaction in rats⁽²⁷⁾. CRP, IL-6, intercellular adhesion molecule-1, MCP-1 and PGE₂ are all markers of inflammation. Adhesion molecules play a key roles in cell-cell and cell-extracellular matrix interactions and are important in the adhesion of leucocytes to activated endothelium⁽²⁸⁾. Excessive expression of adhesion molecules may induce an inflammatory response and tissue injury⁽²⁹⁾. MCP-1 is involved in the recruiting peripheral leucocytes⁽³⁰⁾. PGE₂ is a product of arachidonic acid metabolism,

which regulates many aspects of the inflammatory and immune systems. Elevated PGE₂ production was observed in patients with chronic inflammatory conditions⁽³¹⁾. The results of the present study were similar to previous reports in that the inflammatory markers were elevated in the diabetic groups. Although most of the inflammatory markers measured did not differ between the two diabetic groups, we did observe that Arg supplementation reduced CRP levels in diabetes. This finding is consistent with a report by Wells *et al.*⁽³²⁾, who found an inverse relationship between Arg intake and levels of CRP. In addition, Arg stimulates the secretion of anabolic hormones such as growth hormone, prolactin and insulin-like growth factor-1⁽³³⁾, and growth hormone was found to have an effect on decreasing serum CRP⁽³⁴⁾. The mechanism of Arg that is responsible for decreasing CRP is unclear and requires further investigation.

RAGE is a member of the immunoglobulin superfamily. RAGE is minimally expressed in normal tissues and the vasculature. However, RAGE is up-regulated when advanced glycation endproduct ligands accumulate. Ligation of RAGE by advanced glycation endproducts results in intracellular signalling which leads to activation of a pro-inflammatory response⁽³⁵⁾. Engagement of RAGE in the diabetic tissues initiates a vicious cycle of ligand-RAGE perturbation which consequently leads to chronic tissue damage. In the present study, we analysed liver and lung RAGE expressions, because up-regulation of RAGE occurs in tissues and cell types that are critical for immune surveillance including the lungs, liver, vascular endothelium, mononuclear phagocytes, dendritic cells and neurons⁽³⁶⁾. Lung tissues presented high levels of the RAGE antigen among various organs examined⁽³⁷⁾. A previous study showed that the kidneys and heart of RAGE-null diabetic mice showed no superimposed injuries. Also, the levels of advanced glycation endproducts in RAGE-null mice were lower than those in RAGE-expressing animals⁽³⁸⁾. As levels of glycaemia did not differ among groups of diabetic animals, the authors concluded that factors accelerating advanced glycation endproduct formation in the hyperglycaemic environment were modulated by RAGE⁽³⁸⁾. In the present study, we used immunohistochemistry staining to quantify the expression of RAGE in the liver and lungs of the diabetic rats. We found that RAGE expression is up-regulated in diabetes, whereas Arg administration resulted in lower liver and lung RAGE expressions in the diabetic group. This result is consistent with the lower plasma advanced glycation endproducts and CRP observed in the DM-Arg group. Since the inflammatory mediator CRP and advanced glycation endproduct formation were reduced in the Arg group, this may consequently have reduced the expressions of RAGE in various organs. Also, lower RAGE expressions may down-regulate the production of CRP.

In summary, the present study showed that dietary Arg supplementation resulted in lower advanced glycation endproducts and inflammatory protein production in a diabetic condition. These results are consistent with the immunohistochemical staining which showed that RAGE expressions in the lungs and liver were significantly lower in the Arg-supplemented diabetic group. These results suggest that supplemental dietary Arg may decrease advanced glycation

endproduct–RAGE interactions and consequently reduce tissue damage in rats with type 2 diabetes.

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M.-H. P. and K.-H. H contributed to the concept of the study and did the data analysis. S.-L.Y. designed the study and prepared the manuscript, and C.-H. W interpreted the data. All authors read and approved the final submitted manuscript.

The authors are not employees or consultants associated with any commercial companies, and there were no conflicts of interest in the present study.

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