Aminoguanidine protects against apoptosis of retinal ganglion cells in Zucker diabetic fatty rats

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Abstract. – AIM: The inhibition of advanced glycation end products (AGEs) and the receptor for AGEs (RAGE) mediated downstream signaling pathways have been suggested to have retinoprotective actions in diabetic retinopathy. Herein, we examined the protective effects of aminoguanidine (AG), an AGEs inhibitor, on diabetes-induced injury of retinal ganglion cells in the Zucker diabetic fatty (ZDF) rats.

MATERIALS AND METHODS: Seven-week-old male ZDF rats were treated with AG (50 mg/kg body weight) once a day orally for 13 weeks. Serum and vitreous concentration of AGEs were examined. Expressions of AGEs and its receptor (RAGE) were assessed by immunohistochemistry. Southwestern histochemistry was used to detect activated nuclear factor (NF)-κB.

RESULTS: At the end of the study, vitreal levels of AGEs were significantly reduced in ZDF rats treated with AG. Similary, immunohistochemical analysis showed that AG significantly reduced the positive areas for AGEs and RAGE. Furthermore, AG strongly inhibited the loss of retinal ganglion cells by apoptosis. AG also suppressed the activation of to NF-κB.

CONCLUSIONS: Our results suggest that AG has retinoprotective properties through not only direct inhibition of AGEs formation but also downregulation of NF-κB.

Key Words:
   Aminoguanidine, Advanced glycation end products, Diabetic retinopathy, Retinal ganglion cells.

Introduction

Retinal neuronal cells undergo functional alterations and cell death under diabetic conditions[1-3]. Neuronal degeneration of the retina is also a critical component of diabetic retinopathy[2, 3]. Inner retinal and ganglion cell death causes permanent impairment of visual function.

Advanced glycation end products (AGEs) are the late products of non-enzymatic glycation. The levels of these products are much higher in patients with diabetes[4]. Elevated AGEs levels closely correlate with the severity of diabetic retinopathy[5,6]. In previous studies, it was shown that AGEs were accumulated in the neural retina[7]. Furthermore, it was reported that AGEs are also directly linked with the apoptotic cell death of retinal neuronal cells[8]. AGEs induced-apoptosis is mediated by increasing oxidative stress or via pro-apoptotic cytokine induced by interaction between AGEs and receptors for AGEs (RAGE)[9-11]. Recently, it was found that enhanced apoptosis of the retinal neuronal cell is also associated with nuclear factor (NF)-κB[12,13]. Although the activation of NF-κB in the retina may be involved in retinal cell death or survival[14,15]. These results demonstrated a possible pro-apoptotic role for NF-κB in diabetic retinopathy. Moreover, these findings suggested that the accumulation of AGEs in retinal tissue and the activation of NF-κB are key factors in the development of diabetic retinopathy. Therefore, based on these results, three potential therapeutic targets for diabetic retinopathy have been proposed: (1) inhibition of AGEs formation, (2) blockade of the AGE-RAGE interaction, and (3) inhibition of the AGE-RAGE mediated downstream signaling pathways.

Aminoguanidine (AG) is a selective inhibitor of AGEs, and has been shown to prevent the development of retinal vascular lesions in experimental animals[16-18]. AG effectively prevents capillary closure, pericyte loss and microaneurysm formation in the diabetic retina[19]. In a multicenter trial, AG significantly reduced urinary albumin and slowed the progression of nephropathy and retinopathy[20]. Despite the various effects of AG on diabetic complications, knowledge of its effect on diabetes-induced injury of retinal ganglion cells is limited. To elucidate this issue, we investigated the preventive effect of AG on the development of diabetic retinopathy using Zucker diabetic fatty (ZDF) rat, an animal model of

type 2 diabetes. Based on the above-mentioned therapeutic target, we evaluated the ability of AG to inhibit AGE formation and the expression of RAGE in retinal tissue. We also determined the possible mechanism of AG on NF-κB activation associated with the loss of retinal ganglion cells.

Materials and Methods

Animals and Experimental Design

Male 6-week-old ZDF rats (ZDF/Gmi-fa/fa) and Zucker lean (ZL) counterparts (ZDF/Gmi-lean) were purchased from Charles River Laboratory (Waltham, MA, USA) and acclimated for 1 week prior to the study. Rats were individually housed in plastic cages and maintained at 24 ± 2°C with a 12 h light:dark cycle and received a diet of Purina 5008 (Ralston Purina, St. Louis, MO, USA) and tap water *ad libitum*. Rats were divided into 3 groups of 8 rats according to their initial blood glucose concentration as follows: (1) normal ZL rats, (2) vehicle-treated ZDF rats and (3) ZDF rats treated with AG (50 mg/kg body weight). AG was administered once a day orally for 13 weeks. The blood glucose level and body weight were monitored consecutively, and glycated hemoglobin was determined by a commercial kit (Unimate HbA1c, Roche Diagnostics, Mannheim, Germany). At necropsy, the eye from each rat was enucleated under deep anesthesia, following an intraperitoneal injection of pentobarbital sodium (30 mg/kg body weight), and fixed in 10% neutralized formalin for 24 h and embedded in paraffin. All procedures involving rats were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and approved by the Korea Institute of Oriental Medicine Institutional Animal Care and Use Committee.

Quantification of AGEs Formation

To determine AGEs formation, serum and vitreous samples were analyzed in a competitive enzyme-linked immunosorbent assay (ELISA). The assay was performed using a monoclonal AGEs antibody (6D12, Cosmo Bio, Tokyo, Japan) according to established protocols[21].

Apoptosis Assay

To evaluate apoptosis in retinal neuronal cells, the TUNEL assay was performed with a kit (Dead-End apoptosis detection system, Promega, Madison, WI, USA) according to the manufacturer's instructions. Apoptotic cells were detected with fluorescein-conjugated streptavidin in the retinal section. For quantitative analysis, TUNEL-positive nuclei were then counted in equal area of each slide.

Immunohistochemical Staining

Immunohistochemistry was performed as previously described[22]. Antibodies were mouse anti-AGEs (Cosmo Bio Co., Tokyo, Japan), Carlsbad, CA, USA) and rabbit anti-RAGE (Santa Cruz, CA, USA). For detection of AGEs and RAGE, the sections incubated with LSAB kit (DAKO, Carpinteria, CA, USA) and visualized by 3,3'-diaminobenzidine tetrahydrochloride. For morphometric analysis, the positive areas or numbers of positive cells per unit area (0.32 mm^2) in a total of 10 fields were determined using Image J software (NIH, Bethesda, MD, USA).

Southwestern Histochemistry for Detection of Activated NF-κB

To localize the NF-κB activity in the retina, *in situ* southwestern histochemistry was performed as described by Hernandez-Presa et al[23]. The number of cell positive to NF-κB activation in the ganglion cell layer was then counted with computer assisted Image J software (NIH). As negative controls, the following were used: (1) absence of probe, (2) mutant NF-κB probe labeled with digoxigenin and (3) competition assays with a 200-fold excess of unlabeled NF-κB followed by incubation with labeled probe.

Statistical Analysis

Statistical evaluation of the results was performed using Student's *t*-test and a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using GraphPad Prism 4.0 software (Graph pad, La Jolla, CA, USA).

Results

Blood Glucose Level

At 20 weeks of age, all ZDF rats were developed hyperglycemia compared to the normal ZL rat. AG induced a minor decrease of blood glucose levels (Table I).

AG inhibits AGEs Formation and Accumulation and Reduces the Expression of RAGE

AG was tested for its ability to inhibit AGEs formation and accumulation in the retina. At the

Table I. Blood glucose level.

	ZL	ZDF	AG
Blood glucose (mg/dl)	92.9±10.8	489.8±38.*	324.8±151.1†

ZL: normal Zucker lean rats, ZDF: vehicle-treated Zucker diabetic fatty rats, AG: Zucker diabetic fatty rats treated with aminoguanidine (50 mg/kg body weight). All data were expressed as mean ± SD. *$p < 0.01$ vs. normal ZL rats, †$p < 0.05$ vs.vehicle-treated ZDF rats.

end of the study, the AGEs levels in both serum and vitreous were remarkably elevated in vehicle-treated ZDF rats compared to normal ZL rats. However, these levels in the AG-treated ZDF rats were significantly decreased compared to vehicle-treated ZDF rats (Figure 1A and B). We next carried out immunohistochemical staining for AGEs. It was apparent that AGEs immunoreactivity was only contained in the large and small retinal vessels of the normal ZL rats, whereas AGEs-positive signals were located in both the retinal vessels and the inner neural retina in the vehicle-treated ZDF rats, indicating that serum AGEs had accumulated in the retinal tissues. However, treatment with AG reduced the AGEs deposited in these regions (Figure 1C). We also examined the inhibitory effect of AG on RAGE expression. Immunohistochemical staining for RAGE showed that the extent of retinal RAGE immunolabeling was greater in the vehicle-treated ZDF rats than in the normal ZL rats (Figure 1D). In quantitative
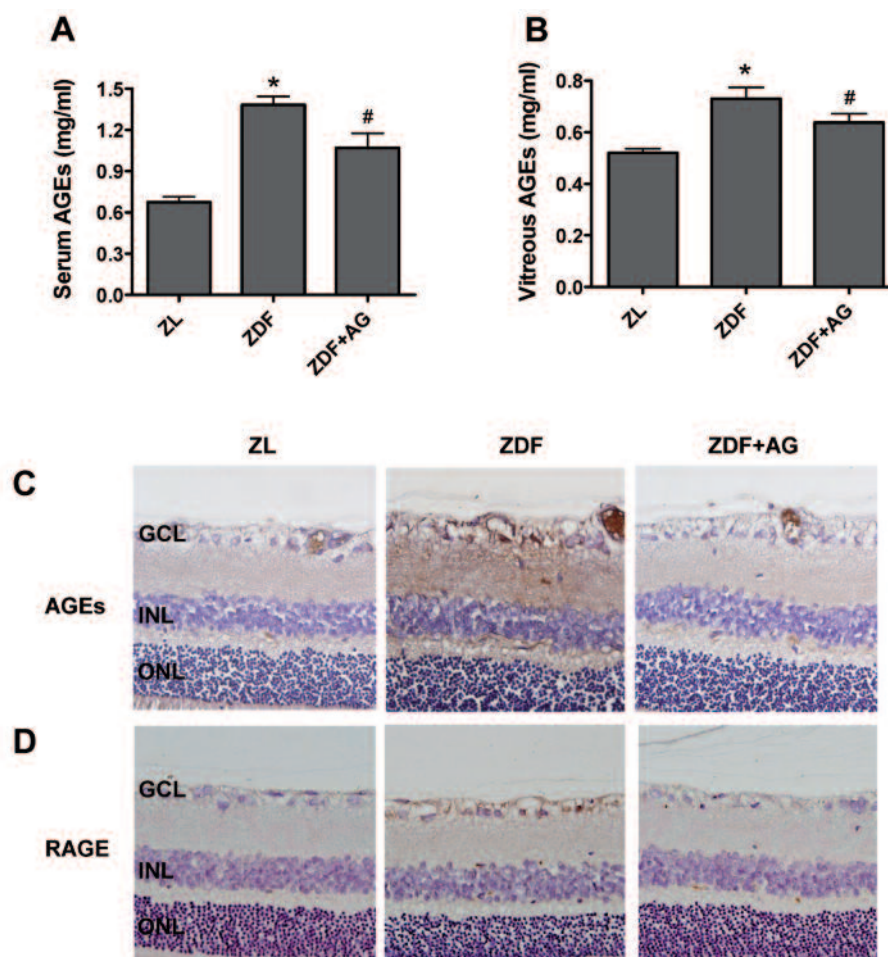

Figure 1. Formation of advanced glycation end products (AGEs) in *(A)* blood and *(B)* vitreous. The values in the graph represent means ± SE, n = 8. *$p < 0.01$ vs. normal ZL rats, #$p < 0.05$ vs. vehicle-treated ZDF rats. Representative immunostaining of *(C)* AGEs and *(D)* receptor for AGEs (RAGE) in retinas from a normal Zucker lean rat (ZL), vehicle-treated ZDF rat (ZDF) and ZDF rat treated with aminoguanidine (ZDF+AG). The sections were visualized by 3,3'-diaminobenzidine tetrahydrochloride substrate staining (brown) and counterstained with Mayer's hematoxylin. GCL, Ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. X200 magnification.

analysis, the expression of RAGE was increased five-fold in the vehicle-treated ZDF rats compared to the normal ZL rats. These changes were reduced by treatment with AG.

Apoptosis in Retinal Neuronal Cells

To characterize the death of neurons in the GCL of vehicle-treated ZDF rats, we applied the apoptosis assay. A significant increase in TUNEL-positive cells in the GCL was observed in vehicle-treated ZDF rats (Figure 2A and C). Treatment of ZDF rats with AG prevented an increase in positive cells similar to those seen in normal ZL rats (Figure 2A and C). The presence of TUNEL-positive cells was not limited to the GCL. The inner nuclear layer demonstrated occasional positive cells, as did the outer nuclear layer of photoreceptor cell nuclei. These results sug-

gest that several ganglion cells were undergoing apoptosis under diabetic conditions, which might lead to histopathological changes, such as the thinning of retinal cell layers.

AG Inhibits the Activation of NF-κB in the Retina

NF-κB is a common downstream signal of AGEs[24]. It also plays an essential role in apoptosis[12,13]. We, therefore, determined whether AG could inhibit the activation of NF-κB in retinal tissue. NF-κB activity was detected in retinal tissue by southwestern histochemistry. This technique allows the localization of activated nuclear factor in the cellular nucleus. Using this novel method, we observed that marked NF-κB activity was mainly found in the nuclei in the GCL and the inner nuclear layer in vehicle-treated ZDF
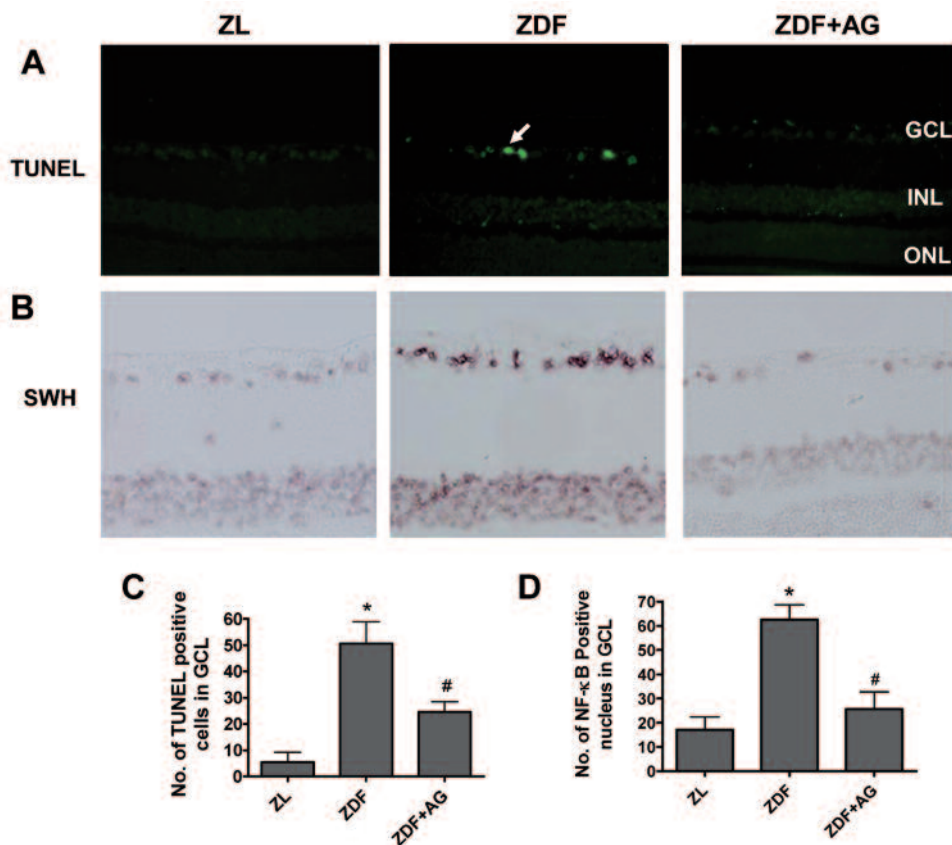

Figure 2. Apoptosis of retinal ganglion cells *(A)* and NF-κB activation *(B)*. The retinal sections were stained with TUNEL (green). Apoptotic ganglion cells (white arrow) were observed in vehicle-treated ZDF rats. NF-κB activation was determined by southwestern histochemistry (SWH). Positive signal mainly detected in nucleus of retinal ganglion cells and inner nuclear cells in the vehicle-treated ZDF rat. X200 magnification. *(C)* Quantitative analysis of TUNEL-positive cells in the retina. *(D)* Quantitative analysis of SWH-positive cells in the retinal ganglion cell layer. All data are expressed as mean ± SE, n = 8. *$p < 0.01$ vs. normal ZL rats, #$p < 0.01$ vs. vehicle-treated ZDF rats.

rats (Figure 2B). In morphometric analysis, the expression of activated NF-κB in vehicle-treated ZDF rats was significantly increased, whereas AG significantly inhibited the expression of activated NF-κB (Figure 2D).

Discussion

In the present study, we examined the preventive effect of AG on diabetes-induced injury of retinal ganglion cells. Two important findings emerged from this work. The first is that AG reduced the AGEs accumulation and RAGE expression in retinal tissue. Yamagishi et al. reported that AGEs have been implicated in the pathogenesis of diabetic retinopathy and that the inhibition of AGEs formation improves diabetic retinopathy[25-27]. AG is a hydrazine compound with a non-toxic nucleophilic hydrazine group, which can react with carbonyl groups lacking electron. Its basic structure is HN–NH–C–NH. AG reacts with products of Amadori rearrangements that contain active carbonyls, such as early glycation products and 3-deoxy-glucose ketones, resulting in inactive forms of the Amadori products that block further rearrangement and dehydrogenation to form AGEs[28]. In addition, the AGE-RAGE interaction elicits ROS generation, the expression of proinflammtory cytokines and the induction of apoptosis[11,25,26]. Thus, the inhibition of AGEs formation or the blockade of the AGE-RAGE axis has been suggested as a novel therapeutic target for diabetic retinopathy[27]. Indeed, AG had a property of AGEs inhibitor in retinal tissue. These results suggest that AG treatment results in the decrease of interaction between AGEs and RAGE and a decline in cellular damage mediated by AGEs.

The second important finding is that AG ameliorated neuronal degeneration by apoptosis. In the adult retina, the death of neuronal cells leads to the development of diabetic retinopathy because neuronal cells cannot replicate[29]. Interestingly, it was reported that the accumulation of AGEs in retinal ganglion cells induced apoptosis[1,8,25,30]. Moreover, AGEs interact with RAGE, inducing subsequent activation of NF-κB and NF-κB-controlled pro-apoptotic molecules, such as TNF-α and nNOS. Our data presenting the upregulation of NF-κB in GCL of diabetic ZDF rat provide strong evidence that NF-κB activation is responsible for the loss of ganglion cells. These results suggest that an-

ti-apoptotic effect of AG is probably due to its inhibitory effect on AGEs and RAGE.

Intensive glycemic control generally inhibited the progression of diabetic retinopathy[31]. However, it was difficult for many patients to maintain the optimal metabolic level. Thus, the development of new drug to modulate the mechanism involved in diabetic retinopathy is needed, even if the blood glucose was not controlled perfectly. In this study, AG inhibited the development of diabetic retinopathy despite continued hyperglycemia.

Conclusions

AG successfully prevented AGEs formation and accumulation in retinal tissue. AG also had anti-apoptotic effects via the suppression of NF-κB activation. Taken together, these results indicate that treatment with AG could be a valuable therapeutic approach in diabetic retinopathy.

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Conflict of Interest
The Authors declare that there are no conflicts of interest.

References

1) MIZUTANI M, KERN TS, LORENZI M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. J Clin Invest 1996; 97: 2883-2890.

2) ASNAGHI V, GERHARDINGER C, HOEHN T, ADEBOJE A, LORENZI M. A role for the polyol pathway in the early neuroretinal apoptosis and glial changes induced by diabetes in the rat. Diabetes 2003; 52: 506-511.

3) BARBER AJ, LIETH E, KHIN SA, ANTONETTI DA, BUCHANAN AG, GARDNER TW. Neural apoptosis in the retina during experimental and human diabetes. Early onset and effect of insulin. J Clin Invest 1998; 102: 783-791.

4) BROWNLEE M. Advanced protein glycosylation in diabetes and aging. Annu Rev Med 1995; 46: 223-234.

5) KOGA K, YAMAGISHI S, OKAMOTO T, INAGAKI Y, AMANO S, TAKEUCHI M, MAKITA Z. Serum levels of glucose-derived advanced glycation end products are associated with the severity of diabetic retinopathy in type 2 diabetic patients without renal dysfunction. Int J Clin Pharmacol Res 2002; 22: 13-17.

6) Miura J, Yamagishi S, Uchigata Y, Takeuchi M, Yamamoto H, Makita Z, Iwamoto Y. Serum levels of non-carboxymethyllysine advanced glycation end-products are correlated to severity of microvascular complications in patients with Type 1 diabetes. J Diabetes Complications 2003; 17: 16-21.

7) Hammes HP, Alt A, Niwa T, Clausen JT, Bretzel RG, Brownlee M, Schleicher ED. Differential accumulation of advanced glycation end products in the course of diabetic retinopathy. Diabetologia 1999; 42: 728-736.

8) Lecleire-Collet A, Tessier LH, Massin P, Forster V, Brasseur G, Sahel JA, Picaud S. Advanced glycation end products can induce glial reaction and neuronal degeneration in retinal explants. Br J Ophthalmol 2005; 89: 1631-1633.

9) Kasper M, Roehlecke C, Witt M, Fehrenbach H, Hofer A, Miyata T, Weigert C, Funk RH, Schleicher ED. Induction of apoptosis by glyoxal in human embryonic lung epithelial cell line L132. Am J Respir Cell Mol Biol 2000; 23: 485-491.

10) Kaji Y, Amano S, Usui T, Oshika T, Yamashiro K, Ishida S, Suzuki K, Tanaka S, Adamis AP, Nagai R, Horiuchi S. Expression and function of receptors for advanced glycation end products in bovine corneal endothelial cells. Invest Ophthalmol Vis Sci 2003; 44: 521-528.

11) Yamagishi S, Inagaki Y, Amano S, Okamoto T, Takeuchi M, Makita Z. Pigment epithelium-derived factor protects cultured retinal pericytes from advanced glycation end product-induced injury through its antioxidative properties. Biochem Biophys Res Commun 2002; 296: 877-882.

12) Romeo G, Liu WH, Asnaghi V, Kern TS, Lorenzi M. Activation of nuclear factor-kappaB induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. Diabetes 2002; 51: 2241-2248.

13) Kowluru RA, Koppolu P, Chakrabarti S, Chen S. Diabetes-induced activation of nuclear transcriptional factor in the retina, and its inhibition by antioxidants. Free Radic Res 2003; 37: 1169-1180.

14) Wu T, Chiang SK, Chau FY, Tso MO. Light-induced photoreceptor degeneration may involve the NF kappa B/caspase-1 pathway in vivo. Brain Res 2003; 967: 19-26.

15) Choi JS, Kim JA, Kim DH, Chun MH, Gwag BJ, Yoon SK, Joo CK. Failure to activate NF-kappaB promotes apoptosis of retinal ganglion cells following optic nerve transection. Brain Res 2000; 883: 60-68.

16) Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M. Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. Proc Natl Acad Sci U S A 1991; 88: 11555-11558.

17) Kern TS, Engerman RL. Pharmacological inhibition of diabetic retinopathy: aminoguanidine and aspirin. Diabetes 2001; 50: 1636-1642.

18) Kern TS, Tang J, Mizutani M, Kowluru RA, Nagaraj RH, Romeo G, Podesta F, Lorenzi M. Response of capillary cell death to aminoguanidine predicts the development of retinopathy: comparison of di-

abetes and galactosemia. Invest Ophthalmol Vis Sci 2000; 41: 3972-3978.

19) Gardiner TA, Anderson HR, Stitt AW. Inhibition of advanced glycation end-products protects against retinal capillary basement membrane expansion during long-term diabetes. J Pathol 2003; 201: 328-333.

20) Vasan S, Foiles PG, Founds HW. Therapeutic potential of AGE inhibitors and breakers of AGE protein cross-links. Expert Opin Inv Drug 2001; 10: 1977-1987.

21) Mitsuhashi T, Vlassara H, Founds HW, Li YM. Standardizing the immunological measurement of advanced glycation endproducts using normal human serum. J Immunol Methods 1997; 207: 79-88.

22) Sohn EJ, Kim CS, Kim YS, Jung DH, Jang DS, Lee YM, Kim JS. Effects of magnolol (5,5'-diallyl-2,2'-dihydroxybiphenyl) on diabetic nephropathy in type 2 diabetic Goto-Kakizaki rats. Life Sci 2007; 80: 468-475.

23) Hernandez-Presa MA, Gomez-Guerrero C, Egido J. In situ non-radioactive detection of nuclear factors in paraffin sections by Southwestern histochemistry. Kidney Int 1999; 55: 209-214.

24) Yamagishi S, Takeuchi M, Matsui T, Nakamura K, Imaizumi T, Inoue H. Angiotensin II augments advanced glycation end product-induced pericyte apoptosis through RAGE overexpression. FEBS Lett 2005; 579: 4265-4270.

25) Yamagishi S, Amano S, Inagaki Y, Okamoto T, Koga K, Sasaki N, Yamamoto H, Takeuchi M, Makita Z. Advanced glycation end products-induced apoptosis and overexpression of vascular endothelial growth factor in bovine retinal pericytes. Biochem Biophys Res Commun 2002; 290: 973-978.

26) Yamagishi S, Hsu CC, Taniguchi M, Harada S, Yamamoto Y, Ohsawa K, Kobayashi K, Yamamoto H. Receptor-mediated toxicity to pericytes of advanced glycosylation end products: a possible mechanism of pericyte loss in diabetic microangiopathy. Biochem Biophys Res Commun 1995; 213: 681-687.

27) Yamagishi S, Nakamura K, Matsui T. Advanced glycation end products (AGEs) and their receptor (RAGE) system in diabetic retinopathy. Curr Drug Discov Technol 2006; 3: 83-88.

28) Singh R, Barden A, Mori T, Beilin L. Advanced glycation end-products: a review. Diabetologia 2001; 44: 129-146.

29) Engerman RL. Pathogenesis of diabetic retinopathy. Diabetes 1989; 38: 1203-1206.

30) Denis U, Lecomte M, Paget C, Ruggiero D, Wiernsperger N, Lagarde M. Advanced glycation end-products induce apoptosis of bovine retinal pericytes in culture: involvement of diacylglycerol/ceramide production and oxidative stress induction. Free Radic Biol Med 2002; 33: 236-247.

31) The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993; 329: 977-986.