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The Antioxidative Effects of *Astragalus* Saponin I Protect Against Development of Early Diabetic Nephropathy

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Abstract. It has been known that oxidative stress plays an important role in the development of diabetic nephropathy (DN). The antioxidative effects of *Astragalus* saponin I (AS I) were studied in vitro and in vivo. In the presence of high glucose and H_2O_2, the total antioxidative capability, catalase, reduced glutathione, and superoxide dismutase level of rat mesangial cells were significantly decreased, and transforming growth factor β1 (TGF-β1) mRNA level, collagen IV, and laminin level were significantly increased. When compared with those in the high glucose group, these 4 indexes of cells incubated in 2.0 and/or 20 μmol/L of AS I were significantly enhanced, and levels of TGF-β1 mRNA, collagen IV and laminin were statistically decreased. By flowcytomery, percentages of S phase of cells incubated in high glucose and H_2O_2 were lowered, while those in AS I were increased. Furthermore, the physical behaviors of rats treated with 12 mg/kg of AS I restored with vigor and weight gaining, while the level of HbAlC was significantly reduced. Thus, AS I has antioxidative effects and is a potential compound worth further study because it may prevent the development of DN.

Keywords: *Astragalus* saponin I, diabetic nephropathy, antioxidative, mesangial cell

Introduction

Radix Astragali, the root of *Astragalus membranaceus* (Fisch.) BUNGE, is a crude drug widely used in Traditional Chinese Medicine. Polysaccharides and saponins, known biologically as active constituents, are the two major chemical compounds extracted from *Astragalus membranaceus*. *Astragalus* saponin I (AS I, Fig. 1), which has various bioactivities including the scavenging effects on O_2^- and ·OH (1), is one of the best known components among them. The extract of Astragalus could inhibit proliferation and matrix over-synthesis of human renal mesangial cells and down-regulate the expression of β1 integrin on its surface (2), and AS I gradually increased the secretion of insulin and C-peptide on rats (3). Recently, our laboratory reported that AS I has therapeutic effects on several pharmacological targets, such as oxidative stress, advanced glycation end products (AGEs), transforming growth factor β1 (TGFβ1), in the development of diabetic nephropathy (DN) and is a potential drug for prevention of early DN (4). There are also other reports consistent with this antioxidative effect of AS I in our experiment (5 – 7).

Hyperglycemia not only generates reactive oxygen

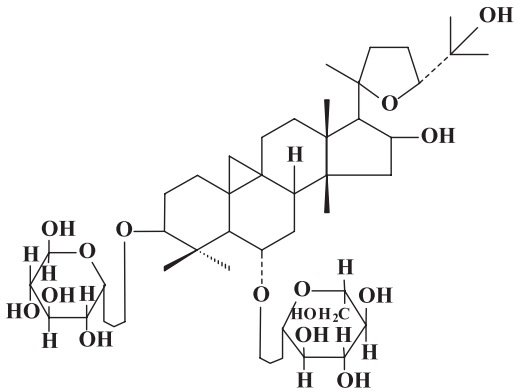

Fig. 1. The chemical structure of *Astragalus* saponin I. The molecular weight of it is 784.

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species (ROS), but also attenuates antioxidant mechanisms, which leads to oxidative stress. This oxidative stress enhances the TGF-β1 synthesis and thereby increases ECM gene expression (8), leading to increased renal albumin permeability. Therefore, oxidative stress is now considered to be one of the major factors closely related to the development of DN. Nitric oxide (NO) is also important in DN as an oxidant and glomerular hemodynamics modulator. For example, inhibition of inducible NO reduces the expression of TGF-β1 (9, 10), which indicates the potential role of NO in the DN mechanism. Thus, hyperglycemia may destroy the balance between ROS and NO, inducing the increase in TGF-β1, and consequently resulting in ECM protein accumulation. Therefore, it is very important to investigate the effects of AS I on oxidative stress, NO, and TGF-β1, which are all related to glucose metabolism.

Mesangial cells are mononucleated cells embedded in ECM and are in contact with the basement membrane and endothelial lining of the glomerular capillaries (11). Functionally, an increase in glomerular filtration rate found in the early phase of diabetes has been proposed to be related to the future development of DN. These abnormalities could be caused by functional changes in diabetic glomeruli, particularly in glomerular mesangial cells because mesangial cells were found to be capable of producing ECM proteins (12, 13) and regulating glomerular filtration rate by their contractility (14). An enhancement of the production of type IV collagen (15) has been shown in mesangial cells cultured under high-glucose conditions. It is considered that these functional changes in mesangial cells in diabetes are caused by the metabolic abnormalities in mesangial cells specific to diabetes. Despite the existing evidences that glucose accounts for the increased accumulation of ECM protein in DN, relatively little is known concerning the antioxidative effects of AS I on ECM protein production.

In our present study, we examined the effects of glucose on the antioxidant defense of the mesangial cells, evaluated the possible antioxidative effects of AS I, and its influences on the parameters that indicate its protective effects against the development of DN on rat mesangial cells, and then we observed its potential therapeutic effects on early stage of DN rats.

Materials and Methods

Drugs

AS I (No. 20020818, which means the product date of Aug. 18, 2002, was the registration number in our laboratory for AS I used in this study) was extracted from *Astragalus membranaceus* (Fisch.) BUNGE (Shandong, China) and stored at the Department of Pharmacology (Nanjing Medical University, China). Powdered herb roots (1 kg) were refluxed with 95% ethanol twice. The filtrate was concentrated in vacuo and then added with a NaOH solution of ethanol and allowed to react at 80°C – 90°C under stirring. The extract was partitioned successively between water and ethyl acetate and then *n*-butanol. The *n*-butanol solution was washed with water to neutral and was concentrated in vacuo to yield the residue. The residue was subjected to column chromatography on silica gel H, eluted with a mixture of chloroform:methanol (4:1), and was finally recrystallized with methanol to give AS I (1.05 g), with the yield of 0.1%.

AS I extracted in our laboratory was authenticated with aspects to its melting point, mass spectrogram, and infrared spectrogram by Professor Fuzhong Zhao (Department of Chemistry, Nanjing Medical University), and its purity was determined by HPLC. The HPLC system consisted of a Shimadzu LC-6A pump and a Shimadzu SPD-6A UV detector (Shimadzu, Kyoto). The analytical column was Phenomenex C18 column (ODS 3,100A, 4.6 × 150 mm ID; Phenomenex, Torrance, CA, USA). The mobile phase was composed of acetonitrile:water (60:40, v:v). The flow rate was 1.0 mL/min. The detector wavelength was 203 nm. The retention times of AS I and impurity peak were 10.8 and 8.5 min, respectively, and the purity of AS I was 97.7%, calculated by the ratio of their peak area.

AS I was dissolved in 1% ethanol for cell culture and suspended in 1% carboxymethyl cellulose (CMC) solution at different concentrations for the purpose of oral administration. Vitamin E (Lot No. 02010801), an antioxidative agent, serving as a positive control drug in cell culture, was kindly provided by Xiamen Cod Liver-oil Factory (Xiamen, Fujian, China). Epalrestat (Lot No. 990921), an aldose reductase inhibitor, serving as a positive control drug for in vivo experiment, was kindly provided by Shanghai Institute of Pharmaceutical Industry (Shanghai, China) and also suspended with 1% CMC solution.

Mesangial cell culture

Rat mesangial cells (No. HBZT-1) were purchased from China Center for Type Culture Collection (Wuhan University, China) and cultured in Dulbecco's Modified Eagle Medium with D-glucose (DMEM, Lot No. 1136551; Gibco, Grand Island, NY, USA) at 37°C containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. For the different experiments, cells were cultured under normal glucose (5.56 mmol/L) and high glucose (25 mmol/L). The cultured mesangial cells were divided into the following 8 groups: the normal standard group, ethanol group, high

glucose group, H_2O_2 group, vitamin E group, low AS I group, moderate AS I group, high AS I group. These groups were incubated in DMEM with 5.56 mmol/L glucose, 1% ethanol + 5.56 mmol/L glucose, 25 mmol /L glucose, 0.1 mmol/L H_2O_2 + 5.56 mmol/L glucose, 0.2 mmol/L vitamin E + 25 mmol/L glucose, 0.2 μmol /L AS I + 25 mmol/L glucose, 2.0 μmol/L AS I + 25 mmol/L glucose, and 20.0 μmol/L AS I + 25 mmol/L glucose, respectively. The media were replaced 2 or 3 times per week. All experiments were performed with cells between passages 5 and 8. After culturing with the different agents for 36 h, cells were harvested for analysis.

Animals and protocol

Male Sprague-Dawley rats (Certificate No. SYXK 2001-0010, weighing 160 – 180 g) were obtained from Laboratory Animal Center of Nanjing Medical University and cared for in accordance with the Guiding Principles for Care and Use of Laboratory Animals of Nanjing Medical University. Early DN was induced by i.p. administration of 60 mg/kg of streptozotocin (STZ, Lot No. P5639; Biomol Research Lab, Inc., Plymouth Meeting, PA, USA), dissolved in pH 4.5 citrate buffer immediately before injection, while rats that received the same volume of citrate buffer (2.5 ml/kg) served as the controlled normal standard. Induction of the diabetic state was confirmed by measuring their blood glucose level at 72 h after the injection of STZ. The rats whose blood glucose concentrations were higher than 13.88 mmol/L were randomly allotted into 5 groups: DN group (treated with CMC solution), low AS I group (treated with 3 mg/kg of AS I), moderate AS I group (treated with 6 mg/kg of AS I), high AS I group (treated with 12 mg/kg of AS I), and epalrestat group (100 mg/kg of epalrestat). The same volume of CMC solution was orally administrated to the normal standard group and DN group. The animals were housed in a controlled environment (24 ± 1°C, with a 12 h light alternating with 12 h darkness cycle, lights on at 7:00 AM) and were allowed food and water ad libitium. After 8 weeks, blood samples were collected for detection.

Measurement of renal function and biochemical indexes

HbA1C was measured by affinity chromatography. The levels of catalase, blood urea nitrogen (BUN), superoxide dismutase (SOD), and reduced glutathione (GSH) were assayed by chemical colorimetry. Different kinds of kits from Jiancheng Bioengineering Institute (No. 20021122; Nanjing, China) were used in all of above analyses.

The NO level of cells was determined by the Griess method (16, 17). Cells were lysed and centrifugated and then 50 μL Griess reagent (equal volume of 1% sulffanilamide in HCl 0.1 mol/L and 0.1% *N*-[-1-naphthyl-ethylenediamine dihydrochloride]) was added to 50 μl of supernatants. Nitrite concentration was determined by spectrophotometry (UV-1600; Rayleigh Analytical Instrument Co., Beijing, China) at 540 nm from a standard curve (0 – 100 μmol/L) derived from $NaNO_2$ (Beyotime Biotechnology, Haimen, Jiangsu, China). The total nitric oxide synthase (tNOS) and inducible nitric oxide synthase (iNOS) level were assayed by chemical colorimetry, whose kits were purchased from Jiancheng Bioengineering Institute (No. 20021122).

Total antioxidative capabilities of rat mesangial cells were determined by the method of ABTS (18). Collagen IV and laminin, main components of ECM, were determined by radioimmunoassay (19), using kits from Shanghai High Biotech Center (No. 20021201; Shanghai, China). The relative quantities of TGF-β1 mRNA were measured by reverse transcription PCR procedure (4).

Cell percentages of S phase

Cell cycle was detected by a quantitative staining method in which propidiumiodine was inserted into DNA. After cultured for 72 h, rat mesangial cells were digested by 0.05% typsin. The mixture was centrifuged at 1,000 rpm for 10 min and the supernatant was removed. The cells were resuspended and adjusted to 10^6/ml; 1.0 ml of this cell suspension was centrifuged again, and then 300 μl of D-Hanks and 2 ml of 70% iced ethanol were added into the pellet to fix the cells. Next, the pelleted cells were centrifuged again with normal saline added. The remaining cells was mixed with 0.5 ml of propidiumiodine and stained at 4°C for 15 min. Then the stained cells were analyzed by a FAC SVantage SE flow cytometer (Becton-Dickinson, NJ, USA). Data were collected at excitation wave of 488 nm, and the various phases of the cell cycle were analyzed by the software Mod Fit 2.0 (Becton-Dickinson).

Statistical analyses

Statistical analysis was performed to compare the effects of AS I on early DN rats using one-way analysis of variance (ANOVA) and *t*-test for different groups. Data are expressed as the mean ± S.D.; the value of $P<0.05$ was considered significant.

Results

The antioxidative effects of AS I on mesangial cells

Figure 2 shows the effects of AS I on the total anti-

oxidative capability, activity of catalase, GSH, and SOD of rat mesangial cells. The levels of all of these indexes in the ethanol group were almost the same as those in the normal standard group ($P>0.05$), indicating that 1% ethanol did not change the oxidative status of the mesangial cells. When compared with those in the ethanol group, the total antioxidative capability, the activity of catalase, GSH, and SOD in the high glucose group and the H_2O_2 group were significantly decreased ($P<0.05$ or $P<0.01$). These indexes in the high AS I group were statistically higher than those in the high glucose group ($P<0.05$ or $P<0.01$).

The effects of AS I on NO in mesangial cells

Table 1 shows the effects of AS I on NO level, tNOS activity, and iNOS activity in rat mesangial cells. There were no significant differences between the ethanol group and the normal standard group ($P>0.05$). When compared with those in the ethanol group, these parameters in the high glucose group and the H_2O_2 group were significantly increased ($P<0.05$ or $P<0.01$). These parameters of NO in the high AS I group were statisti-

Table 1. The effects of AS I on NO level, tNOS activity, and iNOS activity in rat mesangial cells

Group	NO (μmol/L)	tNOS (U/mg·protein)	iNOS (U/mg·protein)
NS	60.12 ± 12.37	49.14 ± 7.80	30.43 ± 3.65
ET	75.29 ± 8.87	49.99 ± 15.60	29.96 ± 12.49
HG	103.29 ± 14.06$^{\#}$	102.27 ± 10.06$^{\#\#}$	86.66 ± 10.91$^{\#\#}$
HP	98.81 ± 20.04$^{\#}$	108.41 ± 22.00$^{\#}$	95.09 ± 23.10$^{\#}$
V_E	80.96 ± 14.05	64.30 ± 11.01*	42.81 ± 10.66**
AL	96.84 ± 9.44	94.25 ± 26.25	70.36 ± 26.45
AM	88.08 ± 19.88	82.12 ± 12.31	62.19 ± 10.09*
AH	76.91 ± 7.96*	69.75 ± 8.99*	49.69 ± 8.49**

$^{\#}P<0.05$, $^{\#\#}P<0.01$, compared with the ET group by the *t*-test. *$P<0.05$, **$P<0.01$, compared with HG group by the *t*-test. Statistical analysis was performed using ANOVA. Data are presented as the mean ± S.D., n = 3.

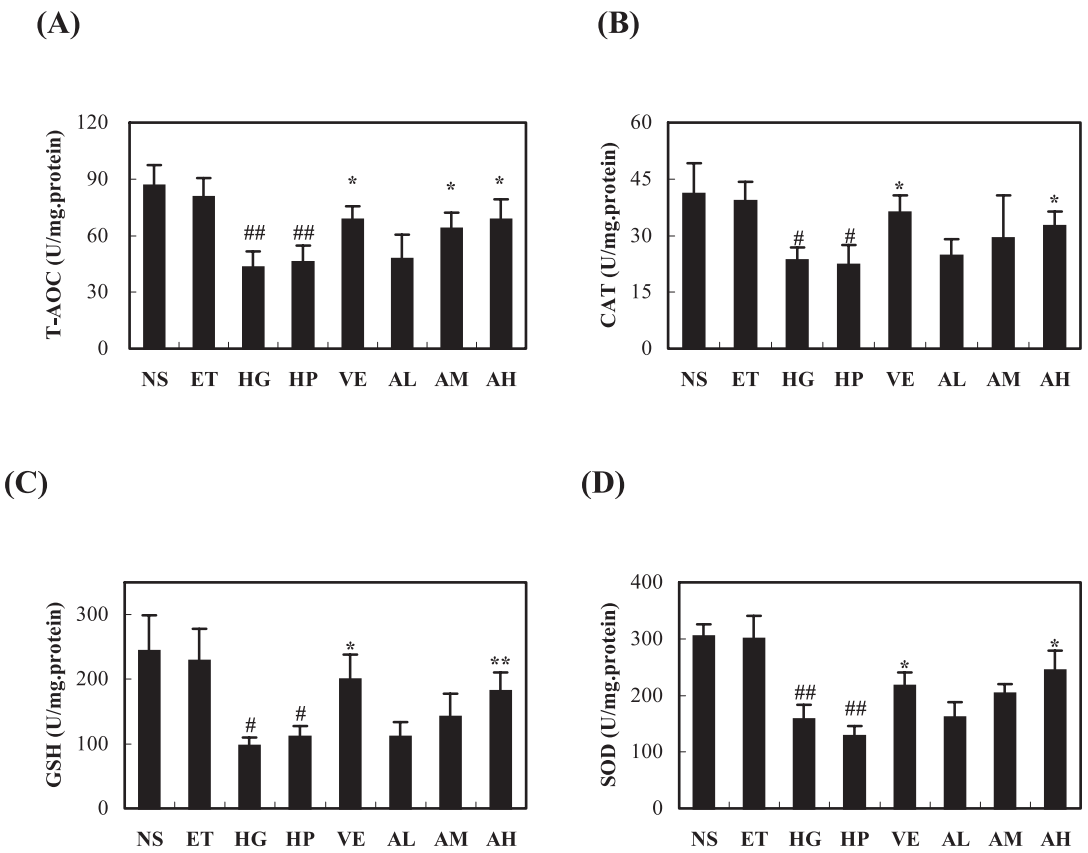

Fig. 2. The effects of AS I on total anti-oxidative capability (T-AOC) (A), activity of catalase (CAT) (B), GSH (C), and SOD (D) of rat mesangial cells. NS, ET, HG, HP, V_E, AL, AM, and AH represent cells incubated in DMEM with 5.56 mmol/L glucose, 1% ethanol + 5.56 mmol/L glucose, 25 mmol/L glucose, 0.1 mmol/L hydrogen peroxide + 5.56 mmol/L glucose, 0.2 mmol/L vitamin E + 25 mmol/L glucose, 0.2 μmol/L AS I + 25 mmol/L glucose, 2.0 μmol/L AS I + 25 mmol/L glucose, and 20.0 μmol/L AS I + 25 mmol/L glucose, respectively. $^{\#}P<0.01$, $^{\#\#}P<0.01$, compared with the ET group using ANOVA by the *t*-test; *$P<0.05$, **$P<0.01$, compared with the HG group by the *t*-test. Data are presented as the mean ± S.D., n = 3.

cally lower than those in the high glucose group (P<0.05 or P<0.01).

Effects of AS I on collagen IV and laminin in mesangial cells

The effects of AS I on the quantities of collagen IV and laminin in rat mesangial cells are shown in Fig. 3. As for the levels of collagen IV and laminin, differences were not significant between the ethanol group and the normal standard group (P>0.05), indicating that 1% ethanol had no effects on collagen IV and laminin in mesangial cells. The collagen IV levels in both the high glucose group and H_2O_2 group were significantly increased (P<0.01), and so was the laminin level in the high glucose group (P<0.05), when compared with those in the ethanol group. Compared with the high glucose group, significant decreases in collagen IV level were found in the low AS I, moderate AS I, and high AS I group (P<0.01), while statistically significant decreases in laminin level of the moderate AS I group and the high AS I group existed (P<0.05).

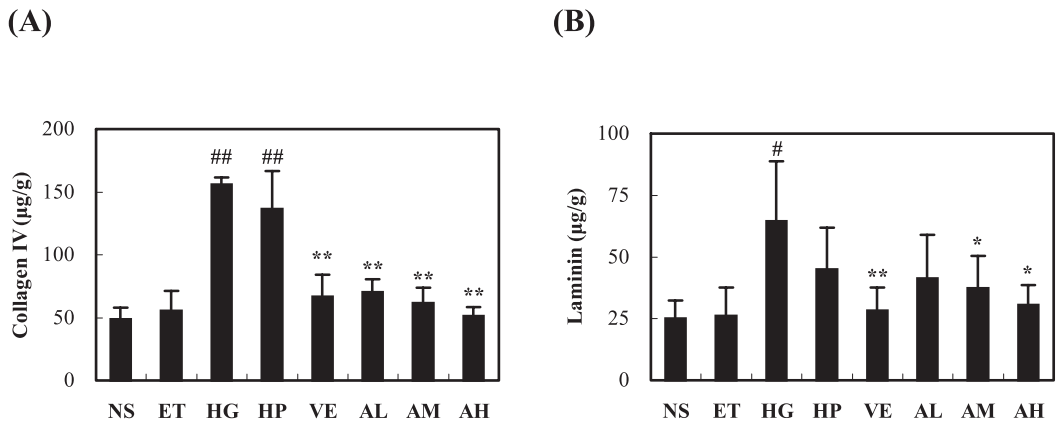

Fig. 3. The effects of AS I on collagen IV (A) and laminin level (B) in rat mesangial cells. Groups of NS, ET, HG, HP, V_E, AL, AM, and AH represent cells incubated in DMEM with 5.56 mmol/L glucose, 1% ethanol + 5.56 mmol/L glucose, 25 mmol/L glucose, 0.1 mmol/L hydrogen peroxide + 5.56 mmol/L glucose, 0.2 mmol/L vitamin E + 25 mmol/L glucose, 0.2 μmol/L AS I + 25 mmol/L glucose, 2.0 μmol/L AS I + 25 mmol/L glucose, and 20.0 μmol/L AS I + 25 mmol/L glucose, respectively. $^{\#}P$<0.01, $^{\#\#}P$<0.01, compared with the ET gruop using ANOVA by the t-test; $^{*}P$<0.05, $^{**}P$<0.01, compared with the HG group by the t-test. Data are presented as the mean ± S.D., n = 3.

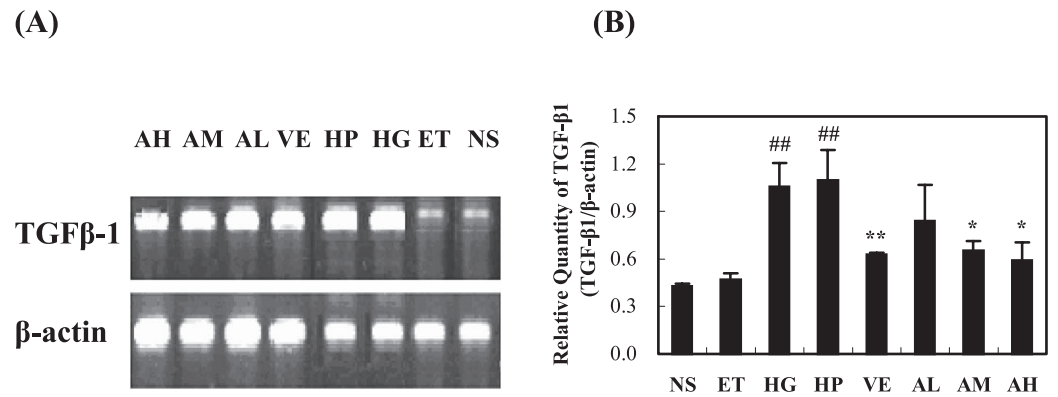

Fig. 4. Effect of AS I on the relative quantity of TGF-β1 mRNA in rat mesangial cells. Groups of NS, ET, HG, HP, V_E, AL, AM, and AH represent cells incubated in DMEM with 5.56 mmol/L glucose, 1% ethanol + 5.56 mmol/L glucose, 25 mmol/L glucose, 0.1 mmol/L hydrogen peroxide + 5.56 mmol/L glucose, 0.2 mmol/L vitamin E + 25 mmol/L glucose, 0.2 μmol/L AS I + 25 mmol/L glucose, 2.0 μmol/L AS I + 25 mmol/L glucose, and 20.0 μmol/L AS I + 25 mmol/L glucose, respectively. A: cDNA samples obtained from mesangial cells were amplified for the detection of TGF-β1 mRNA. β-Actin was used as the internal standard in each sample. B: RT-PCR data of relative quantification of TGF-β1 mRNA performed by densitometric analysis. $^{\#\#}P$<0.01, compared with the ET group using ANOVA by the t-test; $^{*}P$<0.05, $^{**}P$<0.01, compared with the HG group by the t-test. Data are presented as the mean ± S.D., n = 3.

Effect of AS I on the relative quantity of TGF-β1 mRNA in mesangial cells

In Fig. 4, the relative quantity of TGF-β1 mRNA in the ethanol group was almost equal to that in the normal standard group, which shows that ethanol did not affect the quantity of TGF-β1 mRNA of cultured cells. Compared with the ethanol group, the relative levels of TGF-β1 mRNA of high glucose group and H_2O_2 group were greatly increased (P<0.01). Both of moderate and high concentration of AS I decreased the level of TGF-β1 mRNA (P<0.05). Vitamin E had the same effect as the high dose of AS I; statistically significant difference was also found in the comparison with the high glucose group (P<0.01).

Effect of AS I on cellular growth periodicity of mesangial cells

The percentage of S phase in the normal standard group was about 63%, while those in the high glucose and the H_2O_2 groups were below 20%. Vitamin E restored the S phase percentage in the high glucose group and the H_2O_2 group to about 30%. Three doses of AS I greatly enhanced the S phase percentage to 27%, 37%, and 43%, respectively. Therefore, there was a close correlation between the dose of AS I and S phase percentage of cells (Table 2).

Table 2. The effect of AS I on cellular growth periodicity of rat mesangial cells

Group	G_0/G_1 (%)	G_2/M (%)	S (%)
NS	21.9	14.9	63.2
HG	64.0	18.5	17.5
HP	63.2	17.0	19.7
V_E	34.7	35.2	30.1
AL	47.6	25.5	27.0
AM	25.5	37.2	37.3
AH	43.8	13.7	42.5

Effects of AS I on physical behaviors and the level of HbA1C, BUN, and GSH in rats

Physical behaviors of DN rats were hypopraxia, cachexia, polyuria, polydipsia, and tardy weight gaining, while the rats treated with 12 mg/kg of AS I were vibrant, vigorous, and had weight gain. The HbA1C level in the DN group (21.20 ± 3.41%) was markedly higher than that in normal standard group (12.31 ± 1.83%) (P<0.01), which indicates that our early DN rat model was successful. The HbA1C levels in low AS I, moderate AS I, high AS I, and epalrestat groups were 20.56 ± 3.49%, 19.28 ± 5.18%, 15.32 ± 4.21%, and 13.14 ± 3.08%, respectively. Significant decreases of HbA1C level in the high AS I group and epalrestat group were found when compared with that in the DN group (P<0.05 or P<0.01) (Table 3).

Discussion

It is well known that oxidative stress plays a major role in the etiology of diabetic complications, including DN. ROS is excessively produced in DN and its injurious effects may contribute to the pathogenesis of DN, which is characterized by variable degrees of decreased renal function and increased progressive accumulation of ECM protein. The antioxidant status is poor in both glucose intolerance and non-insulin-dependent diabetes, and it is possible that antioxidant therapy mitigates or retards the progress of glucose intolerance (20). There are also other experiments that have shown that H_2O_2 causes mesangial cell contraction and induces cell proliferation (21, 22), indicating a pathogenic role for ROS in the development of DN. The levels of N(epsilon)-(hexanonyl)-lysine (HEL) and dityrosine, which are related to lipid peroxide-derived protein covalent modification and protein cross-linking, are significantly increased in diabetic Akita mice (23). Moreover, the glucose induced increases in fibronectin and collagen IV gene expression can be partially reversed by the addition of two structurally unrelated

Table 3. Effects of AS I on HbA1C, BUN, and GSH in serum of rats

Group	Number of rats	HbA1C (%)	BUN (mmol/L)	GSH (mg/L)
NS	10	12.31 ± 1.83	7.73 ± 0.96	258.3 ± 17.6
DN	10	21.20 ± 3.41$^{\#\#}$	11.16 ± 0.56$^{\#\#}$	165.4 ± 7.9$^{\#\#}$
AL	10	20.56 ± 3.49	11.63 ± 0.65	179.3 ± 6.4**
AM	11	19.28 ± 5.18	7.23 ± 0.89**	197.4 ± 9.7**
AH	10	15.32 ± 4.21*	7.65 ± 0.81**	244.4 ± 17.5**
EPS	10	13.14 ± 3.08**	10.78 ± 1.81	165.2 ± 9.0

$^{\#\#}P$<0.01, compared with NS group by the t-test. *P<0.05, **P<0.01, compared with the DN group by the t-test. Statistical analysis was performed using ANOVA. Data are presented as the mean ± S.D.

antioxidants, trolox and α-lipoic acid, in porcine mesangial cell (24). Studies have shown that antioxidative agents, including taurine, *d*-α-tocopherol, vitamin C, and vitamin E, ameliorate the symptoms of DN rats such as preventing glomerular dysfunction, decreasing albuminuria, glomerular TGF-β1 level and glomerular volume, preventing glucose-induced lipid-peroxidation, and collagen production.

In our study, besides the catalase, GSH, and SOD activity, we also measured the total antioxidative capability. This major antioxidant defense system consists of ascorbate, protein thiols, bilirubin, and α-tocopherol. Body fluid contains "preventive" antioxidants, ceruloplasmin, and transferrin, which are the iron-scavenging proteins and prevent iron availability, contributing to the total antioxidant capacity. Therefore, the total antioxidative capability is a common indicator for oxidative status. The fact that GSH, SOD, catalase activity, and the total antioxidative capability were all significantly increased by AS I in vitro is consistent with other reports, including our former data in vivo (4 – 7). Therefore, we strongly suggest that AS I has effects on antioxidative capability in mesangial cells.

In our present in vivo experiment, AS I (12 mg/kg) statistically reduced the HbA1C level, a persuasive parameter demonstrating the blood glucose level for a relative long period. HbA1C is also one kind of amadori product, which appears in the early stage of DN, and its end product is AGEs. HbA1C is formed via non-enzymatic glycation which is specifically enhanced through the presence of oxidatants, and the ability to form glycoxidation products in peptide and protein structures finally modulates or induces biological reactivity (25). Therefore, the decreasing effect of AS I on HbA1C in vivo contributes to its antioxidative effect. Furthermore, the anti-HbA1C and antioxidative characteristics of AS I may have a synergetic effect on therapy for DN.

In addition to its effect on HbA1C in our experiment, 3 doses of AS I (3, 6, 12 mg/kg) still greatly lowered the blood glucose, microalbuminuria, kidney index, and oxidative status in DN rats (4). In the complicated mechanisms of DN development, the inhibitory effect of AS I on the pathway of hyperglycemia-oxidative stress-AGEs/TGF-β1/ECM is assuredly important. Therefore, we are justified to propose a close relationship between the oxidative status and the pathogenesis of DN. Among all the above effects of AS I closely related to the mechanisms of DN pathology, its antioxidative effect is one of the most important factors, probably due to its ability to scavenge active oxygen species, inhibiting the generation of ROS (1). TGF-β1 is another pivotal factor, which is greatly increased by oxidative status and significantly influences mesangial cell proliferation and ECM level: both of them contribute to the mechanism of DN development.

Recent studies have suggested that increased NO generation and action may be associated with glomerular hyperfiltration and increased vascular permeability early in diabetes (26, 27). Since inhibition of inducible NO reduces the expression of TGF-β, the profibrogenic cytokine, and glomerulosclerosis (15, 16), the present study has reevaluated the effect of high glucose and H_2O_2 on the iNOS pathway in relation to ECM accumulation in rat mesangial cells. Our results suggest that AS I is a probable iNOS inhibitor, and its clinical application for treatment of DN deserves further study.

Renal hypertrophy in early DN is mainly caused by hyperplasia and/or hypertrophy of mesangial cells and renal tubule epithelial cells. There are two restricted points in the cell growth cycle, the first being the G_1/S period and the second being the G_2/M period. High glucose excites the biphase growth of these kinds of cells, and then renal hypertrophy occurs when cell cycle is arrested at the G_1 phase (28). Thus, the percentage of S phase usually acts as an indicator for cell proliferation. Our results indicate that high glucose or oxidative stress reduces the cell percentage of S phase and stops the cell growth cycle at the G_1 phase, inducing the cell hypertrophy. AS I can restore the decreased S phase percentage, facilitating the cell in the G_1 phase to pass successfully the restricted point of cell growth, and preventing the cell from hypertrophy. Furthermore, by radioimmunoassay, we found that high glucose or oxidative stress greatly enhanced the quantity of collagen IV and laminin in rat mesangial cells, while AS I significantly decreased the collagen IV and laminin level. As we know, both collagen IV and laminin are the main components of ECM in the kidney, and their increases are always accompanied with the cell hypertrophy. In addition to our former experiments in vivo, which were focused on morphological parameters, such as the collagen IV level in the cortex, the relative grade of mesangium hyperplasia and thickness of glomerular base membrane, our data suggested that AS I decreases the quantity of ECM and prevents renal hypertrophy, and this kind of effect is probably or partially due to its regulative function on mesangial cell growth cycle.

To summarze, with the data of the in vitro experiments, AS I is proved to have an antioxidative effect and also anti-NO, anti-TGF-β1, and collagen IV and laminin down-regulation effects. It could also restore the cell percentage of the S phase from abnormal level of cultured rat mesangial cells. Furthermore, AS I also has ameliorative effects on physical behaviors and HbA1C on early stage of DN rat. Thus, AS I has antioxidative

effects and the antioxidant treatment could be a potential therapeutic approach for DN.

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