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Original Article

Effect of aminoguanidine on plasminogen activator inhibitor-1 and receptor of advanced glycation end-product in the liver of streptozotocin-induced diabetic rats

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ABSTRACT

Objectives: Advanced glycation end products (AGEs) play an important role in the development and progression of diabetic complications. The receptor for AGE (RAGE) is the ligand-binding site of AGE that initiates and accelerates the atherosclerotic process. Plasminogen activator inhibitor-1 (PAI-1) is a prothrombotic factor that has been proposed as a biological marker for prognostic assessment, monitoring of microvascular and macrovascular complications in diabetes. The purpose of this study is to investigate the effects of aminoguanidine on RAGE and PAI-1 expression levels in the liver of streptozotocin-induced diabetic rats.

Methods: Diabetes was induced in rats by intraperitoneal injection of streptozocin (STZ, 50 mg/kg). On day 3, diabetic rats were administered 50, 100, and 200 mg/kg/day of aminoguanidine. The expression of PAI-1 and RAGE in the liver tissue was evaluated using real-time PCR.

Results: PAI-1 and RAGE gene expression levels were higher in the liver of the diabetic rats compared to the control group. Aminoguanidine at 50, 100, and 200 mg/kg decreased PAI-1 and RAGE gene expression in the liver ($p<0.001$ at all doses). However, these genes were downregulated only at a dose of 200 mg/kg in healthy rats ($p<0.0001$). In addition, hepatic AGE protein levels were significantly decreased following treatment of the diabetic rats with aminoguanidine ($p<0.001$). There was also a significant correlation between AGE protein concentration and the expression of PAI-1 and RAGE.

Conclusion: In summary, the data of the present study suggest that aminoguanidine reduced the expression of PAI-1 and RAGE in the liver of the diabetic rats.

Keywords: Diabetes, aminoguanidine, Plasminogen activator inhibitor-1

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Introduction

Diabetes represents a range of diseases that affect the body's ability to properly control blood sugar. Diabetes affects vasodilation, vascular cell growth and proliferation, and their permeability (1, 2). Uncontrolled diabetes precedes these physiological conditions and can lead to further micro- and macrovascular complications (3, 4). Increased platelet reactivity leads to activation of the prothrombotic agents and inactivation of fibrinolytic factors, resulting in an increased risk of thrombosis in these patients (5). Advanced glycation end products (AGEs) are one of the confounding factors that influence glycative stress-induced coagulation factor activity (6). These products alter protein functions, leading to dysregulation of the normal signaling pathways. Binding of AGEs to the receptor for advanced glycation end product (RAGE) activates the AGE-RAGE signaling pathway and increases oxidative stress (7). Increased oxidative stress via this pathway may increase the inflammatory responses and increase the risk of thrombosis. This leads to inflammation and disruption of tissue physiology, ultimately leading to disease progression. RAGE is a member of the cell surface immunoglobulin superfamily. Downregulation of RAGE is associated with atherosclerosis (8).

Plasminogen activator inhibitor-1 (PAI-1) is another prothrombotic factor that prevents fibrinolysis, increases pathological fibrin sedimentation in tissues, and controls cell replication and angiogenesis (9). It is an acute-phase protein that is influenced by hormones, as well as inflammatory and growth factors (10). PAI-1 is a suggestive biological marker for prognostic assessment, disease monitoring, and therapeutic targeting (11). Glycosylation of PAI-1 neutralizes the activity of PAI-1 inhibitors (12). However, research in this area has reached different conclusions (13-15). Therefore, there is a need for new perspectives on therapeutics that focus on cellular and molecular signaling pathways.

Aminoguanidine, the most commonly used inhibitor to reduce AGE production in previous studies, had no effect on endothelial nitric oxide synthase (eNOS). It inhibits aortic H_2O_2 production, vascular NAD(P)H oxidase (NOX)-dependent O_2 production, and hypercontraction in diabetic mice (16, 17). To our knowledge, no studies have examined the effects of aminoguanidine on PAI-1 and RAGE in the liver tissue of a rat model of streptozotocin-induced diabetes.

Materials and Methods

Diabetic rats and drug intervention

Male Rattus norvegicus (250–300 g) were harvested and maintained under normal pathogen-free, humidity, light, and temperature conditions. The Ethics Committee of Zanjan Medical College approved the use of animals and experimental procedures (IR.ZUMS.REC.1398.433). All procedures were applied according to ARRIVE guidelines. Diabetes was induced by intraperitoneal injection of streptozocin (STZ, 50 mg/kg) dissolved in 0.9% saline immediately prior to dosing once daily for 2 days (18). Diabetes was confirmed with a glucometer (Lifescan) at baseline and 3 days after STZ injection. On day 3, diabetic rats were given 50, 100, and 200 mg/kg/day of aminoguanidine dissolved in 0.9% saline intraperitoneally for 30 days. A control group received the same dose (CH: Healthy controls receiving no treatment. H50: Healthy rats received 50 mg/kg/day of aminoguanidine. H100: Healthy rats received 100 mg/kg/day of aminoguanidine. H200: Healthy rats received 200 mg/kg/day of aminoguanidine). One month later, rats were euthanized with ketamine, the liver tissue was removed, the surrounding connective tissue was cleaned, and it was stored in RNA-late buffer at −80 °C for future experiments.

Gene expression analysis

Total mRNA was extracted from liver tissue using the RNX plus kit (SinaClon Co., Iran) according to the manufacturer's instructions. The quantity and quality of extracted RNA were assessed at 260 nm using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and agarose gel electrophoresis (1.5% w/v), respectively. Finally, cDNA was synthesized in a reaction volume of 20 µl using a synthesis kit (BIOFACT™ Co., Korea). Gene expression was measured in duplicate using the real-time polymerase chain reaction (PCR) method on the ABI StepOne™ Sequence Recognition System (Applied Biosystems, California, USA). The mixture contained 2.5 µL RNA, 1 µL primer (oligo dT), 1 µL random hexamers, 10 µL RT-Pre Mix, and 5.5 µL RNA-free water for cDNA synthesis. After centrifugation, the mixture was then incubated at 70°C for 5 minutes. After 30 minutes of incubation at 50 °C, the samples were stored in a thermal cycler at 95 °C for 5 minutes. Finally, samples were kept on ice for 12 minutes. Primers were designed using Gene Runner software version 3.05 (Hastings Software Inc., USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. Primer sequences are shown in Table 1. The mix for each real-time PCR reaction contained 1 µl of cDNA, 0.5 µl of each forward and reverse primers, and 6.5 µl of SYBR® Green I Master Mix (Roche). The amplification profile included one cycle of 95 °C for 10 min and 40 two-step cycles of 95 °C for 20 s and 72 °C for 30 s. Data precision was determined using NRT and NTC controls. Results were analyzed

with the LinRegPCR 11.0 software (Heart Failure Research Centre, The Netherlands).

Evaluation of proteins in the liver

Liver AGE protein was measured by the ELISA method (Bioassay Technology Laboratory Co., Shanghai, China) according to the manufacturer's instructions. Tissues were rinsed with PBS, cut into 1–2 mm pieces, homogenized in 20 ml of 1X PBS using a tissue homogenizer, and stored overnight at -20 °C. An equal volume of RIPA buffer containing protease inhibitors was added and allowed to dissolve for 30 minutes at room temperature with gentle agitation. Homogenates were centrifuged at 5000 x g for 5 min. The supernatant was immediately removed and analysis was performed immediately using an ELISA kit. The OD of the samples was read at 450 nm.

Statistical analysis

Statistical analyses were performed using SPSS software (version 24), and graphs were generated using GraphPad Prism (version 8). Normal distribution was assessed using the Kolmogorov-Smirnov test. Differences between mean groups were compared using ANOVA followed by a post hoc Tukey test. Statistical significance was determined for $p < 0.05$.

Results

The effects of aminoguanidine on PAI-1 expression in the liver of diabetic rats

The results showed that the liver of diabetic rats had a higher expression of the PAI-1 and RAGE gene than controls. Aminoguanidine significantly decreased PAI-1 gene expression only at a dose of 200 mg/kg/day in control rats ($p < 0.001$). At other doses, aminoguanidine had no significant effect on PAI-1 expression. In diabetic rats, all doses of aminoguanidine significantly decreased PAI-1 gene expression compared to diabetic controls ($p<0.001$) (Fig. 1). Moreover, the reduction of PAI-1 gene expression by aminoguanidine treatment was significantly greater in diabetic rats compared to controls at the dose of 200 mg/kg/day ($p<0.001$) (Fig. 2). PAI-1 gene expression was decreased by 39% and 81% in control and diabetic rats, respectively, at the dose of 200 mg/kg/day of aminoguanidine.

The experiment on the effects of aminoguanidine on RAGE expression revealed that aminoguanidine significantly decreased the RAGE gene expression at the dose of 200 mg/kg/day in diabetic and healthy rats ($p < 0.001$). At other doses, there was no significant difference in RAGE expression between the healthy rats and controls. In diabetic rats, all aminoguanidine doses significantly decreased RAGE gene expression

Table 1: Information about the primers used in the study.

Gene	Primer sequence	Length of target (base pair)	Primer sequence length (base pair)
PAI-1	F: 5′CCGCCTCCTCATCCTGCCTAAG3′ R: 5′ TGTGAAGTCGGCCTGGGTTGAG3′	112	22 22
RAGE	F: 5′ TGACCTGTGCCATCTCTGCCC3′ R: 5′ CAGGGAGGAGCAGCACAGGG3′	99	21 20
GAPDH	F: 5′ GCCGCCTGGAGAAACCTGC3′ R:5′GGAAGAATGGGAGTTGCTGTTGAAG3′	140	19 25

PAI-1: plasminogen activating receptor; RAGE: receptor for advanced glycation end products; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

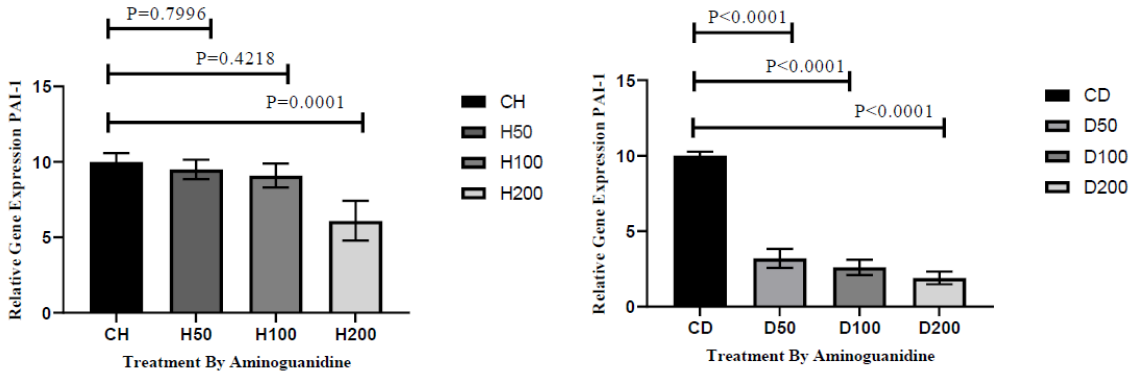

Figure 1: Effect of aminoguanidine on PAI-1 expression in the liver of control and diabetic rats. CH: healthy controls without treatment; H50: healthy rats received 50 mg/kg/day aminoguanidine; H100: healthy rats received 100 mg/kg/day aminoguanidine; H200: healthy rats received 200 mg/kg/day aminoguanidine; CD: diabetic controls without treatment; D50: diabetic rats received 50 mg/kg/day aminoguanidine; D100: diabetic rats received 100 mg/kg/day aminoguanidine; D200: diabetic rats received 200 mg/kg/day aminoguanidine. $p<0.05$ is significant.

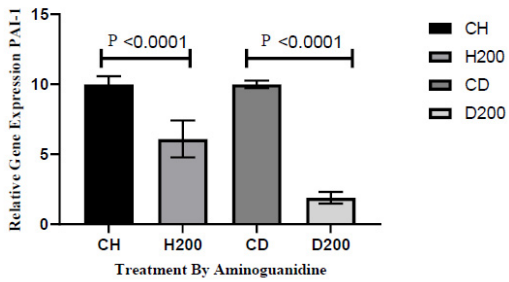

Figure 2: Comparison of PAI-1 expression at the dose of 200 mg/kg/day. CH: healthy controls without treatment; H200: healthy rats received 200 mg/kg/day aminoguanidine; CD: diabetic controls without treatment; D200: diabetic rats received 200 mg/kg/day aminoguanidine.

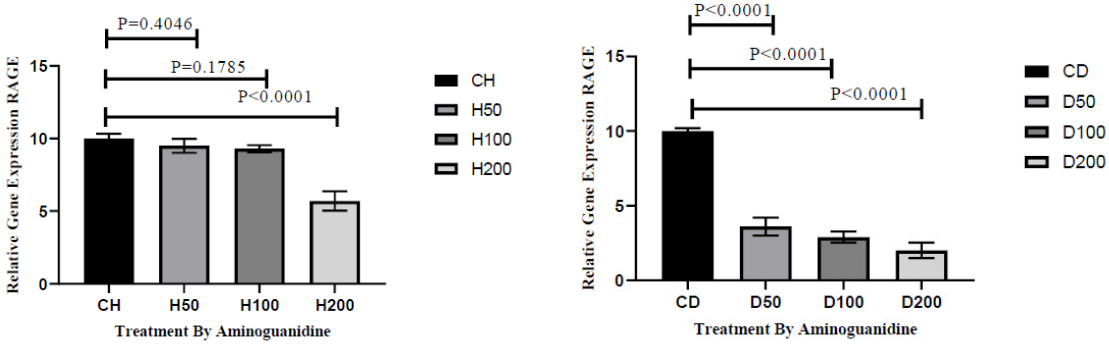

Figure 3: Effect of aminoguanidine on RAGE expression in the liver of control and diabetic rats. CH: healthy controls without treatment; H50: healthy rats received 50 mg/kg/day aminoguanidine; H100: healthy rats received 100 mg/kg/day aminoguanidine; H200: healthy rats received 200 mg/kg/day aminoguanidine; CD: diabetic controls without treatment; D50: diabetic rats received 50 mg/kg/day aminoguanidine; D100: diabetic rats received 100 mg/kg/day aminoguanidine; D200: diabetic rats received 200 mg/kg/day aminoguanidine. $p<0.05$ is significant.

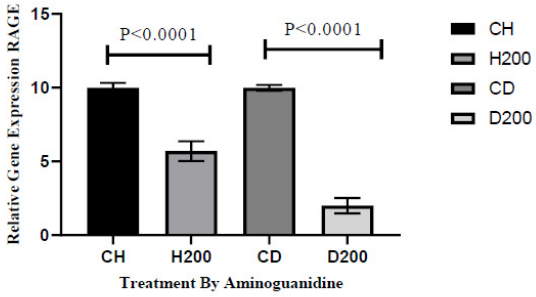

Figure 4: Comparison of RAGE expression at the dose of 200 mg/kg/day. CH: healthy controls without treatment; H200: healthy rats received 200 mg/kg/day aminoguanidine; CD: diabetic controls without treatment; D200: diabetic rats received 200 mg/kg/day aminoguanidine.

compared to diabetic controls ($p<0.001$) (Fig. 3). Moreover, the reduction of RAGE gene expression with aminoguanidine treatment was significantly greater in diabetic rats compared to controls at the dose of 200 mg/kg/day ($p<0.001$) (Fig. 4). RAGE gene expression was decreased by 43% and 80% in control and diabetic rats, respectively, at the dose of 200 mg/kg of aminoguanidine.

AGE protein concentration

As shown in Table 2, hepatic AGE concentrations were significantly higher in diabetic rats in comparison with the control, and aminoguanidine treatment at the dose of 200 mg/kg/day was able to reduce AGE concentration in the liver of these animals ($p<0.001$). Furthermore, a correlation analysis was performed between the expression of PAI-1 and RAGE and AGE concentration in diabetic rats treated with aminoguanidine. As shown in Fig. 5, a significant correlation was observed between the mentioned parameters in diabetic rats.

Discussion

AGEs are a heterogeneous group of molecules that are mechanically formed in the nonenzymatic processes of protein glycation and are associated with

Table 2: AGE concentration in the liver of study groups

Groups	CH	H50	H100	H200	CD	D50	D100	D200
AGE concentration, mg/ml	0.058±0.005	0.054±0.002	0.052±0.003	0.043±0.007	0.073±0.005	0.067±0.006	0.065±0.004	0.048±0.005

CH: healthy controls without treatment; H50: healthy rats received 50 mg/kg/day aminoguanidine; H100: healthy rats received 100 mg/kg/day aminoguanidine; H200: healthy rats received 200 mg/kg/day aminoguanidine; CD: diabetic controls without treatment; D50: diabetic rats received 50 mg/kg/day aminoguanidine; D100: diabetic rats received 100 mg/kg/day aminoguanidine; D200: diabetic rats received 200 mg/kg/day aminoguanidine

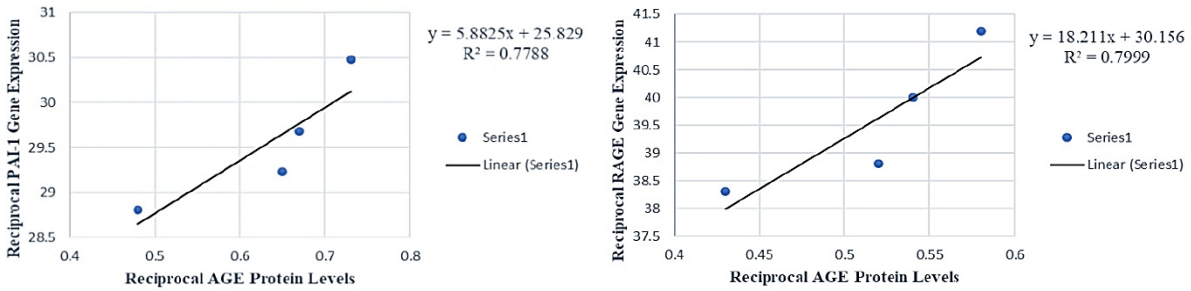

Figure 5: Correlation between liver AGE concentration with the PAI-1 and RAGE expression in the diabetic rats received aminoguanidine.

the development and progression of diabetes (19). In this process, a time- and dose-dependent reaction of protein amino groups with aldehyde or ketone subunits of reducing sugars leads to the formation of stable Amadori products, which ultimately lead to AGEs formation through dehydration and rearrangement pathways. Rapid accumulation of AGEs increases the production of reactive oxygen species (ROS), leading to severe damage and dysfunction, resulting in various organ failures (20). Considering the strong correlation between AGE production and macrovascular and microvascular complications in diabetes, the development of new molecular studies to search for precise therapeutic agents appears to be necessary. Previously, aminoguanidine, a low-molecular weight, water-soluble drug, was introduced as a carbonyl group and methylglyoxal scavenger (21). It is well established that aminoguanidine scavenges or traps reactive carbonyl intermediates produced by the Maillard reaction, mainly dicarbonyl compounds such as hydroxyaldehyde and methylglyoxal (22). Methylglyoxal impairs the insulin signaling by suppressing stimulated phosphorylation of insulin receptor substrate 1 (IRS-1) and inhibiting phosphatidylinositol-3-kinase (PI3K) activity (23). In the present study, the aim was to investigate the effect of aminoguanidine on the AGEs/RAGE axis in the liver of the diabetic rats.

In the present study, aminoguanidine downregulated PAI-1 and RAGE gene expression in both healthy and diabetic rats, and decreased AGE protein levels in the liver of STZ-induced diabetic rats. In the healthy group, aminoguanidine affected gene expression levels only at a dose of 200 mg/kg/day. However, all doses of aminoguanidine (50, 100, and 200 mg/kg) decreased PAI-1 and RAGE gene expression in diabetic rats. In support of these findings, one study suggested that treating diabetic rats with aminoguanidine for 56 days led to a reduction in proteinuria (24). In another study, it was reported that aminoguanidine treatment increased AGE-R1 expression in gastrocnemius skeletal muscle and decreased AGE levels in liver and kidney tissues of high-fructose water-fed rats (25).

In summary, the data of the present study revealed that aminoguanidine treatment led to a decrease in PAI-1 and RAGE expression in the liver of diabetic rats. However, further investigations are required to confirm the effect of aminoguanidine on the expression of these genes in diabetic models.

Conflict of Interest

The authors have no conflict of interest.

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