Original Article

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Aminoguanidine Induced Up-Regulation of Sphingosine-1 Phosphate Receptor-1 (S1PR1) in Heart Tissue of Diabetic Rats

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ABSTRACT

Background & Objective: Cardiac microvascular complications are a significant concern in diabetes, often related to dysfunction of the sphingosine-1-phosphate receptor-1 (S1PR1). Aminoguanidine (AG) is recognized for its capability to alleviate these complications by inhibiting advanced glycation compounds and enhancing vascular function in diabetic rats. Therefore, this study seeks to investigate the therapeutic potential of AG by assessing its impact on the gene expression of S1PR1 in the heart tissue of diabetic rats.

Materials & Methods: Thirty-four diabetic and healthy rats were stratified into eight groups, including diabetic rats, diabetic rats administered with varying doses of AG 50, 100, and 200 mg/kg, healthy rats treated with the same AG doses, and untreated healthy controls. RNA extraction and cDNA synthesis were performed using heart tissue samples, followed by real-time PCR analysis. The fold change in S1PR1 gene expression was then assessed and compared among diabetic rats treated with varying AG doses and their corresponding control groups.

Results: The analysis demonstrated a significant reduction in S1PR1 gene expression in diabetic rats compared to controls. However, AG treatment improved S1PR1 expression, which was correlated with the administered dose, with a notable upregulation observed in rats treated with 200 mg/kg of AG compared to other groups ($P<0.001$).

Conclusion: Considering the significance of the S1PR1 pathway in inhibiting microangiopathy, augmenting S1PR1 gene expression through AG treatment may hold promise in preventing diabetes-related cardiovascular complications.

Keywords: Sphingosine-1-Phosphate Receptor-1 (S1PR1), Aminoguanidine, Diabetes, Cardiovascular Complications

Introduction

Over the past few years, a noticeable shift has occurred in the global burden of diabetes, particularly affecting lower- and middle-income nations. The rate of occurrence of type 2 diabetes expanded from around 415 million cases in 2015 to 462 million patients in 2017, with projections indicating a further increase to 592 million patients by 2035. This surge in diabetes cases is accompanied by a concerning rise in associated mortality, with over 1 million deaths attributed to diabetes annually **(1, 2)**.

It is widely accepted that diabetes plays a significant role in cardiovascular complications, particularly impacting vascular and microvascular endothelial cells. Studies have demonstrated its association with conditions including myocardial infarction (MI), cardiac microvascular disease, and stroke, with primary involvement of cardiac microvessels in diabetic cardiopathy **(3-5)**.

Sphingosine-1-phosphate (S1P) emerges as a crucial player in sphingolipid metabolism, exerting its effects through interaction with five known G-protein coupled receptors (S1PR1-5). These interactions modulate different cellular mechanisms, for instance, apoptosis, migration, proliferation, and angiogenesis **(6, 7)**. Notably, S1PR1 is the most abundant receptor on the cell surfaces of cardiomyocytes and endothelial cells, essential for vascular development and maturation. Activation of S1PR1 by S1P inhibits angiogenesis, preserves vascular stability, and prevents VEGF-induced vascular sprouting. Dysregulation of S1PR1 leads to microangiopathy and increases vascular permeability, rendering microvessels vulnerable to pathological angiogenesis in diabetes patients. Hence, S1PR1 holds the potential as a biomarker for the early diagnosis of cardiovascular diseases related to diabetes **(8-10)**.

Aminoguanidine (AG) is among the drugs utilized to prevent diabetic complications in animal models. Its protective effects in diabetic rats are attributed to its inhibition of nitric oxide (NO) production, an important intermediate in controlling vascular resistance and vasodilation. AG reduces NO levels by interfering with sugar-protein cross-linked compounds, thereby exerting favorable therapeutic effects in diabetic patients **(11)**. Given the pivotal role of S1PR1 in maintaining vascular barrier function and preventing microangiopathy in diabetes, the current research aims to assess the impact of AG on the expression of the S1PR1 gene in an animal model of diabetes. While previous research has shown that AG can inhibit advanced glycation end products (AGEs) and improve vascular function, its impact on Sphingosine-1-Phosphate Receptor-1 (S1PR1) gene expression in the heart tissue of diabetic models has been less explored. This study demonstrates a dose-dependent upregulation of S1PR1 with AG treatment, providing insights into its potential to address diabetic microvascular complications and paving the way for future studies on the therapeutic benefits of modulating S1PR1 expression in diabetes.

Materials and Methods

Animal Preparation

Diabetic and control rats were prepared according to the methodology previously described by Alipour et al. **(12)**. In brief, rats in the diabetic group underwent a single dose of Streptozotocin (STZ) administered intraperitoneally (Merck, Germany). Rats demonstrating blood glucose levels above 250 mg/dl were classified as diabetic. Subsequently, saline and a range of AG doses (50, 100, 200, and 400 mg/kg, Merck, Germany) were administered daily via intraperitoneal injection over one month **(Table 1).** All experimental protocols were undertaken in compliance with the Institutional Animal Care & Use Committee (IACUC) standards and approved by the Zanjan University of Medical Sciences Ethics Review Board (IR.ZUMS.REC.1394.114).

RNA Extraction and cDNA Synthesis:

Heart tissues from the 8 experimental groups, as detailed in Table 1, were utilized for RNA extraction. Total RNA extraction was carried out according to the protocol provided by the Easy Blue kit (iNtRON Company, USA). The PrimeScript RT reagent kit from Takara (Japan) was employed for cDNA synthesis by the manufacturer's directions.

Evaluation of S1PR1 gene expression:

Primer designing was executed utilizing AllelID7 (Primer Biosoft) and Primer Express software programs (ABI) in an exon-exon junction manner. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was designated as the reference gene. Gradient PCR was used to determine the optimal annealing temperature. SYBR Green (Takara, Japan) was employed in the real-time PCR analysis, using 500 ng of cDNA and 0.3 µM of specific primers on a StepOne instrument (ABI, USA).

Statistical Analysis:

Data analysis was conducted with GenEX5 software (MultiD, Sweden) and Statistical Program for Social Sciences (SPSS) software version 20 (SPSS Inc., USA). The relative change in gene expression across various groups was determined using the Pfaffl formula **(13)**. The non-parametric Mann-Whitney test was employed for comparing samples within each group. LinRegPCR software was utilized for raw data analysis and to determine the efficiency of real-time PCR.

Table 1. **Rat groups that were used in this study**

Type	Number
Normal rat treated with 200 mg/kg/day of AG (control)	4
Normal rat treated with 100 mg/kg/day of AG (control)	4
Normal rat treated with 50 mg/kg/day of AG (control)	3
Diabetic rat treated with 200 mg/kg/day of AG	5
Diabetic rat with 100 mg/kg/day of AG	3
Diabetic rat treated with 50 mg/kg/day of AG	6
Healthy normal rat (control)	5
Diabetic rat (control)	4

Results

According to Table 2, the evaluation of lipid, uric acid, and triglyceride profiles for each group of rats revealed notable differences among the diabetic and control groups. In the diabetic group, characterized by rats receiving a single dose of STZ, significant alterations in these metabolic markers were identified compared to the control group.

Table 2. **Lipid, uric acid, and triglyceride profiles for each group of rats**

Group	Sample No.	Cholesterol mg/dL	Uric acid mg/dL	Triglyceride mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Ca/P
Healthy Normal	1	44	2.3	95	12	3	1.2
	2	46	2.5	94	13	3	1.2
	3	44	1.6	70	12	3	1.1
	4	45	1.9	81	13	2	1.3
	5	40	2	82	12	3	1.2
	Mean±SD	**43.8±2.2**	**2±0.3**	**84.4± 10.3**	**12.4±0.54**	**2.8±0.44**	**1.2±0.07**
Diabetic	1	73	3.2	68	30	7	1.3
	2	65	3.3	190	41	5	1.17
	3	70	3	110	41	6	1.5
	4	65	2.9	90	30	5	1.2
	Mean±D	**68.2±3.9**	**3.1±0.18**	**114.5±53.1**	**35.5±6.3**	**5.7±0.9**	**1.2±0.1**
Diabetic- Treated With 50 mg/kg of AG	1	44	4	80	30	7	1.1
	2	68	1.9	87	29	4	1.8
	3	76	3.3	84	41	4	1.4
	4	70	2.4	89	31	5	1.3
	5	68	3	75	32	6	1.3
	6	68	3.5	81	32	6	1.4
	Mean±SD	**65.6±11**	**3.01±0.7**	**82.66±5**	**32.5±4.3**	**5.33±1.2**	**1.38±0.2**
Diabetic- Treated With 100 mg/kg of AG	1	65	6	146	27	4	1.3
	2	48	4.5	101	23	8	0.1
	3	84	7.8	168	31	9	1.3
	Mean±SD	**65.6±18**	**6.1±1.6**	**138.3±34.1**	**27±4**	**7±2.6**	**0.9±0.6**
Diabetic- Treated With 200 mg/kg of AG	1	50	1.6	67	23	4	1.4
	2	63	1.5	185	27	5	1.7
	3	65	2.1	122	29	6	2.2
	4	62	1.8	120	29	7	1.6
	5	60	1.9	150	31	8	1.8
	Mean±SD	**60±5.8**	**1.78±0.2**	**128.8±43.4**	**27.8±3**	**6±1.5**	**1.74±0.2**

Normal- Treated With 50 mg/kg of AG	1	57	0.7	33	23	3	1.8
	2	38	0.8	39	10	1	2
	3	52	0.6	19	17	3	1.3
	Mean±SD	**49±9.8**	**0.7±0.1**	**30.3±10.2**	**16.6±6.5**	**2.3±1.1**	**1.7±0.3**
Normal- Treated With 100 mg/kg of AG	1	44	0.8	76	13	3	1.4
	2	45	0.9	69	13	3	1.2
	3	47	1.9	69	13	4	1.6
	4	47	1.3	87	15	3	1.6
	Mean±SD	**45.7±1.5**	**1.2±0.4**	**75.2±8.5**	**13.5±1**	**3.2±0.5**	**1.4±0.1**
Normal- Treated With 200 mg/kg of AG	1	64	2.4	25	20	4	1.1
	2	67	2.4	83	14	4	1.5
	3	48	1.7	67	18	3	1
	4	51	2	54	16	3	1.7
	Mean±SD	**57.5±9.3**	**2.1±0.3**	**57.2±24.5**	**17±2.5**	**3.5±0.5**	**1.3±0.3**

For gene evaluation, the temperature 61°C was considered as the optimum temperature for S1PR1 amplification. The sharp melt curve of products confirmed the specific amplification of the products **(Figure1)**. According to the LinRegPCR software (V11, Netherlands), 85% efficiency was achieved for all gene amplifications.

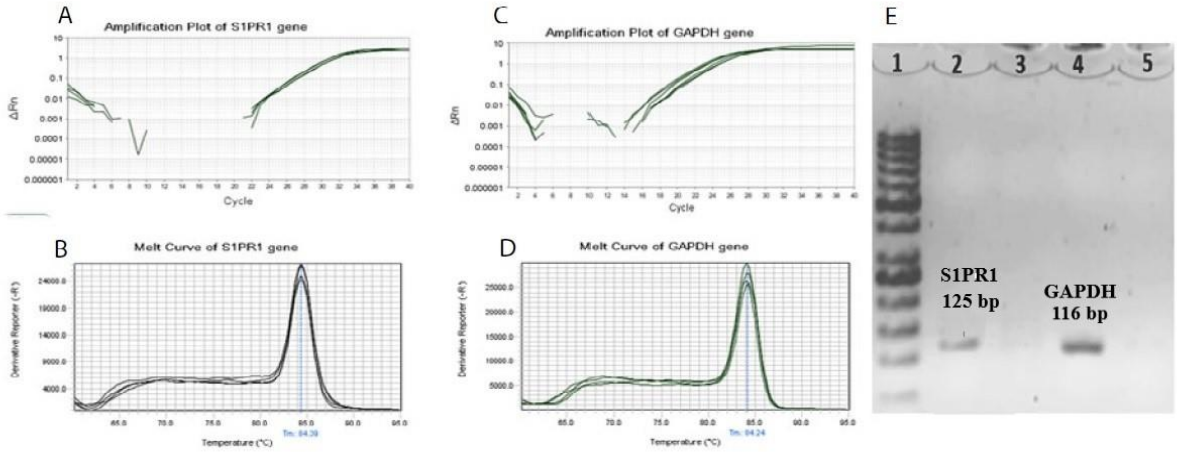

Figure 1. Qualitative RT-PCR of S1PR1 and GADPH genes by SYBR Green method. A & C: amplification plot, B & D: melting curves and E: 2% agarose gel electrophoresis (lane1: 50bp marker, lane2: S1PR1 (125 bp) and lane3: GADPH (116 bp) products. Lane 3 &5 negative controls).

S1PR1's gene expression in diabetic groups treated with AG compared to healthy control groups with no treatment.

In comparing S1PR1 gene expression between diabetic and healthy normal groups, a notable reduction in S1PR1 expression was evident in diabetic rats relative to the healthy normal group (p= 0.0001, data not shown). Furthermore, upon normalization of S1PR1 gene expression in diabetic rats versus the healthy normal group without any treatment, a dose-dependent increase in S1PR1 expression was observed with AG administration (200>100>50 mg/kg). Notably, the difference reached statistical significance only for diabetic rats given doses of 100 and 200 mg/kg of AG in comparison with the other groups ($p<0.05$) **(Figure 2).**

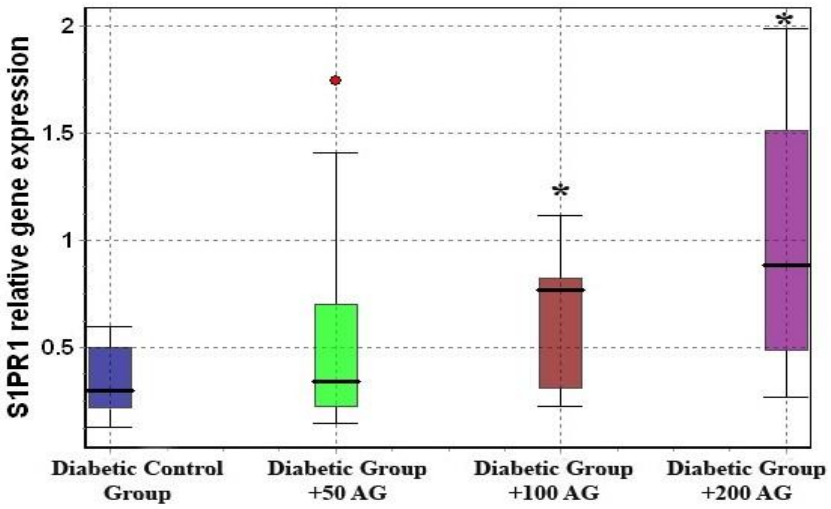

Figure 2. S1PR1's gene expression in diabetic rats compared with control diabetic group without any treatment. After making diabetic rats, they were treated with AG, 50, 100 and 200 mg/kg doses. According to the box plots there is a significant difference between rats treated with 100 and 200 mg/kg dosage of AG compared to the other groups ($p<0.05$). Outlier is shown by a dot.

S1PR1's gene expression in diabetic groups treated with different doses of AG compared to healthy control groups treated with the same drug dosage.

After normalizing the S1PR1 gene expression in diabetic rats given AG at 50, 100, and 200 mg/kg to their respective healthy control groups treated with the same dosage, we compared the fold change in S1PR1 expression among all three different diabetic groups. Our findings unveiled a significant difference in S1PR1 gene expression between the diabetic group administered 200 mg/kg of AG and the other groups ($p<0.001$) **(Figure 3).**

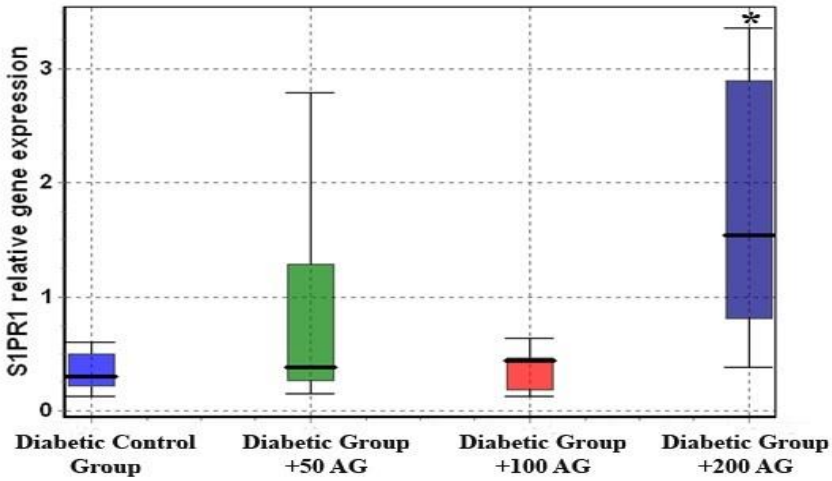

Figure 3. S1PR1's gene expression level in diabetic rats treated with different doses of AG compared to healthy control rats treated with the same dose of AG drug. The amount of expression for healthy rats treated with same doses of the drug is assumed 1. Diabetic rats treated with 200 mg/kg dose of the drug showed a significant difference in S1PR1's gene expression compared to the other groups ($p <0.001$).

Discussion

Research has underscored the pivotal role of dysregulated S1PR1 signaling in precipitating cardiac microvascular complications, including microangiopathy, vascular permeability, and heightened vulnerability of microvessels in diabetic patients. Consequently, S1PR1 emerges as a potential biomarker for diagnosing diabetes-related cardiovascular diseases **(10-12)**. Conversely, the activation of S1PR1 is associated with enhancing vascular barrier function and mitigating microangiopathy **(14)**. Hence, the evaluation of S1PR1 and S1PR3 offers distinct advantages in the early diagnosis of cardiac microangiopathy in diabetes **(10)**.

Based on our findings, we observed a decrease in the expression profile of the S1PR1 gene in diabetic rats versus healthy controls. However, following treatment with AG, a dose-dependent rise in the expression level of the S1PR1 gene was observed in diabetic rats. This suggests that augmenting S1PR1 gene expression through AG treatment may potentially mitigate cardiovascular complications associated with diabetes.

Chen et al. displayed a notable decrease in the S1PR1 expression profile in human umbilical vein endothelial cells (HUVECs) in response to high blood sugar levels. However, this effect was reversed upon treatment of HUVECs with the pAd-S1PR1 adenoviral vector. This suggests that the decreased expression of the S1PR1 gene is implicated in hyperglycemia-induced endothelial cell dysfunction **(15)**. Furthermore, Yin et al. reported on the potential therapeutic effect of FTY720, a sphingolipid drug, in improving cardiac microvascular function by upregulating S1PR1 gene expression **(16)**. This finding aligns with our results, where we demonstrated that administering the AG drug to diabetic rats increased S1PR1 expression, potentially influencing heart function. Previous studies have shown that AG improves vascular elasticity and reduces the permeability of blood vessels in diabetic rats **(17, 18).** Acting as an antioxidant and anti-inflammatory compound, AG can reduce levels of AGEs, inhibit nitric oxide synthase, and prevent the function of vascular endothelial growth factor (VEGF). Taslidere et al. observed a notable decline in blood sugar levels in rats treated with AG and a loss of glycogen in treated diabetic rats **(19)**. Elbe and colleagues investigated the regenerative impact of AG on the aorta in diabetic rats, showing a drop in blood glucose levels, a considerable decrease in the thickness of the tunica media, and prevention of elastic fibril disorganization **(20)**.

Our investigation discovered a reduction in S1PR1 gene expression in diabetic rats compared to healthy controls. However, following treatment with AG, the expression level of S1PR1 improved in diabetic groups while remaining unchanged in healthy rats compared to untreated healthy ones. Therefore, AG may not specifically affect S1PR1 expression levels in healthy normal rats.

Conclusion

The potential of AG to mitigate diabetes-related cardiovascular complications by modulating S1PR1 gene expression presents promising therapeutic avenues. Our study revealing the reversal of decreased S1PR1 expression in diabetic rats following AG treatment suggests a mechanism through which AG may improve cardiac microvascular function in diabetes. AG emerges as a promising candidate for preventing diabetes-associated cardiovascular complications, meriting further investigation into its clinical application. Nevertheless, additional pathological and molecular studies are warranted to elucidate the cardioprotective processes of AG on heart tissue in diabetic rats.

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None.

Authors' Contributions

- MKh: Contributed to the design of methodological procedures, data interpretation, statistical analysis, and manuscript writing.
- RP: Conducted all molecular experiments.
- MA: Provided expertise in the design and execution of animal experiments.
- MF: Played a key role in study design and data interpretation. Reviewed and offered critical insights on the manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Ethics Approval and consent to participate

All experimental protocols were undertaken in compliance with the Institutional Animal Care & Use Committee (IACUC) standards and approved by the Zanjan University of Medical Sciences Ethics Review Board (IR.ZUMS.REC.1394.114).

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