2-Arachidonoylglycerol Attenuates Fibrosis in Diabetic Mice via the TGF-β1/Smad Pathway

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Research Article

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Abstract

Purpose: Diabetic cardiomyopathy (DM) is the cause of late cardiac dysfunction in diabetic patients. Myocardial fibrosis is the main pathological mechanism, which is associated with transforming growth factor-β1 (TGF-β1) expression up-regulation. 2-Arachidonoylglycerol (2-AG) is an endogenous cannabinoid that can effectively improve myocardial cell energy metabolism and cardiac function. Here, we evaluated the protective effect of 2-AG on diabetic cardiomyopathy.

Methods: Male C57BL/6J mice were injected with 2-AG intraperitoneally for 4 weeks (1μg/kg/day) after 12 weeks of diabetic modeling. After 4 weeks, heart function was evaluated by echocardiography. Heart structure was assessed by hematoxylin and eosin staining. Cardiac fibrosis was analyzed using immunohistochemistry, Sirius red stain and Western blot.

Results: After modeling in diabetic mice, cardiac ultrasonography showed decreased cardiac function, and pathological findings showed that myocardial fibrosis. 2-AG could effectively inhibit the up-regulation of TGF-β1 and Smad2/3, improve myocardial fibrosis and ultimately improve cardiac function in diabetic mice.

Conclusion: 2-AG reduces cardiac fibrosis via the TGF-β1/Smad2/3 pathway and is a potential pathway for the treatment of cardiac dysfunction in diabetic mice.

1. Introduction

As a globally prevalent disease, the number of patients with diabetes has doubled over the past 20 years[1]. Diabetic cardiomyopathy is defined as diffuse myocardial fibrosis and impaired systolic function in the absence of valvular disease, hypertension, and ischemic heart disease[2]. This fibrosis usually causes changes in the pumping and electrophysiological functions of the heart, which in turn induces heart failure and sudden cardiac arrest[3].

Collagen fiber is an important component of the extracellular matrix of cardiomyocytes, which supports the structure of the ventricle to maintain its geometry and function[4]. Long-term Collagen deposition can lead to myocardial fibrosis and decreased ventricular compliance[4, 5]. In particular, the deposition of type I Collagen fibers leads to ventricular rigidity[6]. High blood sugar and fatty acid resistance induces the deposition of Collagen, which promotes the development of diabetic cardiomyopathy[5]. TGF-β1 regulates Collagen deposition[7]. A selective increase in TGF-β1 in cardiomyocytes stimulated by high glucose triggers overexpression of the Collagen-promoting gene by activating downstream Smad2/3[8]. Furthermore, fibrosis of the myocardium during diabetes is exacerbated.

2-AG was the second endocannabinoid discovered. It is able to regulate blood sugar and improve energy metabolism and is a potential diabetes treatment[9]. Previous studies have suggested that 2-AG in the heart may regulate cardiac function[10]. Application of 2-AG in diabetic cardiomyopathy improves
inflammation in cardiomyocytes\textsuperscript{[11]}. Recently, Soren V et al. showed that 2-AG induces resistance to liver fibrosis, suggesting that 2-AG may have antifibrotic effects\textsuperscript{[12]}. However, in diabetic cardiomyopathy, whether 2-AG has this antifibrotic effect is still unclear. In this study, we examined the effect of 2-AG in treating cardiac dysfunction in diabetic cardiomyopathy by relieving myocardial fibrosis and explored its mechanism.

2. Materials And Methods

2.1 Animals

Male C57BL/6J mice (20-22g) were obtained from the Animal Experimental Center of Zhejiang University, and the mice were housed in an SPF environment. All mice have free access to water and food.

The mice were randomly divided into the following groups: CON group, DM group, and 2-AG group. Mice in the DM and 2-AG groups were fasted overnight and injected intraperitoneally with 100 mg/kg streptozotocin (STZ) (dissolved in 100 mM citrate buffer, pH 4.5, purchased from Sigma, USA). Mice in the CON group received an injection of the same volume of citrate buffer. On the 3rd and 7th day, two consecutive fasting (for 8 h) blood glucose measurements were obtained by the tail vein. Mice with 8 h fasting-blood glucose $>$11.1 mM were considered diabetic and continued feeding for 12 weeks. After 12 weeks, the mice in the 2-AG group were intraperitoneally injected with 2-AG (dissolved in physiological saline, purchased from Tocris, USA) at 1 µg/kg for 4 consecutive weeks, and blood glucose and body weight were measured weekly during the administration. After 4 weeks, the mice were sacrificed by an overdose of 100 mg/kg ketamine hydrochloride (Ketanest, Pfizer, Germany) and 16 mg/kg xylazine hydrochloride (Rompun 2%, Bayer, Germany). Cardiac function was measured, the heart samples were weighed, and serum and heart tissue were collected for further analysis.

2.2 Echocardiographic evaluation

Cardiac function was determined noninvasively by transthoracic echocardiography before sacrifice\textsuperscript{[13]}. Doppler analysis was performed using a SONOS 5500 ultrasound (Philips Electronics, Amsterdam, The Netherlands) with a 15 MHz linear array ultrasound transducer to determine cardiac function.

2.3 Detection of serum lipids

Blood samples were collected and centrifuged at 3000 rpm/min for 15 minutes at 4 °C to separate the supernatant (serum). The levels of TG, T-CHO, LDL-C, and HDL-C in the serum were measured according to the kit instructions (Nanjing Jiancheng, China).
2.4 Histological analysis

The heart tissue was fixed in 4% paraformaldehyde overnight, dehydrated and embedded in paraffin, sectioned at 5-μm thickness, and mounted on glass slides. Pathological lesions were assessed by hematoxylin and eosin staining (H&E Assay Kit, Beyotime, China). Fibrosis was assessed using Sirius red staining (Sirius Heart Stain Kit, Solarbio, China). Images were observed and acquired using a Nikon microscope (Nikon, Japan).

2.5 Immunohistochemical analysis

Three sections were deparaffinized, rehydrated in gradient xylene and ethanol, antigen repair was performed by microwaving in 0.1 mol/L citrate buffer (pH 6.0), and the sections were then allowed to stand in a 3% hydrogen peroxide solution. After blocking with 2.5% BSA (Sigma, USA), the sections were incubated with anti-Collagen I (1:200, Abcam, UK) at 4 °C overnight and then secondary antibody (1:200, Santa Cruz, USA), colored by 3,3′-diaminobenzidine tetrahydrochloride (DAB; ZSGB-Bio, China), followed by sealing in neutral resin after dehydration in ethanol xylene Images were taken under a Nikon microscope.

2.6 Western blot analysis

The myocardial tissue was homogenized and lysed, and the total protein concentration was quantified by using a BCA protein assay kit (Thermo, USA). A total of 30-50µg of protein was loaded and separated by 10% SDS–polyacrylamide gel electrophoresis (PAGE). Then, the protein was transferred from the gel to a polyvinylidene fluoride membrane. After blocking with 5% skim milk and 0.05% Tween 20, the membrane was incubated overnight in primary antibody (TGF-β1:1:1000; Abcam; 92486; UK; Smad2/3:1:1000; CST; 8685; USA; p-Smad2/3:1:1000; CST; 8828; USA; Collagen I:1:1000; Abcam; 34701; UK; and β-actin:1:1000; CST; 4970; USA). Bands were detected with a specific horseradish peroxidase-conjugated secondary antibody (CWBio) (1:10000, Biosharp, China) and visualized by enhanced chemiluminescence reagents (Thermo, USA). Protein expression was quantified using ImageJ software.

2.7 Statistical analysis

All data are expressed as the mean ± standard deviation, and statistical analysis was performed using GraphPad Pro Prism 7.0. One-way ANOVA was used, and then multiple comparison tests with a Tukey correction were performed, with p < 0.05 considered a significant difference.

3. Results
3.1 Effect of 2-AG on blood glucose, blood lipids and body weights of DM mice

In this study, after 12 weeks of diabetes modeling, 2-AG treatment was given for 4 weeks. Blood glucose and body weight were measured weekly during the study, and blood lipids were measured after serum collection. Compared with the levels in the CON group, the serum HDL levels in the DM and 2-AG groups decreased significantly. Interestingly, the LDL level in the DM group was significantly higher than that in the CON group, and 2-AG reversed this trend. There was no significant difference between TC and TG among the groups (Table 1). There was a significant increase in blood glucose in the DM and 2-AG groups, but there was no difference between the two groups (Fig. 1A). In addition, 2-AG treatment for 4 weeks improved DM-induced weight loss (Fig. 1B).

|                | CON(n = 8) | DM(n = 8) | 2-AG(n = 8) | P value |
|----------------|------------|-----------|-------------|---------|
| HDL-C(mmol/L)  | 1.94 ± 0.42| 1.49 ± 0.29*| 1.47 ± 0.25*| 0.0144  |
| LDL-C(mmol/L)  | 0.22 ± 0.02| 0.33 ± 0.09*| 0.20 ± 0.07##| 0.0084  |
| TG(mmol/L)     | 0.65 ± 0.14| 0.70 ± 0.21 | 0.69 ± 0.08 | 0.7945  |
| T-CHO(mmol/L)  | 1.68 ± 0.27| 1.46 ± 0.27 | 1.60 ± 0.28 | 0.2938  |

T-CHO: total cholesterol; TG: triglyceride; HDL-C: high density lipoprotein; LDL-C: low density lipoprotein

*p < 0.05, compared with CON group; ##p < 0.01, compared with DM group.

3.2 Effect of 2-AG on cardiac function and ventricular remodeling

To investigate whether 2-AG improves cardiac function in diabetic mice, we measured cardiac function by transthoracic echocardiography. As shown in Fig. 2A, 2D, and 2E, the LVEF and LVSF values of the mice in the DM group were lower than those in the CON group, while the values of these two indicators were significantly increased in the 2-AG group compared with those of the DM group. The overall view of the HE-stained heart cross-section also showed that the left ventricular cavity was significantly enlarged in the DM group, and the thickness of the left ventricular sac was reduced, while 2-AG reversed this effect (Fig. 2B). The cross-sectional area of myocardial cells in the DM group was significantly reduced compared with that of the CON group, and 2-AG improved the cross-sectional area of the myocardium caused by DM (Fig. 2C, 2G). In addition, we found that the heart weights in the DM group decreased significantly, and 2-AG reversed this effect (Fig. 2F).
3.3 Effect of 2-AG on myocardial fibrosis

To further explore the reasons for the improved heart function, we performed Sirius red staining. Sirius red staining showed an increase in the positive area in the DM group compared with that of the CON group, and 2-AG improved this effect (Fig. 3A, 3B). To verify the cause of fibrosis, immunohistochemistry analysis of Collagen I was performed (Fig. 3C, 3F). The results showed that the heart tissue in the DM group had significant positive cells compared to that of the CON group, while 2-AG reduced this effect.

3.4 Effect of 2-AG on the TGF-β signaling pathway

As the TGF-β1/Smad2/3 pathway is a key factor in regulating fibrosis, we used Western blot analysis to detect myocardial protein expression. As expected, the results showed that the expression levels of TGF-β1, Smad2/3 and p-Smad2/3 were significantly upregulated in the DM group (Fig. 4A, 4B, 4C, 4D), while 2-AG inhibited the expression of these proteins. Next, we measured the expression of Collagen I. 2-AG downregulated Collagen I expression, which was consistent with the immunohistochemistry results (Fig. 4E).

4. Discussion

2-AG, a ligand for endocannabinoid receptors, has important protective effects in pathophysiological conditions such as shock and myocardial infarction\cite{14}. Our study demonstrates that 2-AG can improve cardiac function in diabetic mice. Myocardial fibrosis is an important pathogenesis of diabetic ventricular remodeling and cardiac pump failure\cite{15}. HE-stained showed that 2-AG reversed ventricular remodeling in mice. Cardiac ultrasound showed that treatment with 2-AG improved left ventricular function decline in diabetic mice. Therefore, we first proposed that 2-AG improves myocardial fibrosis in diabetic mice.

TGF-β1 is a key mediator in fibrosis\cite{7}. Our detection of TGF-β1, Smad2/3, and Collagen I protein showed that hyperglycemia in diabetes upregulated the protein expression of TGF-β1 and activated phosphorylation of downstream Smad2/3. The activated Smad2/3 undergoes nuclear translocation, enters the nucleus, and directs the transcription and translation of collagen, and Smad7 negatively regulates the activation of the fiber gene\cite{16, 17}. Increased collagen and increased cross-linking in the extracellular matrix lead to cardiac sclerosis, which causes changes in cardiac pump function\cite{18}. Collagen I is a major contributor to this process\cite{6}. Treatment with 2-AG reduced the upregulation of TGF-β1, and expression of the downstream proteins Smad2/3 and Collagen I was also downregulated. Therefore, the potential mechanism of endogenous cannabinoid 2-AG on cardiomyocyte fibrosis in diabetic mice may be by reducing the expression of Collagen I protein through the TGF-β1/Smad2/3 pathway, thereby reducing collagen deposition and reducing the degree of heart stiffness to improve ventricular function.
High LDL-C concentration in the serum is one of the major risk factors for coronary heart disease$^{[19, 20]}$. LDL deposits into microvascular endothelial cells, causing persistent inflammation in blood vessels$^{[21]}$. Long-term reductions in LDL-C are important indicators of proper lipid management in patients with heart disease or diabetes$^{[22, 23]}$. Our study showed that 2-AG significantly reduced the increase in LDL caused by diabetes. This suggests that the protective effect of 2-AG on diabetic heart disease is not only by reducing the fibrosis of cardiomyocytes but also by improving the lesions of small blood vessels. 2-AG has a regulating effect on blood lipids and seems to have no effect on blood glucose. This suggests that 2-AG can only maintain the homeostasis of blood glucose but does not act as a hypoglycemic drug.

Interestingly, we found that the thickness of the ventricular wall and the diameter of the myocardial cells in diabetic mice were also significantly reduced, which suggested that the content and quality of the left ventricular myocardium had a significant decrease. However, there was a significant increase in left ventricular wall thickness and myocardial cell diameter in diabetic mice after the use of the endogenous cannabinoid 2-AG. Previous studies have suggested that cardiomyocytes in diabetic mice usually exhibit hypertrophy and thickening of the left ventricular wall$^{[24]}$. Some results were similar to ours$^{[25, 26]}$. We believe this may be related to metabolic changes in cardiomyocytes. Diabetes causes a disorder in myocardial lipid and carbohydrate metabolism. Long-term insulin resistance can lead to a decrease in glucose metabolism in cardiomyocytes, but the increase in fat metabolism leads to a decrease in energy metabolism efficiency$^{[27]}$. A low-calorie diet may cause insufficient energy in the hearts of diabetic mice, which causes atrophy of the cells. In our study, after STZ administration, the mice did not receive additional calorie treatment, and plasma lipid concentrations proved this. Therefore, the low lipid content may be the cause of myocardial atrophy and the reduction in ventricular wall thickness in our diabetic mice. 2-AG can improve the disorder of carbohydrate and lipid metabolism in the myocardium, increase myocardial weight, which is manifested as an increase in body weight. However, the metabolism by which 2-AG regulates left ventricular myocardial energy and affects cardiac pump dysfunction in diabetic mice still needs further investigation.

Our study showed that the endogenous cannabinoid agonist 2-AG is a potential drug for the treatment of diabetic myocardial fibrosis and cardiac pump function damage, and the downregulation of Collagen I protein expression due to inhibition of the TGF-β1/Smad2/3 pathway may be a potential mechanism for this. Whether 2-AG can treat fibrosis caused by other diseases remains to be further studied.

**Abbreviations**
### Declarations

#### Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

#### Conflicts of interest

The authors declare that they have no conflict of interest.

#### Availability of data and material

When necessary, raw data could be provided.

#### Code availability

unavailable

### Authors' contributions

Dr. Zhengjie Chen responsible for study design, conduct of the study, data collection, data analysis and manuscript preparation. Dr. Liangyu Zheng participated in data collection. Dr. Gang Chen responsible for
study design, conduct of the study, data analysis and manuscript preparation.

**Ethics approval**

The protocols used for all animal studies were approved by the Zhejiang University Animal Policy and Welfare Committee and complied with the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

**Consent to participate**

This research does not involve human experiments.

**Consent for publication**

All authors agree to publish in Cardiovascular Drugs and Therapy.

**References**

1. E.J. Benjamin, P. Muntner, A. Alonso, et al., Heart Disease and Stroke Statistics-2019 Update: A Report From the American Heart Association, Circulation, 139 (2019) e56-e528.
2. S. Rubler, J. Dlugash, Y.Z. Yuceoglu, et al., New type of cardiomyopathy associated with diabetic glomerulosclerosis, Am J Cardiol, 30 (1972) 595-602.
3. K. Khavandi, A. Khavandi, O. Asghar, et al., Diabetic cardiomyopathy--a distinct disease?, Best Pract Res Clin Endocrinol Metab, 23 (2009) 347-360.
4. J.S. Janicki, G.L. Brower, The role of myocardial fibrillar collagen in ventricular remodeling and function, J Card Fail, 8 (2002) S319-325.
5. A. Aneja, W.H. Tang, S. Bansilal, et al., Diabetic cardiomyopathy: insights into pathogenesis, diagnostic challenges, and therapeutic options, Am J Med, 121 (2008) 748-757.
6. K.T. Weber, Y. Sun, S.C. Tyagi, et al., Collagen network of the myocardium: function, structural remodeling and regulatory mechanisms, J Mol Cell Cardiol, 26 (1994) 279-292.
7. K.L. Walton, K.E. Johnson, C.A. Harrison, Targeting TGF-beta Mediated SMAD Signaling for the Prevention of Fibrosis, Front Pharmacol, 8 (2017) 461.
8. Y. Zhang, L. Cui, G. Guan, et al., Matrine suppresses cardiac fibrosis by inhibiting the TGFbeta/Smad pathway in experimental diabetic cardiomyopathy, Mol Med Rep, 17 (2018) 1775-1781.
9. M. Alhouayek, J. Masquelier, G.G. Muccioli, Controlling 2-arachidonoylglycerol metabolism as an anti-inflammatory strategy, Drug Discov Today, 19 (2014) 295-304.
10. C.R. Hiley, Endocannabinoids and the heart, J Cardiovasc Pharmacol, 53 (2009) 267-276.
11. D. Chanda, Y. Oligschlaeger, I. Geraets, et al., 2-Arachidonoylglycerol ameliorates inflammatory stress-induced insulin resistance in cardiomyocytes, J Biol Chem, 292 (2017) 7105-7114.

12. S.V. Siegmund, T. Qian, S. de Minicis, et al., The endocannabinoid 2-arachidonoyl glycerol induces death of hepatic stellate cells via mitochondrial reactive oxygen species, FASEB J, 21 (2007) 2798-2806.

13. Y. Pan, Y. Wang, Y. Zhao, et al., Inhibition of JNK phosphorylation by a novel curcumin analog prevents high glucose-induced inflammation and apoptosis in cardiomyocytes and the development of diabetic cardiomyopathy, Diabetes, 63 (2014) 3497-3511.

14. C.R. Hiley, W.R. Ford, Cannabinoid pharmacology in the cardiovascular system: potential protective mechanisms through lipid signalling, Biol Rev Camb Philos Soc, 79 (2004) 187-205.

15. S. Boudina, E.D. Abel, Diabetic cardiomyopathy, causes and effects, Rev Endocr Metab Disord, 11 (2010) 31-39.

16. Y. Li, J.L. Dong, Y.H. Shang, et al., Anti-inflammatory effects of hederagenin on diabetic cardiomyopathy via inhibiting NF-kappa B and Smads signaling pathways in a type-2 diabetic mice model, Rsc Advances, 9 (2019) 26238-26247.

17. S.Q. Wang, D. Li, Y. Yuan, Long-term moderate intensity exercise alleviates myocardial fibrosis in type 2 diabetic rats via inhibitions of oxidative stress and TGF-beta1/Smad pathway, J Physiol Sci, (2019).

18. J. He, M.T. Quintana, J. Sullivan, et al., MuRF2 regulates PPARgamma1 activity to protect against diabetic cardiomyopathy and enhance weight gain induced by a high fat diet, Cardiovasc Diabetol, 14 (2015) 97.

19. B. Wu, Z. Yu, T. Tong, et al., Evaluation of small dense low-density lipoprotein concentration for predicting the risk of acute coronary syndrome in Chinese population, J Clin Lab Anal, (2019) e23085.

20. B.A. Ference, D.L. Bhatt, A.L. Catapano, et al., Association of Genetic Variants Related to Combined Exposure to Lower Low-Density Lipoproteins and Lower Systolic Blood Pressure With Lifetime Risk of Cardiovascular Disease, JAMA, (2019).

21. R. Stocker, J.F. Keaney, Jr., Role of oxidative modifications in atherosclerosis, Physiol Rev, 84 (2004) 1381-1478.

22. R.K. Wadhera, D.L. Steen, I. Khan, et al., A review of low-density lipoprotein cholesterol, treatment strategies, and its impact on cardiovascular disease morbidity and mortality, J Clin Lipidol, 10 (2016) 472-489.

23. G. Russo, B. Pintaudi, C. Giorda, et al., Age- and Gender-Related Differences in LDL-Cholesterol Management in Outpatients with Type 2 Diabetes Mellitus, Int J Endocrinol, 2015 (2015) 957105.

24. X.Y. Hu, T. Bai, Z. Xu, et al., Pathophysiologica Fundamentals of Diabetic Cardiomyopathy, Compr Physiol, 7 (2017) 693-711.

25. O. Bilim, Y. Takeishi, T. Kitahara, et al., Diacylglycerol kinase zeta inhibits myocardial atrophy and restores cardiac dysfunction in streptozotocin-induced diabetes mellitus, Cardiovasc Diabetol, 7
26. O. Nemoto, M. Kawaguchi, H. Yaoita, et al., Left ventricular dysfunction and remodeling in streptozotocin-induced diabetic rats, Circ J, 70 (2006) 327-334.

27. B. Rodrigues, M.C. Cam, J.H. McNeill, Metabolic disturbances in diabetic cardiomyopathy, Mol Cell Biochem, 180 (1998) 53-57.

Figures
Figure 1

The effect of 2-AG on blood glucose and body weight. Blood glucose and weight were measured weekly during the study. A. Blood glucose levels in the DM and 2-AG groups were significantly higher than those in the CON group. B. Mice in the 2-AG group had significantly improved DM-induced weight loss. ## p < 0.01, compared with DM group; ### p < 0.001, ** p < 0.01, * p < 0.05 compared with CON group, n = 8.
Figure 2

2-AG improves cardiac function and ventricular remodeling in diabetic mice. A. Representative images of M-type echocardiograms. B. Overall view of HE-stained. C. Representative diagram of HE-stained cross section of the heart. D. LVEF statistical chart. E. LVFS statistical chart. F. Heat weights. G. Statistical map of myocardial cross-sectional area. LVEF = left ventricular ejection fraction, LVFS = left ventricular
shortening fraction. \( \# p < 0.05, \## p < 0.01, \### p < 0.001 \), compared with the DM group; \( \** p < 0.01, \*** p < 0.001 \), compared with the CON group, \( n = 7 \).

**Figure 3**

2-AG inhibits myocardial fibrosis and collagen deposition in diabetic mice. A. Representation of the Sirius red-stained heart; B. A representative image of Collagen I immunohistochemical staining; C. Statistical graph of the positive area of Sirius red staining; D. A statistical map of immunohistochemical protein expression. \( \** p < 0.01, \*** p < 0.001 \) compared with the CON group; \( \# p < 0.05 \) compared to the DM group, \( n = 7 \).
Figure 4

2-AG inhibits activation of the TGF-β1/Smad signaling pathway in myocardial fibrosis. A. Western blot of TGF-β1, Smad2/3, p-Smad2/3, Collagen I, and β-actin. B, C, and D are statistical representations of the relative expression of TGF-β1, Smad2/3, p-Smad2/3, Collagen I and β-actin, respectively. *p < 0.05, **p < 0.01, ***p < 0.001 compared with the CON group; # p < 0.05, ## p < 0.01, ### p < 0.001, compared with the DM group, n=6.