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ORIGINAL RESEARCH

Metformin Delays the Development of Atherosclerosis in Type 1 Diabetes Mellitus via the Methylglyoxal Pathway

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ABSTRACT

Introduction: The aim of our study was to determine the effect of metformin administration on juvenile type 1 diabetes mellitus and atherosclerosis in apolipoprotein E null ($ApoE^{-/-}$) mice and to explore the mechanism involved.

Methods: Eighteen male $ApoE^{-/-}$ mice were injected with streptozotocin to induce diabetes (diabetic group) and 18 mice who received no streptozotocin injection were assigned to the control (non-diabetic) group. Six mice in each group were then orally administered metformin, simvastatin, or vehicle, respectively, following which the mice were euthanized and tissue samples collected.

Results: Fasting plasma glucose, low-density lipoprotein-cholesterol, and triglyceride concentrations were significantly higher in the three diabetic groups than in the three non-diabetic groups. Plasma N^{ϵ}-(carboxymethyl)lysine and N^{ϵ}-(carboxyethyl)lysine concentrations were higher in the diabetic mice than in the

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non-diabetic mice, but metformin treatment reduced these concentrations more effectively than simvastatin. All three diabetic groups demonstrated obvious arterial plaques, but these were largest in the vehicle-treated diabetic group. The expression of extracellular nitric oxide synthase was highest in the simvastatin-treated non-diabetic group, and in diabetic mice it was higher in the simvastatin-treated group than in the other two groups. No significant expression of AMP-activated protein kinase (AMPK) was measured in the three diabetic groups, but a low level of AMPK expression was detected in the non-diabetic groups.

Conclusions: Metformin can limit the development of atherosclerosis secondary to diabetes in young diabetic mice. A possible mechanism is the removal of methylglyoxal, thereby reducing the formation of advanced glycation endproducts, rather than by lowering the blood glucose level.

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Keywords: Atherosclerosis; Metformin; Methylglyoxal; Type 1 diabetes mellitus

Key Summary Points
The apolipoprotein E (ApoE) knockout mouse model of diabetes mellitus and juvenile atherosclerosis was successfully established.
Metformin can prevent atherosclerosis secondary to type 1 diabetes mellitus.
It may achieve its effect of reducing the terminal products of late glycosylation by removing acetone aldehyde, rather than by lowering the blood glucose level

INTRODUCTION

The prevalence of type 1 diabetes mellitus (T1DM) in pediatric populations has been increasing globally over the last decade [1]. Long-term hyperglycemia and metabolic disorders are associated with T1DM and lead to serious diabetes complications. As a result, the life expectancy of patients with T1DM is 11–13 years shorter than that of the general population [2]. The main cause of death in diabetic patients is cardiovascular complications that involve atherosclerosis [2]. Previous studies have shown that atherosclerosis is present in children with T1DM [3] and that the disorders in vascular structure and function which characterize early atherosclerosis can be reversed. Treatments that minimize glycated hemoglobin (HbA1c) concentrations are the most effective approaches for reducing the development of atherosclerosis, but they cannot completely prevent it. Therefore, it is important that an effective T1DM treatment regimen is developed to protect the cardiovascular system. Recently, metformin has been shown to reduce the degree of atherosclerosis in patients with T1DM without affecting HbA1c levels or the required insulin dose [4, 5].

In this study, we induced T1DM in apolipoprotein E knockout ($ApoE^{-/-}$) mice, characterized the degree of atherosclerosis in the mice while still young, and then determined the effect of metformin treatment on the progression of atherosclerosis and the associated mechanisms.

METHODS

Experimental Animals

Thirty-six male $ApoE^{-/-}$ mice on a C57BL/6 background [6–8] were purchased at the age of 4 weeks from the Laboratory Animal Center of Nanjing University [Nanjing, China; Introduction agreement (2012) No. 470] and kept on a normal chow diet. All experiments were approved by the ethics committee of the Second Hospital of Shandong University [KYLL-2017(KJ)P-0016] and followed national guidelines for the care and use of animals [9]. All sections of this report adhere to the ARRIVE guidelines for reporting animal research. At 8 weeks of age, 18 of the male $ApoE^{-/-}$ mice were injected with 55 mg/kg/day streptozotocin

(STZ) [10, 11] (S8050; Solarbio, Shanghai, China) for 5 consecutive days, then observed for the following 2 weeks, after which they were fasted for 12 h, with no restriction on drinking water, and their fasting plasma glucose (FPG) values measured using blood obtained from the tail tip. Mice with two FPG measurements of $\geq$ 11 mmol/l (FPG1) were regarded as diabetic, weighed (body mass 1), and randomly allocated to groups A, B, and C. Any mice that had not reached the FPG threshold were given an additional injection of STZ (10–20 mg/kg intraperitoneal) 3 days later. The remaining 18 mice did not receive an injection of STZ and were considered to be the non-diabetic (control) group and randomly allocated to groups D, E, and F. Groups A and D were then administered 100 mg/kg/day metformin (IM0140; Solarbio), groups B and E were administered 5 mg/kg/day simvastatin (IS0170; Solarbio), and groups C and F were administered normal saline orally for 4 weeks.

Following this treatment, the mice were fasted for 12 h, with no restriction on drinking water, and then weighed (body mass 2). The mice were then euthanized, and blood was collected from their retro-orbital sinuses for further analysis. FPG (FPG2), triglyceride (TG), and low-density lipoprotein-cholesterol (LDL) levels were determined using an automated biochemical analyzer (AU680 Chemistry Analyzer; Beckman Coulter, Brea, CA, USA). Plasma N^{ϵ}-(carboxyethyl) lysine (CEL) (CSB-EQ027210H; Cusabio, Shanghai, China) and N^{ϵ}-(carboxymethyl)lysine (CML) (CEB977Ge; Cusabio) concentrations were measured by enzyme-linked immunosorbent assays.

Atherosclerotic Lesion Assessment

The aorta was excised from the root to the abdomen, and the connective and adipose tissues were carefully removed. The entire aorta was stained with oil red O for the assessment of atherosclerotic lesions [6–8]. The plaque area stained by oil red O was assessed relative to the total luminal surface area using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Silver Spring, MD, USA). To further characterize the atherosclerotic lesions in the aortic root, we prepared serial cryostat sections (thickness 8 μm) from three different regions of the aortic root. Some of these sections were stained using hematoxylin and eosin [12]; the remaining sections were used for immunohistochemical analysis. Air-dried sections were fixed in acetone and stained with anti-endothelial nitric oxide synthase (eNOS) antibody (ab5589; Abcam, Shanghai, China) or AMP-activated protein kinase (AMPK) antibody (ab32047; Abcam, Shanghai, China) using immunohistochemistry. All images were analyzed using the Image-Pro Plus 6.0 software. In each case, the mean staining intensity at four to five locations for each mouse was used for the analysis.

Statistical Analysis

All data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way analysis of variance, followed by Student–Newman–Keuls multiple comparison tests in SPSS version 13.0 software (IBM Corp., Armonk, NY, USA). A $P < 0.05$ was considered to represent statistical significance.

RESULTS

Baseline Characteristics

Prior to injecting mice with STZ , there were no statistically significant differences in body mass of the ApoE$^{-/-}$ mice in the diabetic and non-diabetic groups. After the STZ injection, the body mass of mice in the diabetic group decreased or remained stable; there was no significant weight gain in the diabetic mice during the 4 weeks of the study ($P > 0.05$). In contrast, the non-diabetic mice showed a significant weight gain ($P = 0.02$).

The mean (± SEM) FPG prior to study initiation was 5.33 ± 0.24 mmol/l. There were significant differences in FPG1 and FPG2 between the diabetic and non-diabetic groups ($P = 0.035$), which is consistent with the successful induction of diabetes in the diabetic group. However, FPG1 and FPG2 were similar in

Table 1 General characteristics of the apolipoprotein E knockout mice

General characteristics	Diabetic groups[a]			Non-diabetic (control) groups[a]		
	A	B	C	D	E	F
FPG1 (mmol/l)[b]	13.5 ± 3.5	15 ± 2.08	11.00 ± 1.70	5.20 ± 0.14	5.6 ± 0.49	4.8 ± 1.16
Body mass 1 (g)[c]	23.95 ± 1.34	20.36 ± 0.92	23.4 ± 0.51	25.25 ± 3.18	24.5 ± 2.12	21.66 ± 4.53
FPG2 (mmol/l)[b]	15.3 ± 8.62	17.7 ± 1.99	9.9 ± 0.15	8.45 ± 1.62	9.30 ± 1.27	9.02 ± 1.96
Body mass 2 (g)[c]	22.62 ± 3.36	22.8 ± 1.9	24 ± 0	27.35 ± 0.91	25.75 ± 1.06	22.76 ± 2.54
LDL (mmol/l)	1.91 ± 0.64	1.57 ± 0.43	2.04 ± 0.36	1.63 ± 0.21	1.26 ± 0.33	1.56 ± 0.43
TG (mmol/l)	1.49 ± 0.45	1.35 ± 0.18	1.95 ± 0.34	1.22 ± 0.39	1.05 ± 0.29	1.32 ± 0.69
CML (μg/d1)	60.1 ± 14.3	79.5 ± 14.3	77.6 ± 11.9	42 ± 11.5	45.9 ± 12.7	43.1 ± 16.8
CEL (pg/m1)	39.6 ± 12.5	57.2 ± 14.7	59.1 ± 16.2	25.1 ± 9.71	24.8 ± 11.2	20.1 ± 9.6

FPG Fasting plasma glucose, *LDL* low-density lipoprotein-cholesterol, *TG* total triglycerides, *CML* N^{ϵ}-(carboxymethyl)lysine, *CEL* N^{ϵ}-(carboxyethyl)lysine

[a] Groups A and D were administered 100 mg/kg/day metformin; groups B and E were administered 5 mg/kg/day simvastatin; groups C and F were administered normal saline orally, all for 4 weeks

[b] FPG measurements were taken to determine the development of diabetes (two FPG measurements of ≥ 11 mmol/l ; FPG1) and after euthanasia (FPG2)

[c] Body weight (body mass) was measured when mice were determined to have developed diabetes (body mass 1) and immediately prior to euthanasia (body mass 2)

groups A, B, and C (diabetic mice) and similar in groups D, E, and F (control mice).

The mean (± SEM) plasma LDL of the diabetic mice was significantly higher than that of the non-diabetic mice (1.84 ± 0.52 vs. 1.48 ± 0.29 mmol/l; $P = 0.043$). Among the diabetic mice groups, the LDL was lowest in the simvastatin-treated group ($P = 0.036$); among all six mice groups, LDL was lowest in the simvastatin-treated non-diabetic group. The mean plasma TG of the diabetic mice was also significantly higher than that of the non-diabetic mice (1.59 ± 0.32 vs. 1.19 ± 0.47 mmol/l; $P = 0.023$).

The plasma CML of the metformin-treated diabetic group was the lowest of all three diabetic groups ($P = 0.047$), and it was higher in the diabetic groups than in the non-diabetic groups ($P = 0.023$). In contrast, plasma CML was similar among all three non-diabetic groups. Similarly, the plasma CEL of the metformin-treated diabetic group was the lowest of the diabetic groups ($P = 0.031$), and the CEL concentration of the metformin-treated non-diabetic group was lower than those of the diabetic groups ($P = 0.01$). However, there were no differences in CEL among the non-diabetic groups (Table 1).

Atherosclerotic Lesions

In the metformin- and simvastatin-treated diabetic groups, the elastic lining of the aorta had been destroyed and the vascular wall showed signs of fibrosis and atherosclerosis. In the saline-treated diabetic group, the intimal elastic layer had been destroyed and the vascular wall showed signs of fibrosis, variations in thickness, and atherosclerosis. No obvious plaque was observed in the three non-diabetic groups (Fig. 1); in contrast, all three diabetic groups had obvious plaques that showed lipid deposition, with the saline-treated group showing the most severe plaque and lipid deposition. A small amount of lipid deposition was observed in the non-diabetic groups (Fig. 2).

Expression of eNOS and AMPK

Endothelial nitric oxide synthase expression was detected in $ApoE^{-/-}$ mouse vascular

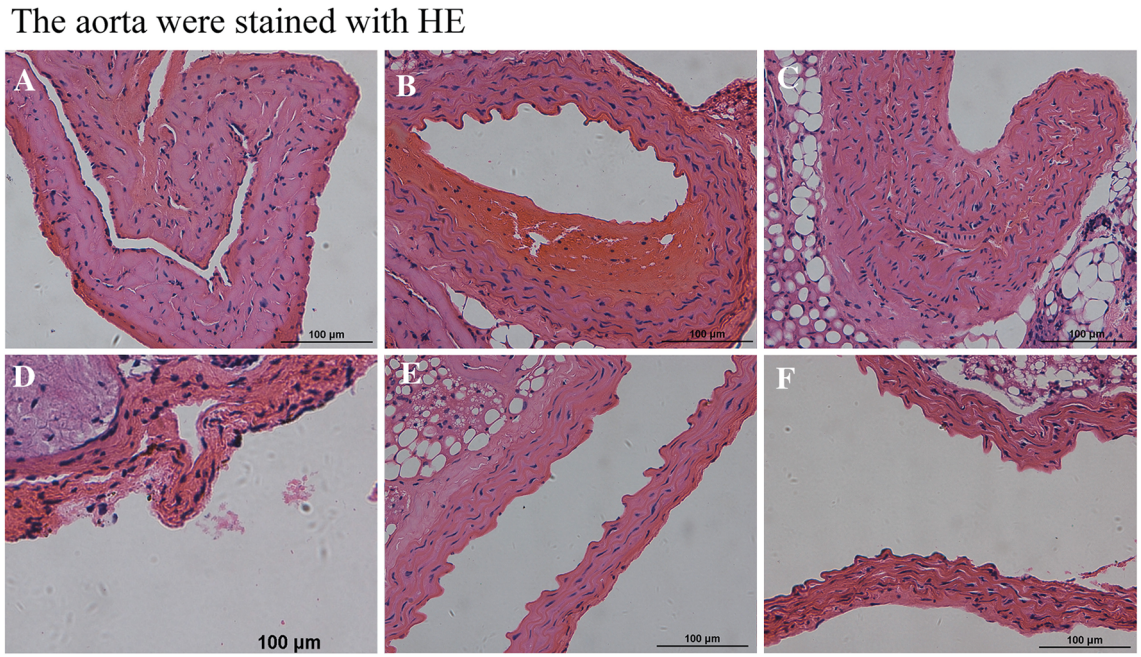

Fig. 1 Mouse aortae stained with hematoxylin and eosin. **A** Metformin-treated diabetic group, **B** simvastatin-treated diabetic group, **C** saline-treated diabetic group, **D** metformin-treated non-diabetic group. **E** simvastatin-treated non-diabetic group, **F** saline-treated non-diabetic group

endothelial cells isolated from the three non-diabetic groups and from the simvastatin-treated diabetic group, with the highest eNOS expression identified in the simvastatin-treated non-diabetic group. The expression of eNOS in the vascular endothelial cells of the non-diabetic groups was significantly higher than that in those of the diabetic groups; among the diabetic groups, the highest expression of eNOS was detected in the simvastatin group ($P < 0.05$), with no significant expression of eNOS in the other two diabetic groups. The expression of eNOS in the metformin- and saline-treated non-diabetic groups was low (Fig. 3). No significant expression of AMPK was observed in the three diabetic groups, AMPK was expressed at a low level in the non-diabetic groups (Fig. 4).

DISCUSSION

In this study, we treated ApoE$^{-/-}$ male mice with STZ to establish a model of T1DM and atherosclerosis. Non-obese diabetic (NOD) mice are a strain of Jcl:ICR mice that are susceptible to diabetic complications and widely used a model of spontaneous non-obese T1DM. However, these mice have strict dietary requirements, high cholesterol-related mortality, small body size, small arteries, and anti-atherosclerotic characteristics; consequently, they do not readily form plaques and are therefore not suitable as a model of both atherosclerosis and T1DM [13]. ApoE is involved in the receptor-mediated uptake of LDL into cells, and ApoE$^{-/-}$ mice fed a high-fat diet develop atherosclerotic plaques that are similar to those of humans in terms of lesion morphology and rupture.This similarity has led to these mice being commonly used to study atherosclerosis [14]. STZ is cytotoxic to β-cells and is widely used to ablate the β-cells of mice, which causes an absolute reduction in insulin secretion, thereby mimicking T1DM in humans. Therefore, we treated ApoE$^{-/-}$ mice with STZ to establish a model of T1DM with atherosclerosis [11]. STZ administration resulted in the expected features of diabetes, such as severe hyperglycemia and weight loss, and atherosclerosis was also successfully

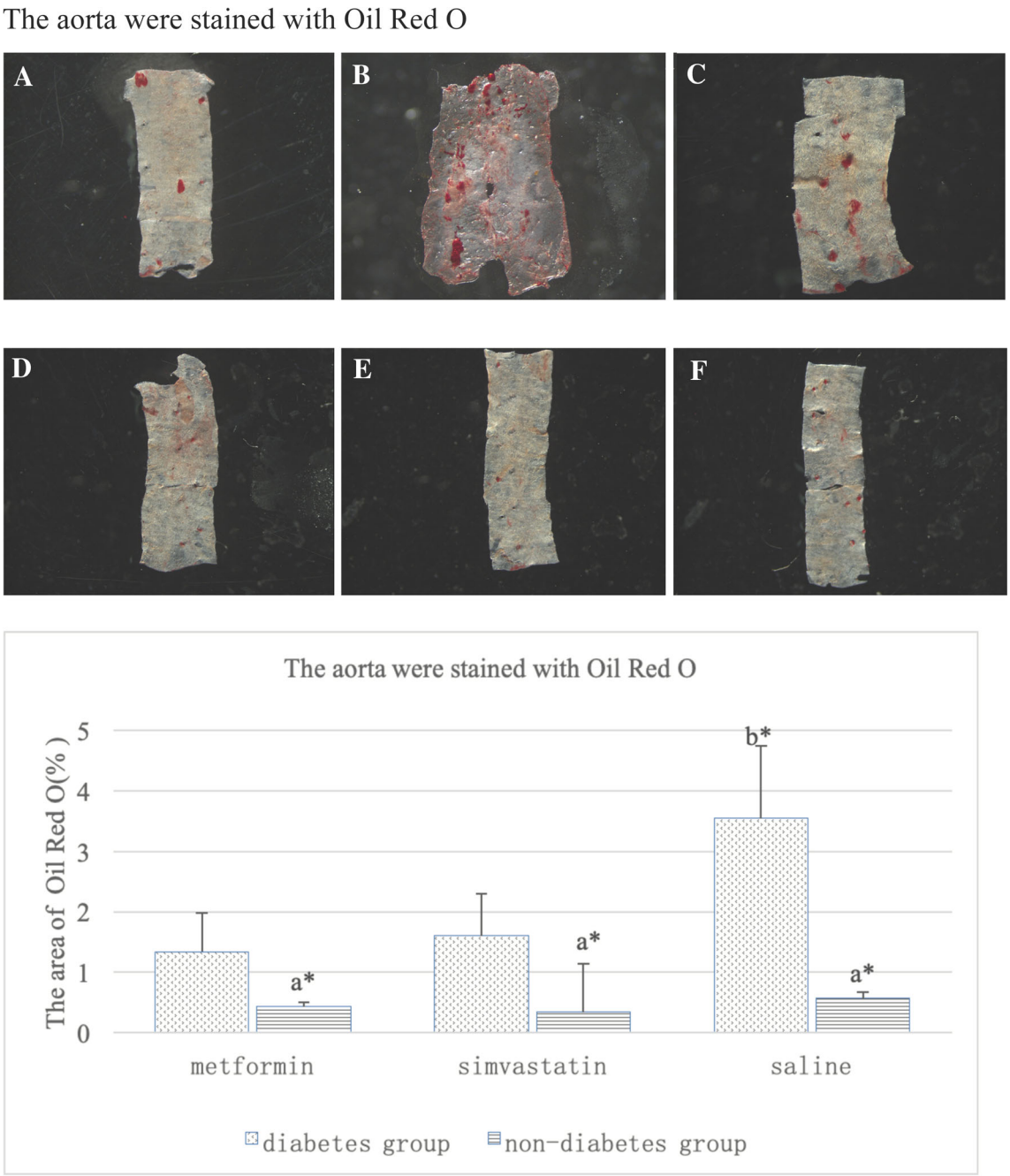

Fig. 2 Mouse aortae stained with oil red O. See caption to Fig. 1 for explanation of figure parts/mice groups (**A**–**F**). Lowercase letters (a, b) with asterisks on bar graph indicate a significant difference at $P < 0.05$ vs. the corresponding diabetic group (a) and a significant difference at $P < 0.05$ vs. the simvastatin- and metformin-treated diabetic groups (b)

established, providing a suitable model to determine the effects of pharmacologic interventions on the development of atherosclerosis.

Obvious plaques formed in the three diabetic groups of mice, but the plaque area of the metformin-treated group was smaller than that

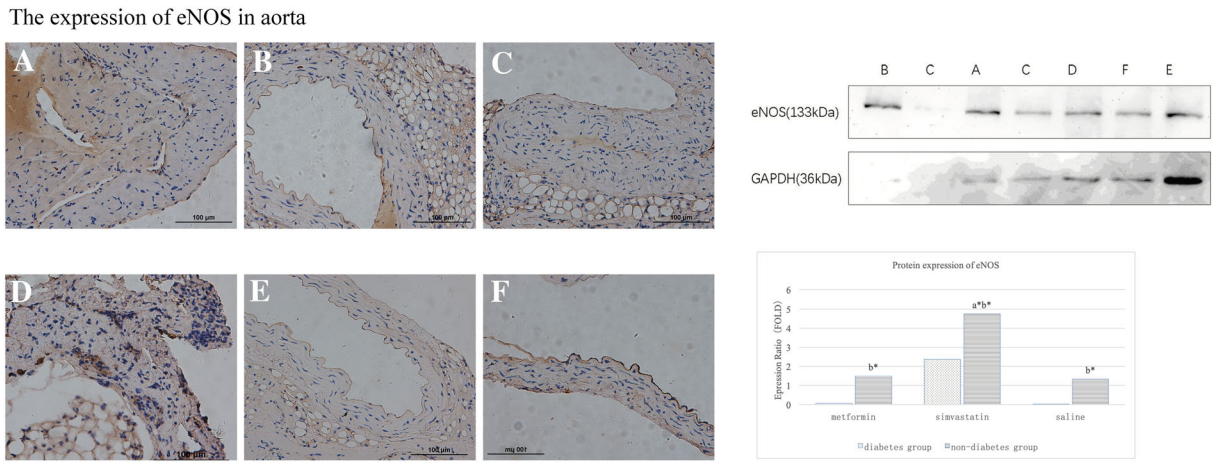

Fig. 3 Expression of endothelial nitric oxide synthase (*eNOS*) in mouse aortae. See caption to Fig. 1 for explanation of figure parts/mice groups (**A**–**F**). Lowercase letters (a, b) with asterisks on bar graph indicate a significant difference at $P < 0.05$ vs. the saline and metformin-treated non-diabetic groups (a) and a significant difference at $P < 0.05$ vs. the corresponding diabetic group (b). *GAPDH* Glyceraldehyde 3-phosphate dehydrogenase

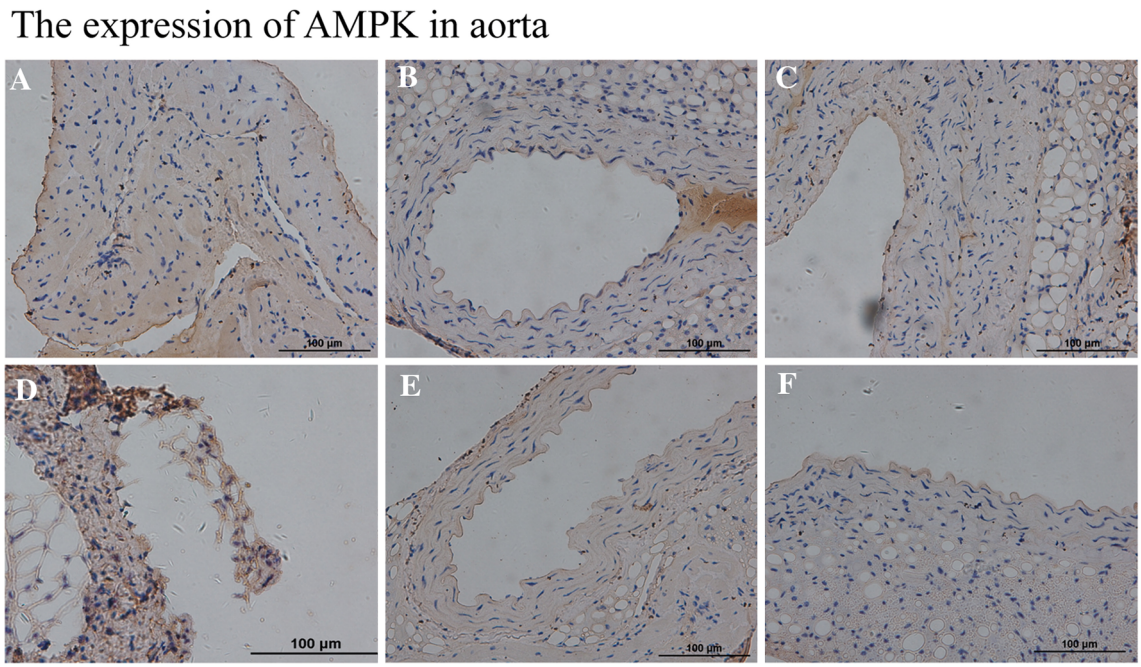

Fig. 4 Expression of AMP-activated protein kinase in mouse aortae. See caption to Fig. 1 for explanation of figure parts/mice groups (**A**–**F**)

of the simvastatin- and saline-treated groups, implying that metformin is more effective at inhibiting plaque formation than simvastatin. However, the FPG of the diabetes-treated group overall was similar to that of the saline-treated group after 4 weeks of treatment, which implies that the anti-atherosclerotic effect of metformin was not achieved secondary to a reduction in blood glucose level. Also, there was no clear

inhibitory effect of metformin on atherosclerosis in the non-diabetic groups of mice.

A previous study showed that metformin protects against the cardiovascular complications of type 2 diabetes by activating the energy-sensing kinase AMPK; phosphorylating eNOS; increasing nitric oxide (NO) synthesis; inhibiting the expression of endothelial cell adhesion molecules, cell proliferation, and migration; and regulating vascular tone and platelet-induced endothelial cell damage [15], which protects vessel endothelia during diastole [16]. Endothelial dysfunction is a key initiating factor in the formation of atherosclerotic lesions, and hyperlipidemia can lead to a reduction in the release of NO by eNOS and a reduction in NO bioavailability [17]. In addition, LDL can upregulate the expression of caveolin protein and stabilize the synthesis of its complexes, resulting in the inhibition of eNOS catalytic activity [18]. Because eNOS is very important for the maintenance of normal endothelial cell structure and function, the upregulation of vascular eNOS expression to promote the generation of NO is a key target in the treatment of atherosclerosis. In the present study, we found that statin treatment slowed the progression of atherosclerotic lesions and upregulated the expression of eNOS protein in ApoE$^{-/-}$ mice. Similarly, an earlier study reported that statins ameliorate atherosclerosis by activating eNOS signaling to protect cell function [19]. In the diabetic mouse group, the expression of eNOS did not increase in the group treated with metformin, but the degree of atherosclerosis was lower than that in the simvastatin-treated group. Therefore, we conclude that the effect of metformin on atherosclerosis in mice with T1DM is not mediated through eNOS.

Previous studies also have shown that metformin may protect against cardiovascular complications of type 2 diabetes by activating AMPK in vascular smooth muscle and inhibiting the signal transducer and activator of transcription (STAT)3 pathway, thereby inhibiting the differentiation of monocytes into macrophages and the production of foam cells, and thus the formation of atherosclerotic lesions [16]. However, in our study no significant expression of AMPK was detected in any of the diabetic groups, suggesting that metformin does not ameliorate atherosclerosis in T1DM by regulating the AMPK pathway.

Metformin can prevent the development of diabetic vascular diseases by inhibiting the formation of advanced glycation end-products (AGEs) during hyperglycemia [20]. AGEs are highly reactive and promote protein cross-linking, which predisposes toward the development of atherosclerosis. A number of animal experiments and clinical studies have shown that aminoguanidine, an AGE inhibitor, has antioxidant and free radical inhibitory effects and reduces the cross-linking induced by AGEs. Metformin has a similar guanidinyl structure to aminoguanidine, and it has been shown that metformin can reduce the tissue concentrations of AGEs in animal models of type 2 diabetes mellitus [21]. In addition, clinical studies have shown that metformin can significantly reduce the concentration of methylglyoxal and its terminal product, methylglyoxal 5-hydro-5-methylimidazolone (MG-H1) and CEL in type 2 diabetes patients [18, 22]. CEL and CML are AGEs that are generated by protein glycosylation. It has also been shown that MG-H1 is a marker of early atherosclerosis in childhood diabetes mellitus [23]. A special type of condensate [triazepinone (TZP]) has been found in the plasma of patients with type 2 diabetes who have been treated with metformin, and its concentration positively correlates with the dose of metformin administered and negatively correlates with the circulating concentration of methylglyoxal. In vitro studies have also shown that the guanidine of metformin combines with the alpha dicarbonyl of methylglyoxal to produce TZP, which can then remove methylglyoxal [24] and thus reduce AGE concentrations, thereby protecting against cardiovascular damage [25]. In the present study, the concentrations of CML and CEL in the metformin-treated diabetic group were lower than those in the other diabetic groups, but still higher than those in the non-diabetic groups. Thus, metformin may achieve its anti-atherosclerotic effect by clearing the methylglyoxal and reducing the production of AGEs.

CONCLUSIONS

We have successfully established a model of T1DM and atherosclerosis and found that metformin can limit the development of atherosclerosis in young diabetic mice. This may be achieved by removing methylglyoxal, thereby reducing the formation of advanced glycation endproducts, rather than by lowering the blood glucose level.

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Authorship Contributions. Wei Song designed the article. Kailin Li established animal model. Guoxin Teng and Linlin Xu researched data for the article. Min Si and Jiang Xue contributed to discussion of content. Aihong Liu wrote the article. Shuang Liang prepared the figures. Guimei Li and Jiang Xue reviewed the manuscript before submission. All authors reviewed and approved the final manuscript.

Disclosures. Aihong Liu, Kailin Li, Linlin Xu, Min Si, Guoxin Teng, Guimei Li, Jiang Xue, Shuang Liang and Wei Song have nothing to disclose.

Compliance with Ethics Guidelines. The experiments were approved by the ethics committee of the Second Hospital of Shandong University (KYLL-2017(KJ)P-0016) and followed national guidelines for the care and use of animals [9]. All sections of this report adhere to the ARRIVE guidelines for reporting animal research.

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