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Active vitamin D3, 1,25-$(OH)_2D_3$, protects against macrovasculopathy in a rat model of type 2 diabetes mellitus

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ABSTRACT. To investigate the protective effect of the active form of vitamin D3, 1,25-$(OH)_2D_3$, on macrovasculopathy in rats with type 2 diabetes mellitus (T2DM), 8-week-old male Sprague-Dawley rats were randomly divided into control group, T2DM group, and treatment group. The T2DM model was established after 6 weeks by administering an intraperitoneal injection of streptozotocin (30 mg/kg). 1,25-$(OH)_2D_3$ was administered by gavage to rats in the treatment group, and an equal volume of peanut oil was administered to rats in the T2DM group. Fasting plasma glucose (FPG), triglyceride (TG), total cholesterol (TC), high-density lipoprotein (HDL-C) and low-density lipoprotein (LDL-C) cholesterols were measured in all rats. The morphology of the thoracic aorta was examined, and the expression of tumor necrosis factor alpha (TNF-α), endothelin (ET), endothelial nitric oxide synthase

(eNOS), CD54, and CD106 in the thoracic aorta was determined by immunohistochemistry. The expression of FPG, TG, TC, and LDL-C in rats from the T2DM and treatment groups was significantly elevated compared with rats from the control group ($P < 0.05$). Compared with that in control group, the expression of TNF-α, ET, eNOS, and CD106 was significantly upregulated in the T2DM group and the treatment group, while the expression of CD54 was increased only in the T2DM group ($P < 0.05$). Moreover, the levels of TNF-α, CD54, and CD106 in rats from the treatment group were lower than those in the T2DM group ($P < 0.05$). These data suggest that 1,25-$(OH)_2D_3$ may protect the macrovessels from injury in T2DM rats by inhibiting the expression of TNF-α, CD54, and CD106.

Key words: T2DM; Active vitamin D3 [1,25-$(OH)_2D_3$]; TNF-α; Macrovasculopathy; Endothelin

INTRODUCTION

In patients with type 2 diabetes mellitus (T2DM), macrovasculopathy accounts for 75-80% of deaths (Laugesen et al., 2012). Macrovascular complications in diabetes are mainly represented by atherosclerosis (AS) of larger arteries. With advances in molecular immunology, accumulating evidence indicates that AS represents a chronic inflammatory process (Boiocchi et al., 2012). Some researchers believe that diabetes is not only a hyperglycemic disease, but also a vascular and inflammatory disease, which increases the risk of coronary heart disease (Husain et al., 2015).

Tumor necrosis factor alpha (TNF-α) is involved in the inflammatory response, and is able to reduce insulin sensitivity, resulting in the development of insulin resistance (Paquot and Tappy, 2005). As an inflammatory factor, TNF-α can act directly on the vascular endothelium to promote thrombosis and the formation of AS plaques. Endothelin (ET) is a potent vasoconstrictor peptide, which promotes the proliferation of smooth muscle cells (Dschietzig, 2014). The synthesis and release of ET is enhanced in response to endothelial injury (Liu et al., 2014; Zhao et al., 2015), and the production of ET by endothelial cells can promote the development of AS (Broder et al., 2013). Nitric oxide (NO) is a vasodilator and endothelial nitric oxide synthase (eNOS) represents the rate-limiting enzyme in the synthesis of NO by endothelial cells (Li et al., 2015). Hyperglycemia can induce the upregulation of eNOS, which in turn enhances NO production (Romero et al., 2012). T2DM is often accompanied by hyperglycemia, hyperlipidemia, and hyperinsulinemia, which all promote oxidative stress in the body and elevated production of free radicals that can damage endothelial cells. After endothelial injury, adhesion molecules such as CD54 and CD106 are upregulated, promoting adhesion of the inflammatory cells to the endothelium to form AS plaques (Shi et al., 2014). This is the key mechanism for the initiation and development of vascular diseases.

Recent studies have demonstrated that the active form of vitamin D3, 1,25-$(OH)_2D_3$, can protect pancreatic β cells, maintain normal insulin secretion and thus normal glucose tolerance (Cândido and Bressan, 2014). In addition, 1,25-$(OH)_2D_3$ can reduce inflammation (Abu El Maaty et al., 2013), modulate the immune system (Yang et al., 2015), and protect

the arterial endothelium thus reducing the level of injury (Dong et al., 2014). This study aimed to analyze the effect of 1,25-$(OH)_2D_3$ on the expression of TNF-α, ET, eNOS, CD54, and CD106 in the arterial endothelium of T2DM rats, in order to investigate the protective effect and the underlying mechanism of 1,25-$(OH)_2D_3$ against macrovasculopathy, to provide theoretical evidence for effective therapeutic approaches in the prevention and mitigation of macrovasculopathy in diabetes.

MATERIAL AND METHODS

Materials

Experimental animals

Thirty-five 8-week-old male Sprague-Dawley rats (purchased from the animal center of Xinjiang Medical University), weighing 180-220 g, were randomly divided into a control group (N = 7), a T2DM group (N = 14), and a treatment group (T2DM + 1,25-$(OH)_2D_3$, N = 14). The experiment was approved by the Animal Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (Approval No. IACUC-20140424017).

Main reagents

Streptozotocin (Sigma-Aldrich, USA), calcitriol (Roche, Shanghai, China), monoclonal antibodies against TNF-α, ET, eNOS, CD54, and CD106 (Boster, Wuhan, China), PV-9000 Polymer Detection System for Immuno-Histological Staining, DAB kit (ZSGB-Bio, Beijing, China), and full-automatic biochemical analyzer (ABX, France) were used in this experiment.

Methods

The experimental rats were housed in standard cages with a 12-h light/dark cycle, and free access to food and water. The rats in the control group were fed a normal diet, and the rats in the T2DM and treatment groups were fed a high-fat and high-sugar diet [10% (w/w) lard, 20% sucrose, 2% cholesterol, 67% normal feed, and 1% pig bile salt]. The 1,25$(OH)_2D_3$ was dissolved in 0.05 mL peanut oil and administered by gavage to rats in the treatment group at a dose of 0.03 mg kg^{-1} day^{-1}. An equal volume of peanut oil was administered by gavage to rats in the T2DM group. Six weeks later, rats were fasted for 12 h. Next, a blood sample was collected from the tail for the fasting plasma glucose (FPG) test, followed by intraperitoneal injection of streptozotocin (dissolved in citrate buffer, pH 4.5) in the T2DM and treatment groups, at a dose of 30 mg/kg, while the control group received an intraperitoneal injection of an equal volume of citrate buffer. One week after the injection of citrate buffer, FPG was analyzed, and the rats were subjected to a glucose tolerance test to examine 2-h plasma glucose (2hPG). Subsequently, glucose was administered by gavage at a dose of 2 g/kg, and blood samples were collected 120 min later for the plasma glucose test. The rats with FPG ≥ 7.0 mM and/or 2hPG ≥ 11.1 mM were selected as the successful T2DM and treatment models, respectively (N = 7 for the control group, N = 10 for the T2DM group, and N = 13 for the treatment group).

Tests and methods

Hematologic parameters

Levels of FPG, total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL-C), and (LDL-C) cholesterols in the rats were analyzed. The rats were anesthetized, and a blood sample of 4-6 mL was collected from the abdominal aorta of each animal. The blood was centrifuged, and the plasma collected to determine the levels of each parameter using an automatic biochemical analyzer.

Pathomorphological examination

Rats were sacrificed, and the thoracic aorta was immediately removed. A vessel fragment of approximately 0.5 cm was cut (at the same position on the thoracic aorta of each rat), fixed in 40 g/L formaldehyde buffer, embedded in paraffin, transversely sectioned into 4-mm thick successive slices, and subjected to hematoxylin-eosin (H&E) staining to examine pathomorphological changes.

Immunohistochemistry

The rat thoracic aorta was cut into 4-5 fragments of the same length, fixed in 40 g/L formaldehyde buffer, and embedded in paraffin. The vessel specimen was sectioned transversely (4 mm in thickness). Three areas on the vessel at 100-mm intervals were selected for sectioning. The sections were subjected to immunohistological staining using a 2-step Power Vision (PV) method to detect the levels of TNF-α, ET, eNOS, CD54, and CD106.

Statistical analysis

Statistical analyses were performed using the SPSS17.0 software. Data are reported as means ± standard deviation (means ± SD). Comparisons between groups were performed using one-way analysis of variance, and $P < 0.05$ was considered statistically significant.

RESULTS

Changes in hematologic parameters

The levels of FPG, TC, TG, and LDL-C in the T2DM groups and treatment groups were higher than those in the control group ($P < 0.05$), while their levels were not significantly different between the T2DM and treatment groups (Table 1). The level of HDL-C was not significantly different among the three groups.

Pathomorphological changes in rat thoracic aorta

Microscopic examination revealed that the intima of the thoracic aorta from control rats was smooth, with intact endothelial cells, the cell monolayer tightly adherent to the internal elastic lamina, and with the vascular smooth muscle cells neatly arranged, as found

in a healthy thoracic aorta. On the other hand, the intima of the thoracic aorta in the T2DM group was disrupted, with local focal roughness, a disrupted endothelium with infiltration of mononuclear cells, and disorganized vascular smooth muscle cells. The lesions in the thoracic aorta of rats from the treatment group were similar to those observed in the rats from the T2DM group, although to a lesser extent. The endothelium of rats from the treatment group was relatively intact and smooth with attenuated damage compared with that of rats from the T2DM group. The adherence and infiltration of mononuclear cells in the endothelium were reduced, and the thickness of the intima was remarkably reduced in the treatment group compared with the T2DM group (Figure 1).

Table 1. Hematologic parameters (mM) in the three groups (means ± SD).

	Control (N = 7)	T2DM (N = 10)	Treatment (N = 13)	*F* value	P value
FBG	5.326 ± 1.072	17.391 ± 1.942*	15.592 ± 2.473*	79.582	0.000
TC	4.635 ± 1.252	18.082 ± 1.861*	17.593 ± 1.414*	194.924	0.000
TG	1.251 ± 0.760	10.154 ± 1.382*	8.922 ± 1.494*	105.298	0.000
HDL-C	2.000 ± 0.521	1.944 ± 0.601	2.540 ± 1.200	1.481	0.245
LDL-C	1.559 ± 0.250	8.293 ± 1.421*	7.713 ± 1.212*	82.900	0.000

*P < 0.05 compared with the control group.

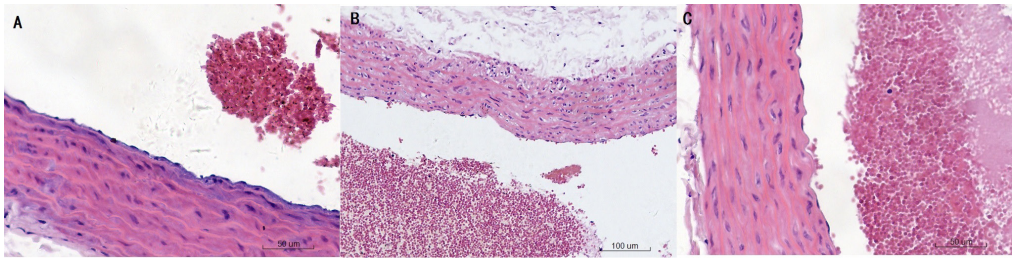

Figure 1. Photomicrographs of retinas from hematoxylin-eosin-stained thoracic aorta of rats from (**A**) control group, (**B**) T2DM **(**type 2 diabetes mellitus **)** group, and (**C**) treatment group.

Immunohistochemical changes in rat thoracic aorta

Immunohistochemical staining for TNF-α, ET, eNOS, CD54, and CD106 revealed that these proteins were mainly expressed in the endothelium of the thoracic aorta. The presence of brownish-yellow particles in the cytoplasm or nucleus was considered as positive staining. The expression of TNF-α, ET, eNOS, and CD106 was increased in the T2DM and treatment groups compared to that in the control group ($P < 0.05$), while the expression of CD54 in the T2DM group was higher than that in the control group ($P < 0.05$). The expression of TNF-α, CD54, and CD106 was significantly reduced in the treatment group compared with that in the T2DM group ($P < 0.05$; Table 2, Figures 2, 3, 4, 5, and 6).

Table 2. TNF-α, ET, eNOS, CD54, and CD106 expression in rat thoracic aorta (means ± SD, number/2*2 mm^2).

	Control (N = 7)	T2DM (N = 10)	Treatment (N = 13)	*F* value	P value
TNF-α	4.68 ± 1.53	11.60 ± 2.93*	8.14 ± 1.54*$^{\Delta}$	14.50	0.00
ET	6.88 ± 1.24	11.1 ± 6.12*	9.57 ± 2.36*	9.236	0.22
eNOS	3.94 ± 1.63	9.56 ± 2.21*	8.46 ± 1.59*	59.77	0.00
CD54	6.0 ± 1.74	8.94 ± 1.56*	6.49 ± 1.97$^{\Delta}$	3.977	0.43
CD106	4.4 ± 1.24	9.96 ± 1.45*	7.77 ± 2.13*$^{\Delta}$	13.15	0.01

*P < 0.05 compared with the control group; ^{D}P < 0.05 compared with the T2DM group. TNF-α (tumor necrosis factor alpha), ET (endothelin), eNOS (endothelial nitric oxide synthase), T2DM (type 2 diabetes mellitus).

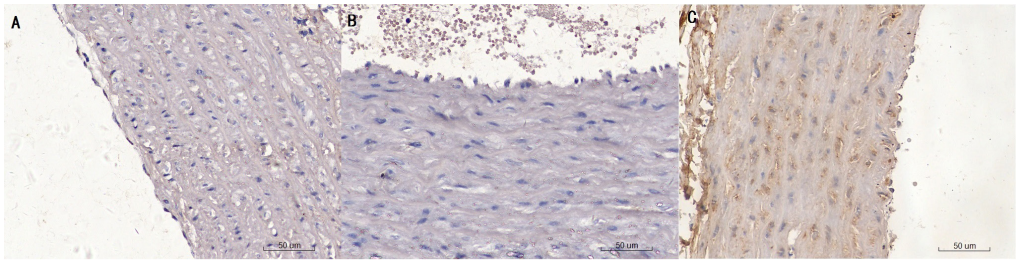

Figure 2. TNF-α (tumor necrosis factor alpha) expression in the thoracic aorta of rats from (**A**) control group, (**B**) T2DM (type 2 diabetes mellitus) group, and (**C**) treatment group. (Immunohistological staining, 400X).

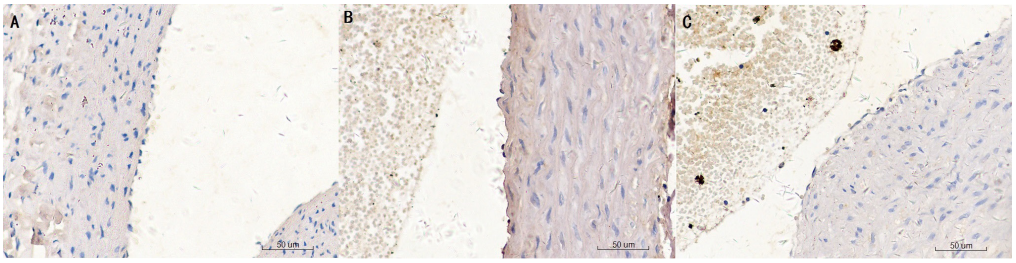

Figure 3. ET (endothelin) expression in the thoracic aorta from rats in (**A**) control group, (**B**) T2DM **(**type 2 diabetes mellitus**)** group, and (**C**) treatment group. (Immunohistological staining, 400X).

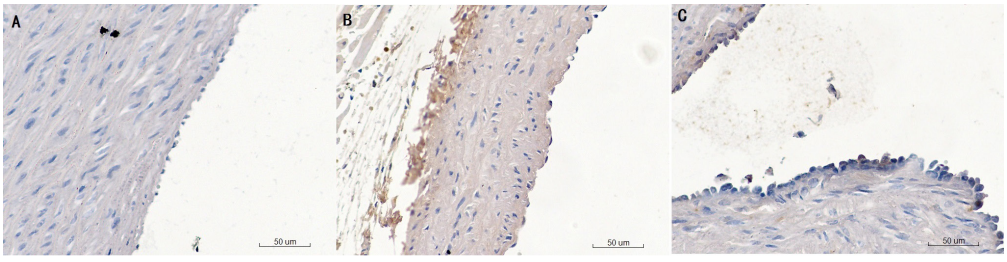

Figure 4. eNOS (endothelial nitric oxide synthase) expression in the thoracic aorta of rats in (**A**) control group, (**B**) T2DM **(**type 2 diabetes mellitus **)** group, and (**C**) treatment group. (Immunohistological staining, 400X).

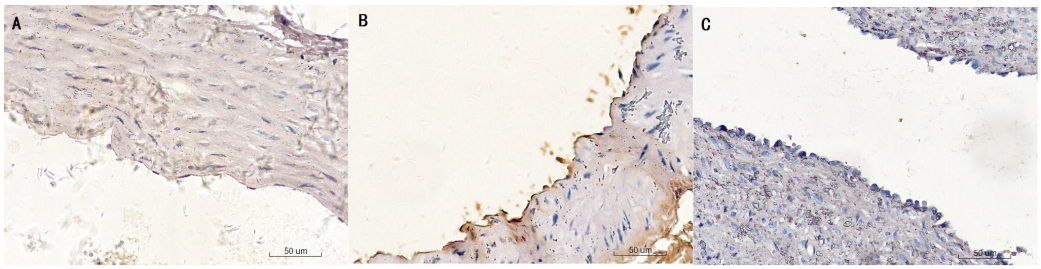

Figure 5. CD54 expression in the thoracic aorta of rats in (**A**) control group, (**B**) T2DM **(**type 2 diabetes mellitus**)** group, and (**C**) treatment group. (Immunohistological staining, 400X).

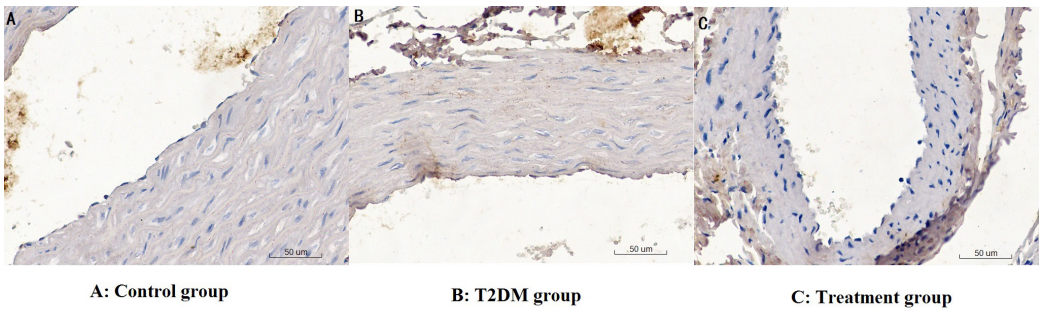

Figure 6. CD106 expression in the thoracic aorta of rats in (**A**) control group, (**B**) T2DM **(**type 2 diabetes mellitus) group, and (**C**) treatment group. (Immunohistological staining, 400X).

DISCUSSION

The results of recent studies have indicated that multiple and complex factors have important roles in the morphological changes associated with diabetes-associated macrovasculopathy (Rahman et al., 2007). However, the protective effect and mechanism of action of 1,25-$(OH)_2D_3$ in the pathogenesis of T2DM have not been reported. In this study, a rat model of T2DM was created to simulate the clinical characteristics of T2DM in humans. The T2DM rats were treated with 1,25-$(OH)_2D_3$ in order to investigate the protective effect of 1,25-$(OH)_2D_3$ on macrovessels in this model.

The results of our study indicated that serum levels of FPG, TC, TG, and LDL-C in rats from both the T2DM group and the treatment group were higher than in those of the control group. However, there was no significant difference in levels of these variables between the T2DM group and the treatment group. Considering that the rats in the T2DM and treatment groups were both on a high-fat, high-sugar diet during model development, while the rats in the control group were fed a normal diet, these dietary differences may explain the changes in levels of FPG, TC, TG, and LDL-C. These results also suggest that the protective effect of 1,25-$(OH)_2D_3$ on macrovessels in diabetic rats is independent of the reduction in blood sugar, lipids, or glycosylation products.

Pathomorphological examination showed that the intima of the thoracic aorta in the T2DM rats was rough, accompanied by shedding of endothelial cells, disorganized vascular smooth muscle cells, and infiltration of mononuclear cells. These results indicate an overall morphological change in the thoracic aorta of T2DM rats, which may have resulted from endothelial inflammation and lipid deposition in the vessels. When compared with the T2DM rats, the rats supplemented with 1,25-$(OH)_2D_3$ displayed attenuated injury to the thoracic aorta, implying that 1,25-$(OH)_2D_3$ could reduce inflammatory responses and, to some extent, alleviate aortic endothelial injury in diabetic macrovasculopathy.

TNF-α ET, eNOS, and CD106 were upregulated in the thoracic aorta of rats in both the T2DM and treatment groups compared to the control group. TNF-α has been shown to reduce insulin sensitivity, resulting in insulin resistance (Zhou et al., 2014). Moreover, as a pro-inflammatory cytokine, TNF-α is involved in the formation of atherosclerotic plaques. Thus, elevated levels of TNF-α suggest that the pathogenesis of AS might be associated with insulin resistance and endothelial inflammation. Upregulation of ET suggests that endothelial injury and abnormalities in hemodynamics may be involved in T2DM-associated macrovasculopathy. Injury to the vascular endothelium directly affects the selective permeability and anti-AS properties of the endothelium, and also interferes with the regulation of vascular tone and leukocyte adhesion (Púzserová et al., 2008). In addition, the upregulation of CD106 indicates the presence of inflammation and adhesion of inflammatory cells onto the vascular endothelium in T2DM-associated macrovasculopathy, thus leading to endothelial injury. In addition, CD106 is known to mediate the adhesion of lymphocytes and other leukocytes to endothelial cells (Yang et al., 2013), suggesting an important role for inflammatory cells, inflammation, and endothelial dysfunction in diabetic macrovasculopathy.

Compared with the T2DM rats, the expression of TNF-α, ET, and CD106 was significantly downregulated in the 1,25-$(OH)_2D_3$-supplemented animals. These results suggest that the protective effect of 1,25-$(OH)_2D_3$ against diabetic macrovasculopathy could depend, in part, on the amelioration of insulin resistance, attenuation of endothelial inflammation, and reduced leukocyte adhesion to the endothelium. In addition, downregulation of CD54 expression suggests that 1,25-$(OH)_2D_3$ is able to downregulate functional antigens on inflammatory cells to suppress their adhesion and infiltration, and can also inhibit the interaction between inflammatory factors and cellulose to reduce inflammation and alleviate endothelial injury via immunoregulation.

In conclusion, expression of TNF-α, CD54, and CD106 was upregulated in a rat model of T2DM revealing that 1,25-$(OH)_2D_3$ could attenuate changes in the expression of these proteins to protect vessels against macrovasculopathy in T2DM. 1,25-$(OH)_2D_3$ was protective against macrovasculopathy in diabetic rats by reducing insulin resistance, attenuating endothelial inflammation, and modulating immune activities.

Conflicts of interest

The authors declare no conflict of interest.

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