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Effects of high glucose on expression of OPG and RANKL in rat aortic vascular smooth muscle cells

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ABSTRACT

Objective: To explore effect of high glucose on expression of osteoprotegerin (OPG) and receptor activator of NF- κ B ligand (RANKL) in rat aortic vascular smooth muscle cells. **Methods:** SD rats were intraperitoneally injected with streptozotocin, OPG and RANKL expression in rat thoracic aortas were detected by immunohistochemical staining. In cultured vascular smooth muscle cells (VSMCs) (A7r5), qRT-PCR and Western blot analysis were used to examine the mRNA and protein levels of OPG and RANKL. **Results:** Our results demonstrated that OPG expression was increased in hyperglycemic rat aortic VSMCs, while RANKL expression was decreased. Besides, *in vitro* experiments high glucose induced OPG expression, but depressed RANKL expression by dose- and time-dependent manner in cultured A7r5. **Conclusions:** Our findings suggested that high glucose could promote the expression of OPG, and inhibit the expression of RANKL in VSMCs, which may be partly be the molecular mechanism of diabetic vascular calcification.

1. Introduction

At present, the vascular calcification is considered to be a serious cardiovascular complication that contributes to the increased morbidity and mortality of patients suffered from diabetes mellitus[1,2]. Diabetic vascular calcification can occur in both the medial and intimal layers of arterial beds[3]. Vascular calcification is an active process affected by many factors and regulated by mutiple genes, which is similar to the physiological mineralization of skeletal tissues, and vascular smooth muscle cells (VSMCS) differentiate into osteoblast like cells is key[4]. A number of factors were reported to regulate differentiation of multiple cell types among bone and blood vessels, and bone metabolism-related factors participate in vascular calcification[5]. osteoprotegerin (OPG)/ receptor activator of NF- κ B ligand (RANKL)/receptor activator of NF- κ B ligand (RANK) system is the recently emerging area that links between the skeletal, immune and vascular biology[6]. Clinical datas have indicated that serum OPG levels is associated with vascular calcification in humans, and determination of serum OPG has been suggested as a prognostic biomarker of cardiovascular disease[7].

In the present investigation, we sought to explore the role of OPG

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and RANKL expression in the VSMCs in hyperglycemia (blood glucose levels >16.6 mmol/L) or high glucose (HG, 25 mmol/L D-glucose) conditions. As results, we found that the up-regulation of OPG and down-regulation of RANKL were detected in VSMCs in streptozotocin (STZ)-induced T1D rat thorocitic arties *ex vivo* and HG-treated VSMCs *in vitro*. Our studies will provide the improved understanding of the mechanism behind diabetic vascular calcification.

2. Materials and methods

2.1. Cell line and regents

Rat aortic smooth muscle cells (A7r5) were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Rabbit anti-rat OPG and mouse-anti rat RANKL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); STZ, D-glucose and D-mannitol were from Sigma Aldrich (Sigma Aldrich, St Louis, MO, USA).

2.2. Establishing rat model of T1D

Male Sprague Dawley (SD) rats, weighing (200±20) g, were purchased from the Center of Experimental Animals of Hainan Medical College, Haikou, China. All procedures were performed in accordance with the Guidelines of the Hainan Council of Animal Care and approved by the Animal Use Subcommittee at the Hainan Medical College. Rats were made type 1 diabetes (T1D) by single intraperitoneally injected STZ (55 mg/kg, Sigma-Aldrich, Shanghai, China). Blood glucose was monitored for up to 3 weeks, and only the rats with blood glucose levels >16.6 mmol/L were involved in the study. At the end of 3 weeks after injection of STZ, rats were sacrificed and the thoracic arterial samples were fixed in neutral formalin for paraffin-embedded sections.

2.3. Immunohistochemistry (IHC)

Immunohistochemistrical staining was performed on the sections of the rat thoracic arterial samples. The process of IHC was performed as stranded protocols. Briefly, After the paraffin sections (5 μm) were deparaffinized and re-hydrated, heat-induced antigen retrieval was performed in sodium citrate buffer (pH 6.0, 10 mM) and endogenous peroxidases were blocked by incubation in 0.3% H_2O_2. Sections then were incubated with primary antibodies against OPG (1:100) and RANKL (1:100) at 4 ℃ overnight and non-immune IgG was used as negative control. Antigenic sites were localized using a SP9000 or SP9003 kit and 3,3'-diaminobenzidine kit (ZSGB-BIO, Beijing, China). Samples with a brown color staining in VSMCs were considered to display positive expression.

2.4. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA). 1 μg of total RNA was used as template to generate the cDNA by oligo(dT18) using Fermentas RT System (cat.#K1622, Thermo Scientific, Guangzhou, China). The pairs of primers (5'-3') synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) as following, OPG forward TTGGCTGAGTGTTCTGGT, reverse TTGGGAAAGTGGTATGCT; RANKL forward CATCGGGTTCCCATAAAG, reverse GAAGCAAATGTTGGCGTA; β-actin forward CTATCGGCAATGAGCGGTTCC, reverse TGTGTTGGCATAGAGGTCTTTACG. PCR was conducted using the LightCycler480 II instrument (Roche, China) Ltd., Guangzhou, China). The total volume was 20 μL, which include 10 μL SYBR Green I PCR Master Mix (TOYOBO, OSAKA, Japan), 0.4 μL forward primer (10 μM), 0.4 μL reverse primer (10 μM), 2 μL cDNA and 7.2 μL ddH_2O. The PCR amplification was as follow, after denaturation at 95 ℃ for 1 min, 45 PCR cycles were performed including 95 ℃ for 15 s, 60 ℃ for 60 s. The relative abundance of target mRNAs were determined from the CT values and plotted as the fold change compared with the control groups.

2.5. Western blotting

Cells were collected and lysed with RIPA lyses buffer (Cat. # P0013C, Beyotime Institute of Biotechnology, Jiangsu, China), and then proteins were quantified using an Enhanced BCA Protein Assay Kit (Cat. #P0010, Beyotime Institute of Biotechnology). Total of 40-50 μg proteins were transferred into PVDF membranes by electrophoretic transfer following electrophoretic separation by SDS - polyacrylamide gel electrophoresis (SDS-PAGE). The membranes were probed with primary antibodies against OPG (1:400, Santa Cruz), RANKL (1:400, Santa Cruz), and β-actin (1:1 000, Santa Cruz) in TBST plus 3% skimmed milk overnight at 4 ℃. After three times washed by TBST, the membranes were incubated with horseradish peroxidase (HRP)-coupled secondary antibodies for 2 hour at room temperature. Bands were visualized using enhanced chemiluminescence (ECL) reagents (Thermo Fisher, Rockford, IL, USA) and analyzed with a BIO-RAD VersaDocTM 5000 MP system (Life Science Research, Hercules, CA, USA). Bands of β-actin were used as loading controls.

2.6. Statistical analyses

The statistical analyses were carried out by using PRISM Software (GraphPad Software, CA, USA). The data were expressed as Mean ±SD. For analysis of differences between two groups, Student's *t*-test was performed. For multiple groups, ANOVA was carried out followed by Student-Newman-Keuls test. The level of statistical significance was set at $P<0.05$.

3. Results

3.1. Up-regulation of OPG and down-regulation of RANKL expression in thoracic arterial samples of streptozotocin-induced T1D rats

The rats with blood glucose levels>16.6 mmol/L were considered as the hyperglycemia after intraperitoneally injected with STZ. Results of immunohistochemistry showed that an obvous increased expression of OPG and decreased expression of RANKL was detected in VSMCs of thoracic arterial samples of hyperglycemic rats when compared with that of normoglycemic rats (Figure 1). These results indicated that 3-week of hyperglycemia would significantly affect OPG and RANKL expression in VSMCs.

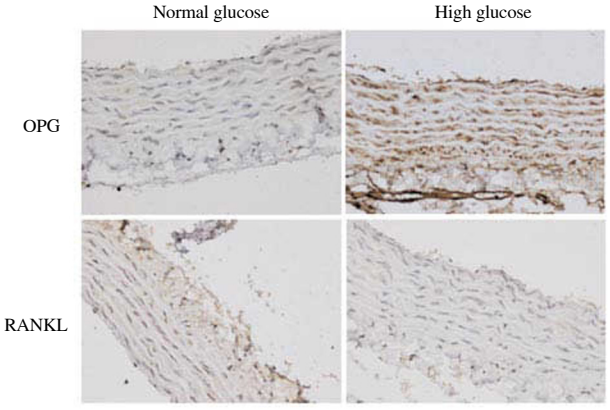

Figure 1. Expression of OPG and RANKL in tunica media of thoracic arties of STZ-induced T1DM (hyperglycemia) rats and normal control (normorglycemia) rats.
Representative photographs of OPG and RANKL protein expression in samples of rat thoracic arties. IHC was used to detect OPG and RANKL protein expression in tunica media of thoracic arties. For the process of IHC, antigens were localized by DAB, tissues were countstained with hematoxylin. Non-immune IgGs were used as negative control. Original magnification: 200×, Bar = 100 μ m.

3.2. Up-regulation of OPG and down-regulation of RANKL in HG-treated cultured VSMCs in vitro

We then explored the role of HG in the OPG and RANKL expression in cultured VSMCs *in vitro*. The VSMCs were treated in NG (normal glucose control), Mtol (osmotic control) and HG medium, 48 hours later, cells were used for analysis the OPG and RANKL expression. Results of qRT-PCR and western blotting showed that HG treatment led to obvious OPG up-regulation and RANKL down-regulation (P<0.01, Figure 2A, B) when compared with NG and Mtol groups, this results indicated that up-regulation of OPG and down-regulation of RANKL was HG-dependent.

3.3. HG influenced OPG and RANKL expression in VSMCs at time-consumed manner

Consequently, VSMCs were treated with HG (25 mmol/L)for 4, 8, 12, 24 and 48 hours, and the results showed that with the extension of time OPG expression was gradually increased both at mRNA and protein level, on the contrary, RANKL expression was gradually decreased (Figure 3A, B), which indicated that HG influenced OPG and RANKL expression in VSMCs by a time-dependent manner.

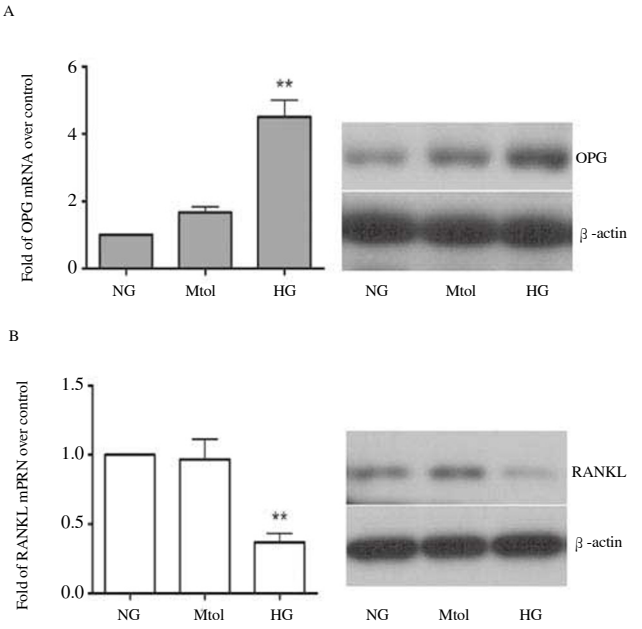

Figure 2. HG induced OPG expression and suppressed RANKL expression in VSMCs *in vitro*.
QRT-PCR and western blotting were used to detect OPG (A) and RANKL (B) expression in VSMCs maintained in media supplemented with normal glucose (NG, 5.5 mmol/L D-glucose), Mitol (Mtol, 25 mmol/L D-mannitol) and high glucose (HG, 25 mmol/L D-glucose) for 48 h. ** P<0.01 *vs.* NG or Mtol.

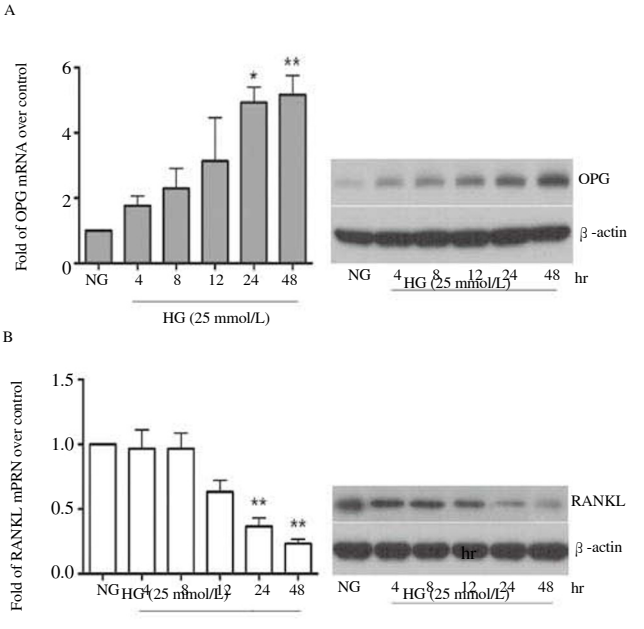

Figure 3. HG influenced OPG and RANKL expression in VSMCs *in vitro*.
qRT-PCR and western blotting were used to detect OPG (A) and RANKL (B) expression in VSMCs maintained NG (5.5 mmol/L D-glucose, 24 hr) and HG (25 mmol/L D-glucose, 4, 8, 12, 24, 48 hr), * P<0.05 *vs.* NG. ** P<0.01 *vs.* NG.

3.4. HG influenced OPG and RANKL expression in VSMCs at concerntration-dependent manner

VSMCs were further treated with HG(48 hours) for 5.5, 12.5, 25 and 50 mmol/L, as shown in Figure 4A & B, with the increase of HG concertration OPG expression was gradually increased both at mRNA and protein level, howerer, RANKL expression was gradually decreased, which manifested that HG affected OPG and RANKL expression in VSMCs by a concentration-dependent manner.

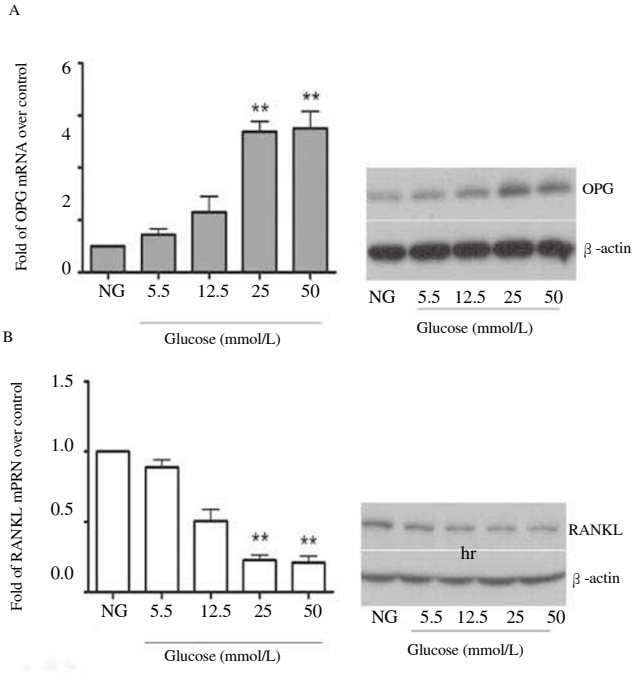

Figure 4. HG influenced OPG and RANKL expression in VSMCs *in vitro*. qRT-PCR and western blotting were used to detect OPG (A) and RANKL (B) expression in VSMCs maintained in media supplemented with various concentration of D-glucose (5.5, 12.5, 25 and 50 mmol/L) for 48 hr, $^{**}P<0.01$ *vs.* 5.5 mmol/L D-glucose (NG).

4. Discussion

Diabetes mellitus (DM) is a chronic disease in which the body either can not use or produce the insulin efficiently[7]. Cardiovascular disease (CVD) remains the leading cause of death among adults with DM, and vascular calcification is a common pathobiological process which correlates with increased morbidity and mortality in patients with diabetes[6]. Diabetes is a condition most always associated with vascular calcification and bone abnormalities[8]. Emerging evidence from the last two decades has shown that vascular calcification is an active and tightly regulated biological process orchestrated by VSMCs adopting an osteoblastic phenotype and that this process bears a lot of similarities to physiological bone mineralization. *In vitro*, Chen *et al* have shown that VSMCs incubated with high concentration of glucose led to an increased expression of the osteoblast transcription factor core binding factor alpha subunit 1 (Cbfa1) and the relative gene expression of ALP and OCN, acceleration the VSMCs calcification[9]. Al-Aly *et al* also found that Ldlr-/- mice fed high fat diet developed hyperglycemia, dyslipidemia and aortic calcification primarily in the tunica media with activation of BMP-2/Msx2-Wnt pathway[10]. Accordingly, the present results suggested that the increased diabetic vascular calcification was partially due to the direct effects of hyperglycemia on the transforming of the VSMCs to osteoblast-like phenotype. However, the mechanism of phenotypic change of VSMCs to osteoblast-like cells VSMCs is not understood well, specifically in diabetic high-glucose conditions.

RANKL, a 316-amino acid transmembrane protein that is highly expressed by T cells in lymphoid tissue and osteoblast /stromal cells in trabecular bone[11-15], binds and activates its RANK, a 616-amino acid transmembrane receptor, which is mainly expressed by osteoclasts and their precusor[16]. RANKL actions are inhibited by OPG, a member of a tumor necrosis factor (TNF) superfamily of proteins that is secreted by osteoblasts. OPG competes with RANK and functions as a decoy receptor to bind to RANKL with high affinity for prevention RANKL/RANK interactions[17,18]. The signaling pathway of RANK/RANKL / OPG has been suggested as the link between vascular and bone metabolism[19,20]. OPG-/- mice not only exhibited a decrease in total bone density but also developed the medial calcification of renal and aortic arteries[21]. Recent outcome studies have indicated that plasma OPG is a strong predictor of cardiovascular disease[22-24]. The cellular source of OPG system in vasculature is mainly ascribe to the endothelia cells[25] and VSMCs[26,27]. Of much interest, in this study we observed an up-regulation of OPG and down-regulation of RANKL expression in the samples both the thoracic VSMCs of STZ-induced T1D and HG-treated VSMCs. Moreover, the results of the experiments conducted *in vitro* revealed that HG induced the increased expression of OPG and decreased expression of RANKL in not only glucose concentration - dependent but also time - consumption manners.

Although complex mechanisms are involved in the initiation and progression of vascular calcification, a central part of which is the phenotypic transition of VSMCs to osteoblast-like cells. The present study demonstrated that hyperglycemia/HG treatment could induce OPG expression and supress RANKL expression, suggesting that RANK/RANKL/OPG system plays a pivotal role in HG-stimulated VSMCs and providing an understanding of molecular and celluar mechanism involved in diabetic vascular calcification.

Conflict of interest statement

We declare that we have no conflict of interest.

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