Protective effect of endoplasmic reticulum stress on calcification of VSMCs induced by high glucose and its effect on expression of GRP78, CHOP, and Caspase-12

Jingping Xu,1 Dawei Wu,2 Xiaoli Li,3 Haiying Zhu,1 Xu Teng4 and Peiying Xu5

Abstract
This article investigates the role of endoplasmic reticulum stress (ERS) in high-glucose-induced vascular smooth muscle cells (VSMCs) calcification and to explore its effects on the expression of GRP78, CHOP, and Caspase-12. VSMCs were treated with high glucose (35 mmol/L) to induce diabetes to see whether high glucose can induce ERS. Changes in alkaline phosphatase activity, calcium deposition, and runt-related transcription factor 2 (Runx2) were measured. After 5 days’ treatment, the alkaline phosphatase activity, calcium content, and Runx2 expression of the bone differentiation marker protein were all up-regulated in the β-glycerophosphate group, the high-glucose group, and the β-glycerophosphate + high-glucose group when compared with the control group. High-glucose treatment of VSMCs can cause ERS and apoptosis and induces transdifferentiation of VSMCs into osteoblast-like cells, resulting in increased basal phosphatase activity, up-regulation of calcium content, and bone differentiation markers in VSMCs. The findings confirm that ERS plays an important role in the calcification of VSMCs.

Keywords
diabetes, endoplasmic reticulum stress, vascular smooth muscle cells, Runx2

Date received: 5 November 2018; accepted: 27 May 2019

Introduction
Diabetes is a common metabolic disorder that seriously threatens human health. Cardiovascular disease is the main cause of death in diabetic patients. Diabetes vascular calcification (DVC) is one of the independent complications of diabetes, which is characterized by high incidence and risk. Moreover, it is the main independent risk factor for the high incidence of cardiovascular disease and high mortality in diabetic patients. Vascular calcification is usually divided into intimal and medial calcification; the former is common in atherosclerosis, while...
the latter refers to calcium and phosphorus deposition in the vascular media, most commonly seen in patients with type 2 diabetes. Traditionally, calcium and phosphorus deposition is a passive degenerative pathological change. At present, most scholars believe that vascular calcification is an active process that is tightly regulated, often accompanied by the characteristics of embryonic skeletal development, that is, vascular smooth muscle cells (VSMCs) are transdifferentiated into osteoblast-like cells. VSMCs play an important role in vascular remodeling. Heterogeneity and phenotypic changes in smooth muscle cells (SMCs) are usually accompanied by a morphological difference, that is, elongated/spindle-like versus spread-out or epithelioid/rhomboid cell shapes. Compared to spread-out SMCs on nonpatterned surfaces, SMCs on micropatterned surfaces demonstrated elongated morphology, significantly lower cell and nucleus shape indexes, less spreading, a lower proliferation rate, and a similar response (but to a lesser extent) to platelet-derived growth factor, transforming growth factor-β and mechanical stretching. It has been reported that high glucose may cause VSMC calcification, which can cause endoplasmic reticulum stress (ERS) and apoptosis. Besides, some scholars believe that high-glucose-induced vascular calcification may be an active pathology in which vascular wall cells and the extracellular matrix work together. The process is similar to the development of the bone. However, it is unclear whether ERS and apoptosis are involved in high-glucose-induced VSMC calcification. To this end, VSMCs in rats were treated with high glucose in this study to simulate the environment of diabetes in the body, and explore the role and mechanism of ERS in calcification of VSMCs induced by high glucose.

Methods

Cell culture and experimental grouping

VSMCs of the rats were provided by the Shanghai Cell Bank of the Chinese Academy of Sciences, cultured in DMEM containing 10% FBS, and then incubated in an incubator at 5% CO₂ at 37°C. The experiment was divided into six groups: the control group, high-glucose group, β-glycerophosphate group, β-glycerophosphate + high-glucose group, 4-PBA pretreatment + high-glucose group (VSMCs were treated with 500 μmol/L 4-PBA for 30 min, then removed, washed twice with PBS, and then treated with 35 mmol/L D-glucose for 24 h), and 4-PBA group (VSMCs were treated with 500 μmol/L 4-PBA for 30 min).

Determination of calcium content

The above six groups of VSMCs were washed with PBS thrice after different treatments, and decalcified by adding 0.6 mol/L hydrochloric acid per well at 37°C for 1 h. Then the calcium content in the supernatant was determined by the methyl thymol blue colorimetric assay kit. The remaining cells were washed thrice with PBS, and then the cells were lysed by cell lysis buffer and then the cells were lysed by cell lysis buffer for 30 min to completely lyse and extract the cytoplasmic protein. The calcium content in the supernatant divided by the cellular protein content is the calcium content.

Determination of alkaline phosphatase activity

VSMCs of the above groups were inoculated into 12-well culture plates after different treatments. Then the culture medium was discarded, and the cells were washed twice with PBS. Thereafter, 1 mL of 1% TritonX physiological saline was added thereto and stored at 4°C for 24 h. Consequently, the cells were fully lysed with ultrasonic treatment for 20 s, and cell disruption was observed under an inverted microscope. Then, it was centrifuged at 10 cm, 12,000 r/min for 10 min, and the alkaline phosphatase activity of the supernatant was determined by an alkaline phosphatase assay kit (phenylidiphenyl phosphate method). Alkaline phosphatase activity is expressed as IU of the supernatant alkaline phosphatase per gram of cellular protein.

Materials and methods

Materials

D-glucose, 4-PBA, and Hoechst 33258 were purchased from Sigma-Aldrich, USA. The alkaline phosphatase assay kit was purchased from Beijing Keruimei Technology Co., Ltd. DMEM and fetal bovine serum (FBS) were purchased from Gibco, USA. Antibodies against GRP78, CHOP, Caspase-12, Runx2, and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were all purchased from Hangzhou Gino Biosystems Co., Ltd.
The expression of protein determined by Western blot

The VSMCs were inoculated into 60-mm culture dishes. After each group was grown to 80% density, the cells were washed with cold PBS after different treatments. Then the cells were stored at 4°C for 0.5 h after adding the lysis buffer, followed by centrifugation at 12,000 r/min for 10 min, and the supernatant was taken and stored in a cryogenic refrigerator for reserve. After the protein was quantified by the bicinchoninic acid (BCA) method, the total protein was electro-transferred onto the polyvinylidene fluoride (PVDF) membrane by SDS-PAGE electrophoresis. After that, it was sealed for 60 min with 5% skim milk, then added with an appropriate ratio of primary antibody, and placed in a shaker at 4°C overnight. The membrane was washed with TBST thrice, 5 min/times. The PVDF membrane was colored with electrochemiluminescence (ECL) luminescent liquid, exposed to an X-ray film in a dark room for scanning, and the gray level was quantified using Imag J. This was repeated five times for each experiment.

Statistical method

Statistical analysis was performed using the SPSS 21.0 software. The measurement data were expressed as mean ± standard deviation (±s). One-way analysis of variance (ANOVA) was used for comparison between the groups, while Student–Newman–Keuls (SNK) test was used for pairwise comparison of the mean value. The test level α was set to 0.05.

Ethical consideration

This study was approved from the institutional ethical review board of The First People’s Hospital of Qinhuangdao, Qinhuangdao, Hebei, China. All the experiments were conducted as per research ethics. The reference No. is 009/ERB/2017.

Results

Induction of VSMCs into osteoblast-like cells

After treatment with VSMCs for 5 days, the alkaline phosphatase activity, calcium content, and Runx2 expression of the bone differentiation marker protein were all up-regulated in the β-glycerophosphate group, the high-glucose group, and the β-glycerophosphate + high-glucose group when compared with the control group (as shown in Figure 1(a)–(d)), suggesting that high glucose can induce VSMCs to differentiate from a contractile phenotype to an osteogenic phenotype.

Effects of PBA on ERS and apoptosis in VSMCs

Compared with the high glucose + 4-PBA group, the expression of ERS marker proteins GRP78 and CHOP were decreased in the 4-PBA group; meanwhile, the expression of ERS pathway apoptotic marker protein Caspase-12 had also declined (Figure 2), and the difference was statistically significant (P < 0.05). Taken together, all these results suggested that high glucose can cause ERS and apoptosis in VSMCs, which may mediate the phenotypic transformation of VSMCs.

Role of ERS in calcification of VSMCs

Compared with the high glucose + 4-PBA group, the alkaline phosphatase activity and the calcium content were decreased in the 4-PBA group (Figure 3(a) and (b)), and the difference was statistically significant (P < 0.05).

After treatment with VSMCs for 5 days, the alkaline phosphatase activity, calcium content, and Runx2 expression of the bone differentiation marker protein were all up-regulated in the β-glycerophosphate group, the high-glucose group, and the high glucose + β-glycerophosphate group when compared with the control group. Compared with the high glucose + 4-phenylbutyric acid (4-PBA) group, the expression of ERS marker proteins GRP78 and CHOP were decreased in the 4-PBA group, while the expression of ERS pathway apoptotic marker protein Caspase-12, alkaline phosphatase activity, and calcium content were also decreased, and the difference was statistically significant (P < 0.05). Tips: high-glucose treatment of VSMCs can cause ERS and apoptosis, and can also induce transdifferentiation of VSMCs into osteoblast-like cells, resulting in increased basal phosphatase activity, up-regulation of calcium content, and bone differentiation markers in VSMCs. On the other hand, 4-PBA not only inhibits ERS and apoptosis of VSMCs induced by high glucose, but also blocks the transdifferentiation of VSMCs into osteoblast-like cells.
osteoblast-like cells, which confirms that ERS plays an important role in the calcification of VSMCs caused by high glucose.

**Discussion**

The results of this study demonstrated that high-glucose treatment (simulating the body’s diabetes status) can induce ERS and apoptosis in VSMCs, and molecular chaperone 4-PBA (ERS inhibitor) can block calcification of VSMCs (important process of vascular sclerosis), implying that ERS may play an important role in the mechanism of VSMCs calcification. It is worth noting that 4-PBA down-regulated the ERS marker protein and also reduced VSMCs apoptosis, delaying the transdifferentiation of VSMCs from SMC contraction phenotype to osteogenic phenotype. The high-glucose-induced calcification of VSMCs mediated by ERS was mainly reported in this study. At present, most scholars believe that vascular calcification is an active process that is regulated by embryonic bone development, that is,
VSMCs transdifferentiated into osteoblast-like cells. In addition, high glucose may cause VSMCs calcification, and this process was a similar bone development process in which VSMCs actively participate. Runt-related transcription factor 2 (RUNX2) or subunit alpha-1 (CBF-alpha-1) is a protein that in humans is encoded by the RUNX2 gene. RUNX2 is a key transcription factor associated with osteoblast differentiation. It has also been suggested that Runx2 plays a cell proliferation regulatory role in cell cycle entry and exit in osteoblasts, as well as endothelial cells. It has been depicted in previous studies that a high-glucose environment can induce calcification of VSMCs in vitro. However, the mechanism of high-glucose-induced calcification of VSMCs is still unclear. On the other hand, high glucose and its metabolite glucosamine can cause ERS in VSMCs, which is related to the formation of atherosclerosis. The results of this study and other scholars have reported that the expression of ERS marker protein and related apoptotic proteins (GRP78, CHOP, and Caspase-12) in VSMCs were up-regulated in a high glucose or diabetic environment, while 4-PBA pretreatment can down-regulate their expression and inhibit the occurrence of apoptosis and calcification, which is manifested by the reduction of Caspase-12 expression. These results indicated that ERS and apoptosis can mediate high-glucose-induced vascular calcification. VC is a regulated process mediated by VSMCs, with similarities to developmental osteogenesis. The exact molecular events responsible for triggering it are unknown. The endoplasmic reticulum (ER) is involved in folding of proteins. ER stress signaling regulates osteogenic differentiation and calcification of VSMCs. ER stress modeled in human primary VSMCs in vitro increased their calcification and was shown to modulate the expression of a number of bone-related genes in VSMCs in vitro.

As a conclusion, high-glucose-induced transdifferentiation of VSMCs into osteoblast-like cells, which are characterized by enhanced alkaline phosphatase (ALP) activity, increased calcium content and up-regulation of bone differentiation transcription factor in VSMCs. Meanwhile, high-glucose treatment-induced ERS and increased apoptosis. On the other hand, 4-PBA can inhibit high-glucose-induced ERS of VSMCs, apoptosis, and calcification, confirming that ERS at least partially mediates calcification of VSMCs induced by high glucose.

Declaration of conflicting interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding
The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iD
Jingping Xu https://orcid.org/0000-0002-4541-1036

References
1. Fadini GP, Albiero M, Menegazzo L et al. (2011) Widespread increase in myeloid calcifying cells
contributes to ectopic vascular calcification in type 2 diabetes. *Circulation Research* 108(9): 1112–1121.

2. Harper E, Forde H, Davenport C et al. (2016) Vascular calcification in type-2 diabetes and cardiovascular disease: Integrative roles for OPG, RANKL and TRAIL. *Vascular Pharmacology* 82: 30–40.

3. Ferramosca E, Bellasi A, Ratti C et al. (2005) Ethiopathogenesis, diagnosis and prevention of vascular calcification in end stage renal disease. *Current Medicinal Chemistry. Cardiovascular and Hematological Agents* 3(2): 165–171.

4. Duan XH, Chang JR, Zhang J et al. (2013) Activating transcription factor 4 is involved in endoplasmic reticulum stress-mediated apoptosis contributing to vascular calcification. *Apoptosis: An International Journal on Programmed Cell Death* 18(9): 1132–1144.

5. Duan X and Zhou YX (2009) Endoplasmic reticulum stress-mediated apoptosis is activated in vascular calcification. *Biochemical & Biophysical Research Communications* 387(4): 694–699.

6. Du Y, Wang Y, Wang L et al. (2011) Cartilage oligomeric matrix protein inhibits vascular smooth muscle calcification by interacting with bone morphogenetic protein-2. *Circulation Research* 108(8): 917–928.

7. Mackenzie NCW, Staines KA, Zhu D et al. (2014) miRNA-221 and miRNA-222 synergistically function to promote vascular calcification. *Cell Biochemistry and Function* 32(2): 209–216.

8. Pugliese G, Iacobini C, Blasetti Fantauzzi C et al. (2015) The dark and bright side of atherosclerotic calcification. *Atherosclerosis* 238(2): 220–230.

9. Farrokhi E, GhatrehSamani K, Hashemzadeh Chaleshtori M et al. (2015) Effect of oxidized low density lipoprotein on the expression of Runx2 and SPARC genes in vascular smooth muscle cells. *Iranian Biomedical Journal* 19(3): 160–164.

10. Song Z, Zhao Y, Wang X et al. (2016) Secondary hyperuricemia in chronic renal failure promotes vascular calcification in rats. *Sheng Li Xue Bao [Acta Physiologica Sinica]* 68(6): 709–715.

11. Bo J, Yin HC, Wang LQ et al. (2013) Expression profile of mitogen-activated protein kinase pathway genes in vascular calcification associated with osteogenic differentiation of smooth muscle cells. *Chinese Journal of Tissue Engineering Research* 17(20): 3618–3625.

12. Yan JY, Zhou Q, Yu HM et al. (2015) High glucose promotes vascular smooth muscle cell calcification by activating WNT signaling pathway. *Journal of Southern Medical University* 35(1): 29–33.