LABORATORY STUDY

Effects of Gingko biloba extract (EGb 761) on vascular smooth muscle cell calcification induced by β-glycerophosphate

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ABSTRACT

Objective To investigate the effects of Gingko biloba extract (EGb 761) on calcification induced by β-glycerophosphate in rat aortic vascular smooth muscle cells. Methods Rat aortic vascular smooth muscle cells were cultured with various concentrations of EGb 761 and β-glycerophosphate for 7 days. Calcium content in the cells, alkaline phosphatase activity, cell protein content, NF-κB activation, and reactive oxygen species production were assayed, respectively. Results The calcium depositions of vascular smooth muscle cells of the β-glycerophosphate group were significantly higher than those of the control group (p<0.01), and were inhibited by EGb 761 in a concentration-dependent manner (p<0.05). Data showed β-glycerophosphate induced the enhanced expression of alkaline phosphatase, up-regulated the NF-κB activity and increased reactive oxygen species production of vascular smooth muscle cells while these decreased when administrated with EGb 761 (p<0.05). Conclusions EGb 761 significantly reduced deposition of calcium induced by β-glycerophosphate in rat aortic vascular smooth muscle cells. It not only reduced the deposition of calcium, but also inhibited osteogenic transdifferentiation, which may be associated with decreasing expression of alkaline phosphatase, down-regulating the NF-κB activity, and reducing reactive oxygen species production of vascular smooth muscle cells, and may have the potential to serve as a role for vascular calcification in clinical situations.

Introduction

Vascular calcification (VC), a major risk factor for cardiovascular mortality, particularly in patients with end stage renal disease (ESRD) and diabetes, is now known to be an active and tightly regulated process involved with phenotypic transition of vascular smooth muscle cells (VSMCs) that resembles bone mineralization.3–6 Hyperphosphatemia, manifested during ESRD and subsequent dialysis in which serum inorganic phosphate (Pi) levels typically exceed 2 mmol/L, is highly associated with the extent of VC and contributes directly to high morbidity and mortality in vascular disease.7,8 Several studies have reported that a high Pi level played an important role in mitochondrial reactive oxygen species (ROS) production, phenotypic modulation of VSMC, and induced vascular calcification.9–13

Recently, herbal therapy has been gaining popularity among clinicians due to their beneficial effects with minimum toxicity. The Ginkgo biloba extract (EGb 761, Beaufour Ipsen Industrie, Paris, France) is a standardized extract of dried leaves that contains 24% flavonol glycosides and 6% terpene lactones, such as ginkgolides A, B, C, J, and bilobalide. It has been used in the treatment of various cardiovascular diseases.14–16 Moreover, several studies have been reported the antioxidant, repressing atherosclerosis, and anti-apoptotic properties of EGb 761 and its constituents.15,17–20 Additionally, it can stabilize the changes in mitochondrial membrane potential occurred due to excessive ROS and peroxynitrite generation and help prevent the disruption of ionic homeostasis.21–23 However, the pharmacological potential and mechanism of action of EGb 761 in VC of ESRD is yet to be explored. Therefore, the present study was designed to evaluate the protective effect of EGb 761 on calcification and the expression levels of calcification-associated factors induced by β-glycerophosphate (BGP, Sigma, St. Louis, MO) in rat vascular smooth muscle cells.
Materials and methods

Cell culture of VSMCs

Rat VSMCs were obtained from the tunica media of an adult male Sprague-Dawley rat (Experimental Animal Center of Shandong University, Jinan, China) thoracic aorta using the explant culture method as follows: the rats were anesthetized with 400 mg/kg chloral hydrate and the thoracic aorta was removed under aseptic conditions. The thoracic aorta was cut into 1-2 mm² pieces following the removal of any residual blood. The tissue pieces were cultured in dishes containing Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life Technologies, Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS; Gibco Life Technologies), 100 U/mL penicillin G and 100 μg/mL streptomycin (all from North China Pharmaceutical Limited by Share Ltd., Shijiazhuang, China) in a 5% CO₂ incubator at 37 °C. Cells that migrated from explants were collected when confluent. The cells were maintained in DMEM supplemented with 15% FBS, and the medium was replaced twice per week. VSMCs were identified by a positive staining of α-smooth muscle actin (Sigma-Aldrich, St. Louis, MO) and used for all the experiments between passages 6-12. The current study was conducted in accordance with the Declaration of Helsinki (2013) and all experimental protocols were approved by the Review Committee for the Use of Animal Subjects of Qilu Hospital of Shandong University (Jinan, China).

Calcification assays

Rat VSMC calcification was induced as previously described.24 Rat VSMCs were randomly divided into a negative control group, high phosphorus, and Egb 761 intervention groups. A negative control group using low glucose DMEM medium containing 10% FBS and a high phosphorus group in which 10 mmol/L β-glycerophosphate was added to normal medium to generate high phosphorus medium. Various concentrations of Egb 761 (1 mg/mL, 5 mg/mL, and 10 mg/mL, respectively) were added to the medium for the Egb 761 intervention groups on the basis of high phosphorus. The stimulating time was 7 days. Control medium was prepared equally but without BGP. The medium was replaced twice a week and Egb 761 was added from the beginning and at each medium renewal of the experiment. For precise biochemical Ca²⁺ measurements, calcium content in the cells was extracted with 6 M hydrochloric acid for 24 h at 37 °C. The o-cresolphthalein complex one method was conducted using a Calcium Assay kit (BioSino Biotechnology, Beijing, China) and normalized to the protein content of the same culture.

Assay of alkaline phosphatase activity

After the cells were cultured for 7 days following treatment, ALP activity was measured using an Alkaline Phosphatase Activity Detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer’s instructions. Cell protein content was measured with a bicinchoninic acid (BCA) protein assay kit (Beijing Solarbio Science & Technology Company Co., Ltd., Beijing, China) and ALP activity was normalized against the total protein.

Assay of NF-κB activation and ROS production

NF-κB activation in the cells was examined using a NF-κB activation detection kit (Santa Cruz Biotechnology Inc., TX), which contains anti-NF-κB P65 mAb and secondary goat anti-rat IgG/FITC mAb. The cells were collected after cultured and induced for 7 days, then they were trypsinized with 0.25% tryptase and 0.01% EDTA. Cells were washed twice with phosphate-buffered saline (PBS; Beijing Solarbio Science & Technology Company Co., Ltd., Beijing, China) and made into unicell suspension, and then anti-NF-κB P65 mAb and secondary goat anti-rat IgG/FITC mAb were added after washed with PBS and incubated for 30 min at 37 °C in order. The cells were washed twice with PBS after centrifugation and 100 μL PBS was added, and then they were analyzed by flow cytometry (COULTER EPICS XL; Beckman Coulter, Inc., Fullerton, CA) after filtration. Cell under fluorescence staining was considered as positive and its ratio was calculated.

It is reported that BGP can immediately induce intracellular and mitochondrial reactive oxygen species (ROS) production approximately three-fold in VSMCs and BGP-induced VSMCs calcium deposition is mainly via mitochondrial ROS.9 Therefore, ROS of VSMCs were examined after cultured and treated for 7 days. Cells were labeled with 10 μM DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) for 30 min and then were washed three times. The amount of ROS was determined as mean fluorescence intensity measured by flow cytometry.

Statistical analysis

Each experiment was repeated independently three times and all the data are expressed as mean ± standard deviation. Means were compared by Student’s t-test. Means between multiple groups were performed using the analysis of variance (ANOVA). Inter-group comparison was made using ANOVA and the Student-Newman-Keuls test. All the statistical analyses were
performed using the SPSS 19.0 software (SPSS, Inc., Chicago, IL). For all the statistical tests, \( p < 0.05 \) was considered to indicate a statistically significant difference.

**Results**

*Effects of BGP and EGb 761 on calcium content of cultured rat aortic VSMCs*

Biochemical Ca\(^{2+}\) measurements were performed on 7 days after the intervention with various media conditions. As shown in Figure 1, the calcium contents of the BGP group were significantly higher than those of the control group (\( p < 0.01 \)), and were inhibited by EGb 761 in a concentration-dependent manner (\( p < 0.05 \)).

*Effects of BGP and EGb 761 on the bone-related factors-ALP of cultured rat aortic VSMCs*

Effects of BGP and EGb 761 on the bone-related factors-ALP of cultured rat aortic VSMCs are shown in Figure 2. In the present study, high phosphorus induced the enhanced expression of ALP (\( p < 0.05 \)), while these decreased when administrated with EGb 761 (\( p < 0.05 \)). The data showing EGb 761 degrade the expression of ALP of rat aortic VSMCs in a concentration-dependent manner.

*Effects of BGP and EGb 761 on NF-\( \kappa \)B activation of cultured rat aortic VSMCs*

Effects of BGP and EGb 761 on NF-\( \kappa \)B activation of cultured rat aortic VSMCs are shown in Table 1. In the present study, high phosphorus up-regulated the NF-\( \kappa \)B activity of VSMCs (\( p < 0.01 \)), and EGb 761 decreased the protein expression of P65 and down-regulated the NF-\( \kappa \)B activity of rat aortic VSMCs in a concentration-dependent manner (\( p < 0.05 \)).

*Effects of BGP and EGb 761 on ROS production of cultured rat aortic VSMCs*

Effects of BGP and EGb 761 on ROS production of cultured rat aortic VSMCs are shown in Figure 3. ROS were determined as mean fluorescence intensity measured by flow cytometry. ROS fluorescence intensity of the BGP group was significantly higher than those of the control group (\( p < 0.01 \)), and was inhibited by EGb 761 (\( p < 0.05 \)).

**Discussion**

Increasing evidence indicates high-phosphate conditions may lead to imbalance among the expression levels of calcification inducers and calcification inhibitors; however, the mechanism of action of hyperphosphatemia in vascular calcification remains
Further studies are required to clarify the mechanism and inhibit its detrimental action.

In the present experimental model, BGP-induced calcification was strongly inhibited by EGb 761 in the media of rat aortic VSMCs in a concentration-dependent manner. Data show EGb 761 attenuated the calcification of cultured rat aortic VSMCs by decreasing the protein expression of P65, down-regulating the NF-κB activity, and reducing intracellular and mitochondrial reactive oxygen species (ROS) production.

Pi is one of the substrates for adenosine-5'-triphosphate synthesis during oxidative phosphorylation and also plays an important role in regulating mitochondrial membrane potential and reactive oxygen species production in isolated mitochondria. In addition, several studies have reported that uremia results in increased oxidative stress, and oxidative stress is related to VC in vitro. It has been reported that ROS plays a central role in high Pi-induced VSMC calcification and hyperphosphatemia-induced VC in CRF rats, and antioxidants can relieve vascular complications in patients with chronic kidney disease. In our study, BGP induced intracellular and mitochondrial ROS production approximately three-fold in VSMCs, which is consistent with the previous report, and EGb 761 inhibited ROS production, decrease the oxidative stress and thus prevented rat aortic VSMCs calcification.

NF-κB is an oxidant-responsive transcription factor that regulates the expression of many genes. The NF-κB/Rel family of transcription factor includes five proteins, p50, p52, p65 (or RelA), RelB, and c-Rel, which exist as homodimers or heterodimers. The most common NF-κB heterodimer is composed of p50 and p65. In resting cells, NF-κB is sequestered in the cytoplasm by its association with proteins belonging to the IκB inhibitor family. Stimuli, such as the proinflammatory cytokines tumor necrosis factor (TNF)-α can activate NF-κB. In the canonical pathway, these stimuli trigger activation of an IκB kinase complex, which in turn phosphorylate NF-κB inhibiters (IκB). This phosphorylation step leads to the ubiquitination and subsequent degradation of IκBs by the proteasome. The NF-κB complex translocates into the nucleus and regulates the expression of genes correlate with proliferation, apoptosis and angiogenesis and activates transcription. For this reason, NF-κB is considered to be connected with multiple aspects of vascular calcification.

Table 1. Effects of BGP and EGb 761 on NF-κB activation.

| Group                  | Ratio of NF-κB activated VSMCs |
|------------------------|---------------------------------|
| Control                | 7.67%±2.52%                     |
| BGP (10 mM)            | 49.33%±5.13%                    |
| BGP + EGb 761 (1 mg/mL)| 40.05%±5.58%                    |
| BGP + EGb 761 (5 mg/mL)| 32.01%±4.97%                    |
| BGP + EGb 761 (10 mg/mL)| 18.04%±5.61%                    |
and promoting p65 nuclear translocation and knocking down p65 by shRNA or over expressing IkBa significantly reduced Pi-induced calcium deposition in bovine aorta smooth muscle cells and demonstrated that activation of NF-κB is essential for Pi-induced SMC calcification. EGB 761 significantly decreased Pi-induced calcium deposition in rat aortic VSMCs by down-regulating the NF-κB activity, as assessed by calcium content and ratio of NF-κB activated VSMCs assay.

The bone-related factor-ALP activity, a vital marker of calcification, markedly enhanced following BGP treatment and decreased when administrated with EGB 761. This clearly shows that the protective effect of EGB 761 on rat aortic VSMCs calcification by not only decreasing the deposition of calcium in the cells but also affecting the osteogenic transdifferentiation.

In conclusion, EGB 761 prevented calcification and further progression of already established calcification in rat aortic VSMCs. It not only reduced the deposition of calcium, but also inhibited osteogenic transdifferentiation, which are consistent with experimental animal studies and with clinical studies linking EGB 761 to beneficial effects on VC. However, the detailed mechanism by which EGB 761 may influence this process is unclear. More research is required to observe the EGB 761 in patients with renal failure and to confirm a possible protective effect of EGB 761 against VC.

Disclosure Statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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