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Zinc and BGP-15 as potential inhibitors of vascular smooth muscle cell calcification induced by high glucose and prolyl hydroxilase inhibitor

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ZINC AND BGP-15 AS POTENTIAL INHIBITORS OF VASCULAR SMOOTH MUSCLE CELL CALCIFICATION INDUCED BY HIGH GLUCOSE AND PROLYL HYDROXILASE INHIBITOR

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1. INTRODUCTION

Cardiovascular diseases are responsible for one-third of all deaths worldwide, in which vascular calcification also plays a significant role as an independent risk factor. In patients with chronic kidney disease (CKD), vascular calcification and anemia occur as primary complications, the prevention and therapy of which are still a major challenge for clinicians today. In the pathogenesis of vascular calcification, vascular smooth muscle cells lose their phenotypic markers while the expression of osteogenic proteins are induced, resulting in the accumulation of calcium and phosphate-containing hydroxyapatite crystals in the extracellular matrix. Recombinant erythropoietin (EPO) has been used primarily in the treatment of anemia as a complication of CKD, with alternatives to prolyl hydroxylase inhibitors (PHIs). PHIs ensure that hypoxia-induced factors (HIFs) are active under normoxic conditions, thus ensuring continuous endogenous EPO synthesis. However, osteoblast-directed transdifferentiation of vascular smooth muscle cells is enhanced due to sustained and continuous HIF activation. Because high serum phosphate (Pi) levels in patients with CKD are essential inducers of the calcification process, the use of PHIs may further enhance vascular calcification. In our work, we investigated how PHI (Roxadustat) affects high Pi-induced calcification of vascular smooth muscle cells. Zinc is one of the essential trace elements, the lack of which contributes to the development of many diseases, including cardiovascular disease. In our work, we investigated the role of zinc in high Pi-induced vascular calcification aggravated by PHI of human aortic smooth muscle cells. We demonstrated that zinc inhibits Pi-induced calcification of vascular smooth muscle cells aggravated by PHI. Hyperglycemia and disorder of glucose metabolism accelerate the progression of vascular calcification. BGP-15 is an insulin-sensitizing drug candidate molecule, which activate the anti-calcification chaperon - heat shock protein 70 (Hsp70) - and may play an important role in the treatment of *diabetes mellitus*. We investigated the effect of BGP-15 on high glucose and/or high Pi induced osteoblast transdifferentiation of human aortic smooth muscle cells. We found that BGP-15 is a potential inhibitor of vascular smooth muscle cell calcification.

2. REVIEW OF LITERATURE

Cardiovascular diseases and complications can be classified as leading death in developed countries. The development and progression of vascular diseases are influenced by especially the dysfunction and activation of endothelial cells, the oxidative modification of low density lipoprotein (Low density lipoprotein, LDL) and the calcification of smooth muscle cells.

2.1.Vascular calcification

Vascular calcification plays pivotal role in atherosclerosis, CKD, diabetes mellitus and several cardiovascular diseases such as myocardial infarction and coronary disease. In previous studies, calcification was considered a passive, irreversible process associated primarily with aging. The precise mechanism is nor completely understood, although data from recent years indicate that vascular calcification is an active, multi-step, cell-mediated, and well-regulated process underlying the mineral metabolism disorder, which is mediated by vascular smooth muscle cells (VSMCs) results in osteoblast transdifferentiation. The risk of developing it is increased by many factors, such as high blood pressure, dyslipidemia, obesity, impaired glucose metabolism, but oxidative stress, anemia, microinflammation also play a role in its development. Vascular calcification follows two distinct patterns: (1) intimal calcification, which is characterized by focal mineralization in the intima layer of the vascular wall in the vicinity of atherosclerotic plaques, and (2) media calcification, which appears as diffuse mineralization in the tunica media layer and does not affect lumen of the artery. In patients with CKD and hemodialysis, media calcification is a primary pathogenic symptom and increases the risk of caridovascular mortality and morbidity by approximately twenty-fold. To describe the mineral and bone metabolism disorder that occurs during CKD, the term CKD-MBD (chronic kidney disease-mineral and bone disorder) was introduced to describe the systemic nature of the disease. In patients with CKD, one of the most common comorbidities, diabetes mellitus, requires close attention because hyperglycemia and impaired glucose metabolism accelerate the process of vascular calcification. Approximately 30% of type I diabetics and 50% of type 2 diabetics develop renal disease, the progression of which is associated with elevated plasma Pi levels, which play a pivotal role as an increased risk factor for the formation of vasculopathy.

2.2.Calcification and osteoblast transdifferentiation of vascular smooth muscle cells

In end-stage renal disease (ESRD), persistently high serum phosphate level has been widely established to induce vascular calcification, because Pi elimination is reduced due to decreased renal function. Previous studies indicated that elevated phosphate induce VSMCs calcification

as well as an osteoblastic phenotypic change. The entry of extracellular Pi into the cell is primarily *via* a membrane transporter, the Pit-1 sodium-dependent phosphate cotransporter. Elevated intracellular Pi concentration induces the expression of markers of osteogenic differentiation (BMP-2 [bone morphogenic protein-2], Runx-2 [Run-related transcription factor], Osterix [Osx], MSX-2 [Homeobox protein-2]). One of the master regulators of the vascular calcification process is Runx-2, which is involved in osteoblast differentiation as a transcription factor and regulates the expression of bone matrix proteins. Alkaline phosphatase (ALP) is a hydrolase enzyme that is activated in the early stages of osteoblast *trans*-differentiation, reducing the level of pyrophosphate, which inhibits the formation of calcium deposits in the extracellular matrix. One characteristic mediator of normal bone formation and vascular calcification is BMP-2 - a cytokine belongs to the Transforming Growth Factor-β (TGF-β) superfamily- whose main function is the transcriptional regulation of osteogenic genes. Osteocalcin (osteocalcin, OC), a vitamin K-dependent non-collagen-type extracellular matrix protein, plays an important role in osteogenesis and mineralization of VSMCs. OC is able to bind with high affinity to calcium and hydroxyapatite as a result of post-translational γ-carboxylation. In addition to the factors inducing the osteoblast-like phenotype change of VSMCs, there are physiologically also endogenous inhibitors that prevent calcification (Fetuin-A, Osteopontin, MGP [Matrix-Gla protein], pyrophosphate [pyroPi]). The results of research over the past few years show that extracellular vesicles (EVs) secreted by cells of the vasculature also play a prominent role in the physiological and pathophysiological processes of the cardiovascular system. EVs are membrane-free particles with a double lipid layer that function as intracellular messengers and can be divided into three groups: exosome (30-100nm), microvesicles (200-1000nm), and "apoptotic bodies" (1-4μm). Circulating EVs are capable of transporting proteins, lipids, and amino acids involved in the regulation of intracellular signaling processes. EVs secreted by VSMCs play an important role in the early stages of vascular calcification. VSMCs also secrete extracellular EVs under physiological conditions that do not contain hydroxyapatite crystals, but contain Fetuin-A and MGP, which are characteristic inhibitors of calcification.

2.3.Effects of prolyl hydroxylase inhibitors in the treatment of anemia in patients with CKD.

In addition to vascular calcification, one of the most significant complications in CKD is anemia, as the process of hematopoiesis is severely impaired and endogenous EPO production is greatly reduced or eliminated. A promising approach to correct CKD-associated anemia is the pharmacologic inhibition of hypoxia-inducible factor (HIF) prolyl-4-hydroxylase domain

(PHD) proteins by PHIs (Roxadustat, Vadadustat). HIFs regulate multiple genes, including erythropoietin (EPO) and proteins promoting iron absorption, iron transport, and heme synthesis. HIF-1 is a heterodimeric transcription factor consisting of an oxygen-sensing α subunit and an β subunit. HIF-1 will be active when the two subunits dimerize and translocate to the nucleus, where they bind to CRE elements of HIF-regulated genes and then activate the target genes together with p300 and CREB-binding proteins as co-activators. Under normoxia, PHD proteins hydroxylate HIFs facilitating their polyubiquitination and proteasomal degradation, thereby inhibiting the activation of HIF-responsive genes. In animal and human clinical studies, inhibition of the PHD enzyme by PHI has been reported to increase endogenous EPO production. Roxadustat is one of the orally bioavailable HIF PHD inhibitors (PHI) promoting the transcriptional activity of HIFs. Roxadustat increases hemoglobin levels in a dose-dependent manner and significantly decreases hepcidin levels in treated patients. In patients with CKD, in addition to increasing hemoglobin levels, Roxadustat increased serum transferrin levels and decreased ferritin levels, suggesting an important role in the normalization of iron metabolism. Simultaneously with the study of PHIs, several studies have demonstrated that HIF proteins also play a role in vascular calcification. The direct relationship and molecular mechanism of hypoxia and vascular calcification are not yet known. The major master regulator of hypoxia is HIF-1α, which enhances glycolysis and reduces oxidative phosphorylation, thereby promoting cell adaptation to the hypoxic environment. Pyruvate dehydrogenase kinase-1-4 (PDK 1-4) are mitochondrial regulators of glucose metabolism that reduce PDH activity, thereby inhibiting pyruvate → acetyl-CoA conversion. Expression of PDK-4 and HIF-1α is increased not only during hypoxia but also during mineralization of vascular smooth muscle. HIF-1α is known to bind to the PDK-4 promoter and thus affects the expression of PDK-4-regulated genes.

2.4. *The role of zinc deficiency in the pathogenesis of cardiovascular and other diseases*

2.4.1. Physiological effects of zinc

Zinc is an essential trace element for all living organisms as it is involved in maintaining cell membrane integrity, cell proliferation / differentiation, and many biochemical processes. The human body contains approximately 2-3 g of zinc, of which 57% is found in skeletal muscle, 29% in bone, and the remainder in the heart, liver, kidney, and plasma. Zinc plays an important role in the optimal functioning of many biochemical processes. Zinc is a divalent cation that is able to bind to nearly 10% of the proteins in our body, thus contributing to the structural stability of proteins and promoting the catalytic activity of more than 300 enzymes. Zinc is involved in

the regulation of chronic inflammatory processes by reducing the production of inflammatory cytokines. It regulates the process of oxidative stress by contributing to the synthesis of antioxidant enzymes. Metallothionenes are small, cysteine-rich, intracellular proteins that bind heavy metal ions, including zinc, selectively and with high affinity, and have significant antioxidant properities. Metallothionenes protect biological structures and RNA from oxidative effects. Zinc is rapidly detached from the metallothionein protein and induces further metallothionein synthesis, thus stabilizing the amount of metallothionenes in the tissue.

2.4.2. Zinc deficiency and its consequences

Zinc plays a central role in the development of many physiological and pathophysiological processes, such as membrane repair mechanisms, oxidative stress, inflammation, and metabolic processes. Zinc deficiency in developing countries is so significant that it is the fifth leading cause of various diseases, affecting approximately 2 billion people worldwide. Zinc deficiency has only been given great importance in the last few decades when it has been associated with diseases such as growth retardation, skin lesions, hypogonadism, and autoimmune diseases. Based on research in recent years, zinc deficiency also plays a role in chronic metabolic diseases such as cardiovascular disease as well as type 2 diabetes. The results of in vitro and in vivo experiments confirm that zinc has a protective role in the process of atherosclerosis, which is a major risk factor for cardiovascular disease. Zinc regulates the formation of atherosclerotic plaques by helping to preserve the integrity of endothelial cells and also regulates lipid peroxidation through various redox signaling processes.

2.5. BGP-15 as a drug candidate for insulin sensitization

BGP-15 (N ′ - (2-hydroxy-3- (piperidin-1-yl) propoxy) -3-pyridinecarboximidamide) is a hydroxy acid derivative could reduce the neuro-, nephrotoxic, and myelotoxic effects of various cytostatics. In recent years, several research groups have explored the beneficial effects of BGP-15, including insulin sensitization and anti-inflammatory effects. The precise mechanism of action and intracellular targets of BGP-15 are unknown, but strongly enhance the expression of heat shock protein expression. It is characterized by the fact that it does not induce stress protein synthesis, but by co-induction, i.e. it enhances the stress response elicited by many effects. The insulin sensitizing effect of BGP-15 has also been demonstrated in several animal models of diabetes. In the genetically insulin-resistant Goto Kakizaki rat (normal weight, insulin resistance), BGP-15 significantly improved insulin resistance in a dose-dependent manner, similar to metformin and rosigliatone, which are antidiabetic drugs used in clinical practice.

Examining the effect of BGP-15 in a New Zaeland rabbit model, it was found that an improvement in insulin resistance was observed only in the group on a cholesterol-rich diet. A human clinical trial demonstrated that BGP-15 also improved insulin sensitivity, glucose utilization, and muscle tissue glucose utilization in patients with insulin resistance. In addition, BGP-15 provides protection against cardiomyopathy and retinopathy as a complication of diabetes.

3. AIMS OF STUDY

Hypothesis 1: In patients with CKD, mineral and bone metabolism disorders and diabetes mellitus are the primary risk factors for the development of vascular calcification. PHIs used to treat anemia as a complication of CKD may accelerate the progression of calcification in patients with CKD through continuous HIF activation.

a. We investigated whether PHI (Roxadustat-FG4592) enhances high Pi-induced calcification and osteoblast-directed transdifferentiation of VSMCs.

b. Have we investigated whether zinc inhibits this pathological process?

c. We investigated whether metallothionein, as a protein with high affinity for zinc, has a role in the mechanism of the inhibitory effect of zinc on vascular calcification.

d. We examined the plasma zinc concentration in patients undergoing hemodialysis and carotid endarterectomy, from which it can be concluded that lower plasma zinc concentration may contribute to the acceleration of the calcification process.

Hypothesis 2: Investigation of the potential anticalcifying effect of BGP-15 in high Pi-induced calcification of VSMCs at normal and high glucose concentrations.

a. We first looked at how high glucose (11 mM) affects high Pi-induced osteoblast transdifferentiation in human aortic smooth muscle cells.

b. Subsequently, we studied the effect of BGP-15 on high Pi-induced calcification of smooth muscle cells at normal (5.5 mM) and high (11 mM) glucose concentrations.

4. MATERIAL AND METHODS

4.1.Cell culture and reagents

Human aortic smooth muscle cells (HAoSMCs) were purchased from Cell Applications (San Diego, CA, United States, Cat.: 354-05a) and Lonza (Allandale, NJ, United States Cat.: CC-2571). The main considerations in the selection of donors were that they did not suffer from known cardiovascular disease, diabetes mellitus, did not smoke, and were from the Caucasian population. All reagents were purchased from Sigma-Aldrich (St. Luis, MO, USA) unless otherwise stated. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 1000mg/L glucose supplemented with 10% fetal bovine seum (FBS, Life Technologies, Vienna, Austria), 100 U/ml peniccilin, 100 μg/ml streptomycin, and neomycin (growth medium, GM). For cell treatment with high glucose medium (11 mmol / l), the growth medium was supplemented with 1000 mg / L glucose. Cells were grown to confluence and used from passages 5 to 7. Medium were changed every 2 days. To induce Pi-mediated calcification GM (normal-, and high glucose conditions) was supplemented with inorganic phosphate (3 mmol/L referred to as a calcification medium) in the form of Na_2HPO_4/NaH_2PO_4 (pH 7.4) for 10 days. To investigate the effect of zinc on vascular calcification, $ZnCl_2$ x $6H_2O$ was dissolved in deionized water and we supplemented the calcification medium with various concentrations of zinc(15-30 μmol/L). To investigate the effect of PHI on mineralization of HAoSMCs, cells were treated with FG4592 (Selleckchem, Houston, United States) in various concentration (5 and 20 μmol/L). To investigate the inhibitory effect of BGP-15 on vascular calcification we supplemented the calcification medium with BGP-15 dissolved in deionized water with various concentrations (15-200 μmol/L).

4.2. Quantification of calcium deposition

4.2.1. Alizarin Red staining

Cells grown on 24-well plates were washed twice twice with phosphate-buffered saline (PBS) pH 7.4 and fixed with 4% paraformaldehyde for 10 min at room temperature. Then, cells were rinsed with PBS and stained with 2% Alizarind Red S for 10 min. Excessive dya was removed by several washes in deionized water. Extracellular calcium deposition was stained in red color.

4.2.2. Colorimetric method for the determination of calcium deposition

Cells grown on 24-well plates were washed twice with PBS pH 7.4 and decalcified with 0.6 mol/L HCl for 30 min at 37◦C. After decalcification, cells were solubilized with a solution of NaOH 0.1 mol/L and SDS 0.1%, and the protein content of samples was measured with

bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, United States). The calcium content of the supernatants was determined by the QuantiChrom Calcium Assay Kit (Gentaur, Brussels, Belgium). The calcium content of the cells was normalized to protein content and expressed as μg/mg protein

4.2.3. Fluorescent staining of extracellular calcium deposit

To analyze extracellular calcium deposition by confocal microscopy, cells were treated as described above trypsinized, plated to glass coverslips and further incubated for 16 h as in growth medium or in calcification medium with or without BGP-15 as described above in the presence of OsteoSense 680EX Fluorescent Imaging Agent (1:100), a near-infrared based bisphosphonate calcium tracer, for 24 h at 37 ◦C (PerkinElmer, Waltham, MA, USA). Cells were then fixed with 4% paraformaldehyde, washed twice with phosphate buffered saline pH 7.4 (PBS). Nuclei were visualized with Hoechst. Samples were investigated with Leica SP8 confocal microscope using Leica Application Software X (Leica, Mannheim, Germany).

4.3. Measurement of intracellular inorganic phosphate

Intracellular inorganic phosphate concentration was determined by colorimetric analysis using QuantiChromTM Phosphate Assay Kit (Gentaur). Cells grown on 12-well plates were washed twice with PBS pH 7.4 and then lysed with solubilization buffer (1% Triton-X 100, 0.5% Igepal CA-630, 10% protease inhibitor. 150 mmol/L NaCl, 5 mmol/L EDTA, 10 mmol/L Tris). Intracellular phosphate concentration of the cells was normalized to protein content expressed as μmol/mg protein.

4.4. RNA Isolation and Quantitative Reverse Transcription – Polymerase Chain Reaction

Cells grown on 24-well plates and total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, United States). The cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, United States) for RT-PCR. Real-time polymerase chain reactions were performed using fluorescent TaqMan probes. TaqMan gene expression assays for BMP-2 (Hs00154192_m1), Msx-2 (Hs00741177_m1), PDK-4 (Hs01037712_m1), α-actin/ACTA2 (Hs00426895_g1), Sp7 (Hs01866874_s1), KLF-5 (Hs001561745_m1), TAGLN/SM22α (Hs0103877_g1), MT1a (Hs00831826_s1), MT21a (Hs02379661_g1), GAPDH (Hs02786624_g1), RNA45S5 (Hs05627131_Gh). Polymerase chain reactions were carried out using the iCycler iQ Real-Time PCR system (Bio-Rad,

Hercules, CA, United States) and the results represent the relative mRNA expression of target genes normalized RNA45S5 and GAPDH mRNA levels.

4.5. Preparation of Whole-Cell Lysates and Nuclear Fractions

Cells grown on 24-well plates were washed twice with PBS pH 7.4 then lysed using RIPA buffer (50 mmol/L Tris [pH 7.5], 150 mmol/L NaCl, 1% Igepal CA-630, 1% sodium deoxycholate, 0,1% SDS) containing 10% Complete Mini Protease Inhibitor Cocktail and 20% PhosSTOP phosphatase inhibitor cocktail, and incubated for 20 min on ice. Lysates were centrifuged at 14,000 × g, 4˚C for 20 min. Supernatants were used as whole-cell extracts.

For nuclear fraction separation, cells were washed with PBS pH 7.4 then scraped with ice-cold hypotonic buffer (20 mmol/L HEPES, 250 mmol/L Sucrose, 10 mmol/L KCl, 2 mmol/L $MgCl_2$) containing 10% Complete Mini Protease Inhibitor Cocktail and 20% PhosSTOP phosphatase inhibitor cocktail on ice. Using 1 ml syringe, the cell suspension was passed through a 28G needle 30 times then swelled on ice for 20 min. Lysates were centrifuged at 15,000 × g, 4˚C for 15 min. Supernatants were discarded and this procedure was repeated 4 times. Finally, the nuclear pellets were dissolved in RIPA buffer supplemented with 10% Complete Mini Protease Inhibitor Cocktail and 20% PhosSTOP phosphatase inhibitor cocktail and centrifuged at 16,000 × g, 4˚C for 15 min. Supernatants were used as nuclear fractions. Protein content was determined using the bicinchoninic acid assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA, United States).

4.6. Western blot

Nuclear translocation of Runx-2, phospho-Runx-2 (Ser451),Msx-2 and Osx were investigated using nuclear and fraction, in case of the other proteins whole cell lysate was electrophoresed in 10 or 12% Tris-glycine SDS-gels, and proteins were transferred to 0.45 μm nitrocellulose membrane (GE Healthcare, Chicago, IL, USA) or 0.22 μm PVDF membrane (Advansta Inc., Menlo Park, CA, United States). Membranes were blocked with 5% w/v milk or 5% w/v BSA for 60 min at room temperature. Afer blocking membranes were incubated with Runx-2 (Cell Signaling Technology, Danvers, MA, United States, Cat.: 12556 [1:1000]), Runx-2 (Ser451) (Bioss Antibodies Inc., Woburn, MA, United States, Cat.:bs-5685R [1:500]), Metallotionein (Abcam, Cambridge, United Kingdom; Cat.: ab12228 [1:1000]), PDK-4 (Abcam, Cat.: ab88063 [1:1000]), Msx-2 (Novus Biologiccals, Centennial, Colorado, US, Cat: NPB1-85445), Annexxin A2 (Cell Signaling Technologies, Danvers, MA, USA, Cat: #8235), MGP (Novus Biologicals, Bio-Techne Ltd., Abringdon, United Kingdom, Cat.: NBP2-45844), Hsp90 (Cell

signalling; Cat.: 4874S [1:5000]), Lamin B1 (Proteintech, Manchester, United Kingdom Cat.: 66095-I-Ig [1:5000]) primary antibody overnight a 4 ˚C. Antigen-antibody complexes were visualized with the horseradish peroxidase chemiluminescence system (Advansta, Menlo Park, CA, United States). Protein bands were normalized to Hsp90, GAPDH and Lamin B1 protein. Quantification of chemiluminesce was done by using Image J software.

4.7. Metallotionein 1 and metallotionein 2 siRNA transfection

Small interfering RNA specific to MT1a (Cat.: 4392420; ID: s194620) and MT2a (Cat.: 4392420; ID: s194629) and negative control siRNA (Cat.: 4390843) were obtained from Ambion (Thermo Scientific, Waltham, MA, United States). Transfection of siRNA into VSMCs was performed using Oligofectamine reagent (Thermo Scientific, Waltham, MA, United States) according to the manufacturer's guide. Briefly, the cells were plated on 24-well plates to form 60–70% confluent monolayers. MT1a and MT2a siRNA were applied at 20 pmol/L concentration and transfection reagent complex were added to the cells for 5 h in OPTI-MEM Reduced Serum Medium then replaced to the growth medium for 16 h then to calcification or normal growth medium until the next transfection procedure. This transfection protocol was repeated every 3 days. Gene expression of MTs was analyzed by q-RT-PCR and immunoblot after 10 days.

4.8. Human samples

Healthy volunteers were recruited without any known diseases including hypertension, dyslipidemia, liver and kidney malfunctions (n = 18, mean age 46 years, F/M 7/11). Stage 5 CKD patients subjected to hemodialysis were selected (n = 28, mean age 55 years, F/M 15/13) from the dialysis unit of the Clinical Centre, Department of Internal Medicine, University of Debrecen, Debrecen, Hungary. Vascular disease group was selected from patients undergoing carotid endarterectomy (n = 15, mean age 72 years, F/M 6/9). Volunteers did not take any zinc supplementation. Blood was drawn in Vacutainers (BD, Franklin Lakes, NJ, United States) using citrate immediately before surgery or the hemodialysis session from CKD patients by venipuncture. Participants gave their informed consent to the study, which was approved by the Regional and Institutional Ethics Committee of the University of Debrecen, Medical and Health Science Center (Nr. DE OEC RKEB/IKEB 3712-2012). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

4.9. Measurement of Plasma Zinc Concentration With Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES)

A five milliliters serum samples were digested two times with 10 – 10 ml Carlo Elba nitric acid 65% (m/m) on water-bath at atmospheric conditions. The digestion was finished with 2 – 2 ml analytical grade hydrogen peroxide 30% (m/m) (Reanal, Hungary). The digested samples were filled to 10 ml with deionized water. At the same time with these sample digestion, we prepared blank solutions from nitric acid and hydrogen peroxide, too. Flame atomic absorption spectrometric (FAAS) analysis was carried out using the following settings. Hollow cathode lamp: Zn Cathodeon lamp, Lamp current: 10 mA, Wavelength: 213.9 nm, Slot burner: 10 cm, Acetylene: 1 L/min, Air: 5 L/min (3 bar), Observation height: 10 mm. The zinc concentrations are the average of three parallel measurements.

4.10. Immunofluorescence staining

Cells were treated as described above in the presence or absence of 50 μmol/L (NG condition) and 150 μmol/L (HG condition) of BGP-15 for 10 days in 24-well plates. Cells were then trypsinized, plated to glass coverslips and further incubated for 16 h as in the growth medium or in calcification medium with or without BGP-15 as described above. Cells were then fixed with 4% paraformaldehyde, washed twice with phosphate buffered saline pH 7.4 (PBS), permeabilized with 0.5% Triton X-100, washed and blocked with 10% normal goat serum and 2% BSA for 60 min in PBS with 0.05% Tween-20 (PBS-T). Slides were then incubated overnight with KLF-5 antibody (Thermo Scientific, Waltham, MA, USA, Cat: 701885) at a dilution of 1:250 overnight at 4 ˚C in antibody dilution buffer (1% BSA in PBS-T), washed, then, further incubated with goat anti-rabbit IgG conjugated to Alexa Fluor 568 (Thermo Fisher Scientific, Waltham, MA, USA) at a dilution of 1:500 for 60 min. Nuclei were visualized with Hoechst. Samples were investigated with Lightning super-resolution microscopy using Leica Application Software X (Leica, Mannheim, Germany).

4.11. Isolation of EVs

To examine the effect of BGP-15 on extracellular vesicle (EV) formation, we exposed VSMCs to 3 mmol/L inorganic Pi under normal glucose and high glucose levels. To avoid a sharp decrease in serum concentration, FBS of the experimental medium was gradually decreased from 5% to 0%. Therefore, cells were cultured in calcification medium with normal and high glucose in the presence of 5% serum for 5 days, 3% for 3 days, and 1% for 2 days in the presence or absence of 50 μmol/L BGP-15 under normal glucose condition, while 150 μmol/L under high glucose condition. Then, 24 h before EV isolation, VSMCs were cultured under serum-free conditions. To limit the number of intact cells during EV preparations, cell culture

supernatants were centrifuged at 400× g for 5 min at room temperature, then supernatants were transferred to new tubes and spinned again at 2000× g for 5 min at room temperature. Supernatants were then transferred to Amicon Ultra MWCO 3000 tubes and concentrated according to the manufacturer's guide. Concentrated supernatants were diluted ten-fold with PBS pH 7.4 and concentrated again. The protein contents were measured with BCA assay. The presence of Matrix Gla protein (MGP) and Annexin A2 in EVs was analyzed by Western blot.

4.12. Statistical analysis

Data are shown as mean ±SEM and analyzed by GraphPad Prism 5.02 software (GraphPad Software Inc., La Jolla, CA, USA). Statistical analysis was performed by the one-way ANOVA test followed by Bonferroni correction. A value of $p < 0.05$ was considered significant.

5. RESULTS

5.1. Zinc inhibits phosphate-induced mineralization of VSMCs in a dose-dependent manner

Zinc has been shown to prevent high Pi induced osteogenic *trans*-differentiation of VSMCs. To examine whether zinc inhibits Pi-mediated mineralization of VSMCs *in vitro* in our experimental conditions, we cultured human VSMCs in calcification medium containing 3 mmol/L inorganic Pi in the presence (15–30 μmol/L) or absence of $ZnCl_2$ for 10 days. In cells cultured in high Pi medium, the amount of extracellular calcium deposition was increased, which was inhibited by zinc in a dose-dependent manner. This was confirmed by Alizarin Red staining, which stains extracellular calcium deposits, and by measuring the calcium content of the extracellular matrix. Active uptake of Pi into the cells via the type III sodium-dependent phosphate cotransporter, Pit-1, is a key factor of Pi-induced calcification of VSMCs. Therefore, we measured Pi uptake by analyzing intracellular Pi levels in calcification medium in the presence or absence of zinc. Pi induced a robust increase in intracellular Pi while at a concentration of 15 μmol/L, zinc significantly lowered Pi uptake.

5.2. PHI enhances Pi induced mineral deposition in VSMCs that is inhibited by zinc

Hypoxia greatly enhances VSMCs calcification and osteochondrogenic *trans*-differentiation, so PHI through HIF stabilization, potentially enhances VSMC mineralization. VSMCs cultured in calcification medium were exposed to PHI FG4592 (5 and 20 μmol/L, respectively) supplemented with 30 μmol/L $ZnCl_2$ for 10 days. To measure the calcium content of extracellular matrix we showed that PHI markedly enhanced Pi induced VSMC mineralization compared to Pi alone, while zinc (30 μmol/L) inhibited enhanced calcification by PHI.

5.3. PHI (FG4592) decreases smooth muscle specific marker expression

Downregulation of smooth muscle-specific markers is a common characteristic of VSMCs calcification. Recent data shows that during hypoxia HIF1α induces phenotypic switch in VSMCs. So we examined whether PHI aggravates Pi induced loss of smooth muscle specific markers such as smooth muscle α-actin (ACTA-2) and smooth muscle protein 22α (TAGLN). Cells were cultured in growth medium in the presence or absence of PHI (5 and 20 μmol/L) or in calcification medium with or without PHI and 30 μmol/L zinc and the calcification process was monitored for 10 days. We showed that Pi induced the downregulation of ACTA-2 and TAGLN expression that was significantly aggravated by PHI in a dose-dependent manner after 10 days and PHI also induced a dose-dependent decrease in smooth muscle markers in growth medium without high Pi. Zinc (30 μmol/L) significantly restored smooth muscle-specific gene

expression in response to Pi as well as to Pi and PHI. These results support the hypothesis that high Pi and PHI synergistically boost calcification of VSMCs, decrease the expression of ACTA and TGLN, which is compensated by zinc

5.4. PHI enhances the expression of osteochondrogenic markers that is inhibited by zinc

In our optimized in vitro calcification model, we examined how PHIs affect the expression of genes important in calcification. In our studies, we examined the expression patterns of BMP-2 (Bone Morphogenetic Protein-2), Msx-2 (Msh homeobox 2), and Osx genes, which play an important role in the pathomechanism of vascular calcification. Based on the obtained results, it can be concluded that PHI increases the expression of the osteogenic genes in a dose-dependent manner compared to the cells treated with Pi, which was significantly inhibited by zinc at a concentration of 30 μmol / L. In our experiments, we observed that PHI alone (without high Pi) did not affect mRNA levels of BMP-2, Msx-2, and Osx.

5.5. PHI influences Ser451 phosphorylation of Runx2

The transcription factor Runx-2 is one of the major regulatory proteins in the pathogenesis of vascular calcification. Runx-2-dependent transactivation of osteogenic genes is regulated by Ser451 phosphorylation of Runx-2. Nuclear translocation and phosphorylation status of Runx-2 were examined by Western blot, in which nuclei were separated from VSMCs. We showed that the nuclear translocation if Runx 2 was not affected either by Pi or Pi and PHI. Examining Ser451 phosphorylation of Runx-2, we showed that Ser451 is dephosphorylated in the presence of high Pi and PHI, a process that was significantly inhibited by zinc at a concentration of 30 μmol / L.

5.6. Role of pyruvate dehydrogenase kinase-4 (PDK-4) in vascular calcification

In addition to regulating glucose and fatty acid metabolism, the mitochondrial enzyme PDK-4 also plays a central role in the process of vascular calcification. High Pi exposure in VSMC cells increased PDK-4 mRNA levels, which was further enhanced by PHI. As a result of high Pi, the expression of PDK-4 protein was increased, which was further increased by PHI. Zinc significantly reduced PDK-4 expression in both Pi and Pi + PHI. These data demonstrate that both transcriptional and post-translational effects of PHI on VSMC calcification can be counteracted by zinc.

5.7. Inhibitory effect of zinc is independent of zinc responsive protein metallothionein

Metallothionenes are low molecular weight proteins that are able to selectively bind various heavy metal ions (zinc, copper, cadmium) and also plays role in antioxidant processes. Vascular calcification has been associated with free radical formation and oxidative stress, so we investigated whether zinc inhibits Pi induced mineralization of VSMC in an MT-dependent fashion. VSMC were cultured in calcification medium containing 3 mmol/L Pi in the presence (30 μmol/L), or absence of zinc for 10 days and MT1/MT2 gene expressions were analyzed by qRT-PCR. Our results showed that exposure of cells to zinc (30 μmol/L), slightly above the physiological concentration, did not induce MT1a and MT2a in our experimental conditions compared to untreated cells. Metallothioneins have been shown to be expressed in atherosclerotic plaques representing a local protective mechanism, so we hypothesized that it has a similar function in our *in vitro* calcification model. To investigate this, in our further experiments we supplemented the calcification medium with a concentration of 100 μmol / L ZnCl2, which significantly increased the mRNA levels of metallothionein 1a and 2a compared to the controls. MT1a/2a expression was silenced with MT1/2 specific siRNAs. Q-RT-PCR and immunoblot analysis of VSMC demonstrated that siRNA treatment effectively decreased MT1/2 expression in cells exposed to zinc (100 μmol/L). Simultaneously with the gene silencing, the cells were cultured in calcification medium alone or supplemented with 100 /mol / L ZnCl 2 for 10 days, and then the calcium concentration in the extracellular matrix was measured. We have shown that zinc inhibits high Pi-mediated mineralization of VSMCs despite silencing of the metallothionein gene.

5.8. Plasma zinc level is reduced in patients undergoing carotid endarterectomy and in CKD patients on hemodialysis

A recent report revealed that reduced zinc levels contribute to the progression of vascular calcification. Plasma zinc concentrations in hemodialysis patients, patients undergoing carotid endarterectomy surgery, and the control group were measured by inductively coupled plasma atomic emission spectrometry. Plasma zinc levels were reduced both in CKD patients and patients with carotid artery stenosis compared to healthy patients. These data suggest that low plasma zinc concentration is a potential risk factor for vascular diseases.

5.9. High glucose aggravates high Pi-induced extracellular matrix calcium deposition and Pi uptake of VSMCs.

Diabetes mellitus, hyperglycemia, and disorder of glucose metabolism are known to enhancevascular calcification. In our optimized *in vitro* calcification model, we analyzed whether the high glucose (11 mM) condition anhences extracellular matrix caldium deposition and Pi uptake of VSMCs. We showed that high Pi increased ECM calcium deposition and Pi uptake of VSMCs, which was significantly aggravated by high glucose.

5.10. BGP-15 mitigates high Pi-induced mineralization of VSMCs in both normal glucose and high glucose conditions

To examine whether BGP-15 inhibits high Pi-induced mineralization of VSMCs under normal glucose and high glucose conditions. VSMCs were cultured in growth medium or in calcification medium containing 3 mmol/L inorganic Pi under normal glucose (5.5 mM) and high glucose (11 mM) conditions in the presence or absence of different concentrations of BGP15 (15–100 μM BGP-15 in normal glucose condition and 50–200 μM in high glucose condition) for 10 days. Calcium deposits in the extracellular matrix were detected by Alizarin Red staining, which showed that BGP-15 significantly reduced high Pi-induced mineralization in a dose-dependent manner even at normal and high glucose concentrations. To quantify extracellular matrix mineralization, we measured the calcium content of the extracellular matrix. We found that Pi alone increased the ECM calcium content, which was aggravated by the high glucose condition. Importantly, BGP-15 dose-dependently lowered calcium deposition in normal glucose and high glucose conditions. The active uptake of Pi into the cells has s a crucial role in Pi-induced calcification of VSMCs. So we showed that Pi markedly increased the intracellular Pi level, which was aggravated by the high glucose condition. BGP-15 dosedependently lowered the Pi uptake in both conditions. As 25 μM of BGP-15 did not affect calcification, we omitted this concentration from further experiments.

5.11. BGP-15 inhibits high Pi-induced loss of smooth muscle cell-specific markers in both normal glucose and high glucose conditions

Downregulation of smooth muscle-specific markers, such as transgelin (TAGLN) and smooth muscle α-actin (ACTA-2), is a common feature of VSMC calcification and osteochondrogenic transition. We showed that high Pi downregulated TAGLN and ACTA-2 expression, which was further decreased by the high glucose condition. Importantly, BGP-15 counteracted Pi-induced loss of TAGLN and ACTA-2 in both conditions.

5.12. BGP-15 inhibits osteochondrogenic gene expression induced by high Pi in both normal glucose and high glucose conditions

VSMCs phenotypic switch toward an osteochondrogenic phenotype involves the expression of osteoblast-specific markers. To decipher the molecular mechanism of how BGP-15 inhibited VSMCs mineralization, we analyzed the expression of several key markers of osteochondrogenic transformation, which are KLF-5, Msx-2, BMP-2, and Sp7. The KLF-5 transcription factor plays a prominent role in the initial phase of mineralization of VSMCs. We showed, that high Pi induced mRNA expression of KLF-5, which was aggraveted by high glucose. BGP-15 inhibited high Pi-induced mRNA expression of KLF-5 in dose-dependent manner in both normal glucose and high glucose conditions. As a transcriptional regulator, MSX-2 plays an important role in the osteoblast transition of VSMCs. We showed that high Pi doubled the mRNA expression of MSX-2 in normal glucose concentrations and more than tripled compared to controls in high glucose condition. BGP-15 lowered high Pi-induced expression of Msx-2 not only in the NG condition, but it also reduced high Pi-induced expression boosted by high glucose in dose-dependent manner. Examining the m RNA expression of BMP-2, which plays an important role in Pi-induced mineralization of VSMC cells, we showed that high Pi increased almost threefold compared to control, while Pi and high glucose more than quadrupled were blunted by BGP-15 in a dose-dependent manner. The Osx is a characteristic transcription factor for osteoblast differentiation and bone formation. We showed that high Pi induced Osx mRNA expression was aggravated by the high glucose condition. Importantly, both Pi and Pi + high glucose-triggered Osx expression were reduced by BGP-15 in a dose-dependent manner.

5.13. BGP-15 inhibits nuclear translocation of osteochondrogenic proteins induced by high Pi.

Osteochondrogenic transdifferentiation of VSMC cells is characterized by nuclear translocation of KLF-5, Msx-2, and Osx proteins. Western blot showed that KLF-5, Msx-2, and Osx proteins were translocated to the nucleus by Pi, which was dose-dependently inhibited by BGP-15. Nuclear translocation of the KLF-5 transcription factor was also confirmed by immunostaining.

5.14. BGP-15 inhibits Pi-induced PDK-4 expression in VSMCs and affects the protein composition of extracellular vesicles.

Pyruvate dehydrogenase kinase-4 (PDK-4) has an important role in vascular calcification. So, we explored whether BGP-15 inhibits PDK-4 induction in response to high Pi in both normal glucose and high glucose conditions. We showed that high Pi induced PDK-4 mRNA levels and protein expression were nearly doubled compared to controls in normal glucose condition,

whereas triple induction was observed at high Pi and high glucose levels.. Importantly, BGP-15 inhibited PDK-4 expression in both conditions.

During mineralization of VSMCs, the secretion of extracellular vesicles (EVs) is increased as a result of elevated extracellular calcium concentration. Extracellular vesicles were isolated and examined for the presence of Matrix Gla protein (endogenous calcification inhibitor protein) and Annexin A2 (calcification enhancing protein) proteins in the EV by Western Blot. We showed that the amount of MGP protein was lower under the effect of high Pi than in the control cells, and its amount decreased further under the effect of high glucose. As a result of BGP-15, the amount of MGP protein remained at the control level even at low and high glucose concentrations. In parallel, the amount of Annexin A2 protein was increased by high phosphate and / or high glucose, which BGP-15 maintained at control levels.

6. DISCUSSION

Anemia is one of the most significant complication in addition to vascular calcification in chronic renal failure (CKD). Recombinant erythropoietin (EPO) is primarily used in the treatment of anemia, an alternative to the increasingly widely used hypoxia inducible factor (HIF) prolyl hydroxylase inhibitor (PHI) drugs (Roxadustat-FG4592), which have outstanding therapeutic potential. Recent evidence suggets that active HIF proteins enhance the progression of high Pi-mediated vascular calcification, a significant risk factor in the pathogenesis of CKD. First, we investigated the relationship between drug-induced hypoxia and vascular calcification. We showed that PHI enhances phosphate-induced osteoblast transdifferentiation of VSMCs, demonstrating that HIF proteins stabilized in a hypoxic environment accelerate the process of vascular calcification. Under hypoxic conditions, HIF-1α is dimerized in the cytosol and translocated to the nucleus as a regulatory protein that enhances the expression of pyruvate dehydrogenase kinase-4 protein, which is also important in vascular calcification. We demonstrated that HIF stabilization by PHI enhances PDK-4 expression in high Pi-induced smooth muscle cell mineralization. Elevated extracellular Pi induces phenotypic switch of VSMCs into osteoblast-like cells enhancing vascular calcification by reducing the expression of VSMC markers (smooth muscle 22α, SM22α; smooth muscle alpha-actin, SM α-actin) and up-regulating osteoblast-specific genes. We confirmed that high extracellular Pi down-regulates smooth muscle markers and induces osteochondrogenic genes. We showed that by stabilizing HIF, PHI FG4592 down-regulates smooth muscle-specific gene expression at the physiological Pi level too. Osteochondrogenic transformation of VSMCs also involves proteins regulating osteoblast development in bones *in vivo* play a pivotal role in the osteoblastic transformation and calcification of VSMCs. BMP-2 is commonly expressed in calcified human atherosclerotic plaques and triggers phosphate-induced calcification in human VSMC. It has been reported that hypoxia induces BMP-2 expression in osteoblasts and microvascular endothelial cells. Our data demonstrated that PHI FG4592 exacerbates Pi-induced expression of osteochondrogenic genes such as BMP2, Msx-2, and Sp7. Runx-2 transcription factor is essential for osteoblast transdifferentiation of VSMCs, however, recent evidence suggests that post-transcriptional modifications of Runx-2 regulate the expression of osteogenic genes. Phosphorylation of Ser451 (Serine 451) reduces Runx-2-dependent transactivation, which we demonstrated in our study. We showed that PHI decreases the phosphorylation of Ser451 in VSMCs in response Pi. However, phosphorylation of Ser451 is not influenced by the calcification medium alone. This suggests that the decreased phosphorylation level of Runx2

S451 might be required the co-presence of several calcification enhancers. Recent reports have shown that oxidative stress and reactive oxidation products are involved in the pathogenesis of vascular calcification, that is effectively reduced by zinc supplementation *in vitro.* We showed that zinc dose-dependently reduces PHI and / or high Pi-induced osteoblastic transdifferentiation of VSMCs. Metallothioneins are abundantly induced by high levels of zinc and cadmium and characterized by potent antioxidant properties. Here we showed that metallotioneinss are not involved in the protective effect of zinc against vascular calcification. Zinc plays an important role in vascular disease because low zinc level might be associated with increased calcification in CKD. In accordance with this study we detect low plasma zinc levels in dialysis dependent-CKD patients and in patients who underwent carotid endarterectomy present compared to control group. Our data supports the importance of monitoring and maintaining physiological plasma zinc levels in patients with increased risk for vascular calcification. Our results described here showed that PHI (FG4592) markedly exacerbates Pi-induced calcification of VSMCs. PHI FG4592 promotes vascular calcification by reducing the expression of smooth muscle cell-specific markers, increasing the expression of osteogenic genes and PDK-4 and modulating the phosphorylation level of Runx-2. Zinc is able to inhibit this pathophysiological process. Overall, one of the potential side effects of PHIs used in the treatment of anemia in patients with CKD is an increase in vascular calcification, which is one of the most significant complication in this group of patients due to inadequate mineral metabolism. The adverse effects of PHIs in this regard can be reduced by zinc supplementation, so in addition to monitoring hemoglobin and EPO levels in patients with CKD, monitoring of plasma zinc levels is also required.

The prevalence of vascular diseases and diabetes mellitus are continually rising worldwide. One of the known complications of diabetes is vascular calcification which necessitates the study of the relationship between the two diseases and the exploration of molecular mechanisms, exploring potential drug candidates that simultaneously treat both hyperglycemia and vascular calcification has high clinical relevance. A new insulin-sensitizing drug candidate molecule, BGP-15, may play an important role in the treatment of diabetes mellitus. The effect of BGP-15 in the pathomechanism of vascular disease as a complication of diabetes mellitus has not been studied in previous studies. Previous research has shown that high glucose promotes the mineralization processes of VSMCs. We showed that high glucose (11 mM) strongly aggravated the osteogenic transdifferentiation and mineralization of VSMCs in response to high Pi. Vascular smooth muscle cells are physiologically characterized by a contractile phenotype as evidenced by highly expressed cell-specific markers such as ACTA-2 and TAGLN.

Exogenous factors such as high extracellular inorganic Pi and hyperglycemia reduce the expression of smooth muscle cell-specific markers and cause phenotypic changes in VSMCs to osteoblast-like cells. We demonstrated that both high Pi and high glucose (11 mM) triggered the loss of SMC-specific markers, and the high glucose condition aggravated high Pi-induced downregulation of SMC markers. We showd that BGP-15 attenuated the loss of SMC-specific markers triggered by high Pi in both glucose conditions. These results indicate that BGP-15 may help prevent the phenotypic switch of VSMCs induced by hyperglycemia and / or hyperphosphatemia. During mineralization and osteochondrogenic transdifferentiation of VSMCs are governed by the activation of osteogenic proteins such as KLF-5, BMP-2, Msx-2, and Sp7. KLF-5 has a critical role in the initiation of high Pi-induced VSMC calcification and its expression is increased in the calcified aorta of adenine-induced uremic rats. In the present study, we showed that high Pi induced KLF-5 expression and nuclear translocation both of which were significantly aggravated by high glucose (11 mM). Importantly, BGP-15 attenuated both Pi and Pi and high glucose-induced KLF-5 expression and nuclear translocation suggesting the therapeutic potential of BGP-15 to improve VSMCs phenotypic switch early at its initiation step. Activation of the BMP-2/Msx-2 axis has a pivotal role in vascular calcification. Msx-2 promotes vascular calcification and enhances vascular mineralization in diabetic atherosclerotic calcification by governing osteochondrogenic reprogramming. Hyperglycemia increases BMP-2 expression, which enhances the calcification of VSMCs. Elevated plasma BMP-2 levels are associated with atherosclerosis burden and coronary mineralization in type 2 diabetes. Based on these results, inhibition of the BMP-2 / Msx-2 pathway may be a therapeutic option in high glucose and/or high Pi-induced vascular calcification. Our results show that BGP-15 significantly inhibits Pi-induced BMP-2 and Msx-2 expression in both normal and high glucose conditions. Sp7/Osterix is the second most important transcription factor in osteoblast differentiation and bone formation. This is supported by the fact that silencing of Sp7 expression inhibits VSMCs calcification in mice. We showed, that BGP-15 effectively inhibited both high Pi and high Pi-mediated mineralization of VSMCs aggravated by high glucose. PDK-4 is involved in the pathogenesis of vascular calcification through inhibition of autophagy and regulation of metabolism. Given that the PDK-4 inhibition is beneficial both in diabetes and vascular calcification, it is reasonable to assume that lowering the PDK-4 activity might have a dual positive effect on diabetic vascular calcification. Our data showed, that PD1-4 expression was nearly doubled by high Pi and tripled by high glucose and high Pi, however, BGP-15 reduced expression to control levels in both conditions.

Evidence shows that smooth muscle cells-derived extracellular vesicles (EVs) play an important role in vascular calcification. Extracellular vesicles contain both calcification inhibitory (MGP) and enhancement (Annexin A2) proteins and during calcification the homeostasis of these proteins are disturbed. MGP (Matrix Gla Protein) protein is known to inhibit arterial calcification and is present in reduced amounts during vascular calcification. Annexin A2 is found in large amounts in extracellular vesicles secreted during vascular calcification. Here, we demonstrated that EVs released by calcifying VSMCs contained elevated Annexin A2 and decreased MGP loads induced by high Pi, which were counteracted by BGP-15.

In previous studies, several anti-diabetic drugs have been reported to mitigate vascular calcification, especially the Metformin. Metformin decreases high Pi-induced osteogenic transdifferentiation via the cAMP-activated protein kinase pathway and the inhibition of pyruvate dehydrogenase kinase-4. These necessitate further research to explore the effect of BGP-15 in our in vitro calcification model. Our results showed that BGP-15 inhibits not only the high Pi-induced osteochondrogenic transformation and mineralization, but also the high Pi-induced calcification process aggravated by hyperglycemia.

Based on our results, we suggest that BGP-15 could be an effective therapeutic candidate against diabetes-associated vascular complications by preventing the loss of VSMCspecific markers, decreasing pro-calcific but increasing anti-calcific EV release, and inhibiting osteochondrogenic gene induction, together with the metabolic reprogramming of VSMCs induced by high Pi in normal glucose and high glucose conditions.

7. SUMMARY

The major inducer of vascular calcification is the disorder of mineral and bone metabolism in patients with chronic renal failure. Its exact mechanism is not clear yet, so there is no available drug therapy for clinicians to reverse this pathophysiological process. In our study we investigated the factors that may accelerate vascular calcification and the potential inhibitors that may inhibit this process.

In our research we investigated the possible role of zinc in the osteoblast transformation of VSMCs induced by high Pi and / or PHI. We demonstrate that PHI enhances Pi-mediated calcification of vascular smooth muscle cells as a result of continuous HIF activation which is inhibited by zinc in a dose-dependent manner. Zinc decreased the expression of osteoblast-specific genes (BMP-2, Msx-2, Sp7 / Osx), while the expression smooth muscle cell-specific markers (ACTA-2, TGLN) remained at the control level. We investigated the role of metallothionein as a primary zinc - binding protein in the mechanism of the inhibitory effect of zinc. We monitored the calcification process after the silencing of metallothionein gene in *vitro* and we found that the inhibitory effect of zinc on mineralization is independent metallotionenin. Decreased plasma zinc levels in patients undergoing hemodialysis and undergoing carotid surgery suggest that zinc may be of great importance in the prevention of vascular disease *in vivo*.

In addition to the dysregulated metabolism of minerals, hyperglycemia also plays role in the progression of vascular calcification. We have shown that high glucose (11 mM) increases Pi-induced osteoblast transdifferentiation in vascular smooth muscle cells compared to normal glucose (5,5 mM). Examining the effect of BGP-15 in our optimized *in vitro* calcification model we found that BGP-15 reduced the expression of osteogenic genes (BMP-2, KLF-5, Msx-2, PDK-4) in a dose-dependent manner and the nuclear translocation of KLF-5, Msx-2, Osx proteins at both normal and high glucose concentrations.

The result of this study demonstrate that in addition to PHI treatment in patients with CKD zinc plasma levels and stage of vascular calcification should be closely monitored and zinc supplementation might be considered.

In addition to the antidiabetic effect of BGP-15, it may be a potential drug candidate for the treatment of diabetes-associated vascular disease.

List of publications

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List of publications related to the dissertation

1. **Nagy, A.**, Pethő, D., Gesztelyi, R., Juhász, B., Balla, G., Szilvássy, Z., Balla, J., Gáll, T.: BGP-15 Inhibits Hyperglycemia-Aggravated VSMC Calcification Induced by High Phosphate.
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   DOI: http://dx.doi.org/10.3390/ijms22179263
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List of other publications

3. Pethő, D., Gáll, T., Hendrik, Z., **Nagy, A.**, Beke, L., Gergely, P., Méhes, G., Tóth, C., Gram, M., Akerström, B., Balla, G., Balla, J.: Ferryl Hemoglobin and Heme Induce A1-Microglobulin in Hemorrhaged Atherosclerotic Lesions with Inhibitory Function against Hemoglobin and Lipid Oxidation.
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