SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

THE ROLE OF HYPOXIA-INDUCIBLE FACTOR ACTIVATION IN GLUCOSE-INDUCED OSTEO-/CHONDROGENIC DIFFERENTIATION OF LENS EPITHELIAL CELLS AND CHRONIC KIDNEY DISEASE-ASSOCIATED HEART VALVE CALCIFICATION

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Introduction

Ectopic calcification is the deposition of calcium- and phosphate-containing salts in soft tissues. It can happen due to high levels of phosphate and/or calcium in the serum (metastatic calcification) or injury or trauma (dystrophic calcification). Although it was previously thought to be a passive process, recent research suggests that it is a regulated mechanism driven by an imbalance between calcification inducers and inhibitors. Ectopic calcification can occur in various soft tissues, including the cardiovascular system, which is the most studied process.

Cardiovascular calcification occurs due to the accumulation of hydroxyapatite crystals in blood vessels, heart valves, and myocardium, leading to heart diseases. It is caused by ageing, hereditary disorders, diabetes, and chronic kidney disease (CKD). The process involves transforming vascular smooth muscle cells (VSMCs) and valve interstitial cells (VICs) into osteoblast-like cells regulated by osteogenic transcriptional factors. The primary mechanisms of calcification include inflammation, apoptosis, ER stress, ROS production, and matrix vesicle release.

Vascular calcification (VC) is a common complication in patients with CKD. Hyperphosphatemia is a significant risk factor for VC, mediated through sodium-dependent phosphate cotransporters (Pit-1/2). Calcification inhibitors, such as Fetuin-A, pyrophosphate (PPi), osteopontin (OPN), and Matrix Gla Proteins (MGP).

The global estimate of diabetes in 2021 was 537 million, with a prevalence of 8.3%. The number is expected to increase to 783 million by 2045. Type 2 diabetes (T2D) is a significant cardiovascular risk factor, and VC is one of its major complications. The molecular mechanism of T2D leading to VC involves hyperglycemia, oxidative stress, inflammation, and advanced glycation end products (AGEs). AGEs accumulate in the blood vessels in T2D, bind to RAGE, and activate signalling pathways involved in VC development. Reactive oxygen species contribute to the formation of AGEs through glycoxidation, accelerating the progression of diabetic complications. Hyperinsulinemia, diabetic nephropathy, and hyperphosphatemia are also involved in the development of diabetic VC.

The eye is a complex sensory organ responsible for vision. It consists of several parts, including the cornea, conjunctiva, sclera, choroid, lens, retina, and optic nerve. Calcium deposition can occur in all layers of the eye, leading to eye calcification, which is associated with diseases such as pseudoxanthoma elasticum (PXE), CKD, and diabetes.

Conjunctival and corneal calcifications (CCC) are common in patients undergoing hemodialysis and are associated with high serum calcium levels and elevated mortality risk. Calcium deposition in the retina, particularly hydroxyapatite, is linked to diseases like age-related macular degeneration (AMD), PXE, and CKD. Retinal arteriolar calcification is seen in patients with chronic renal failure, while AMD and PXE patients develop calcifications in the subretinal pigment epithelial layer.

The HIF-1 pathway is essential for the cell's response to hypoxia. HIF-1 is a transcription factor composed of two subunits, HIF-1α and HIF-1β. In normoxic conditions, the HIF-1α subunit is degraded by the VHL complex, which requires hydroxylation by PHDs. PHD2 is the primary enzyme responsible for this reaction. In hypoxia, HIF-1α subunit dimerizes with HIF-1β to form transcriptionally active complexes. HIF's activity is regulated by FIH1, which prevents the recruitment of co-activators to the C-terminal domain. HIF pathway activation leads to the transcriptional activation of various genes involved in angiogenesis, metastasis, cell survival, and glycolysis. High HIF-1α expression is observed in diabetic patients and rats with VC. Hyperglycemia impairs hypoxia-induced VSMCs growth through HIF-1α inhibition. Hypoxia and high phosphate levels can cause arterial calcification by converting VSMCs into an osteo-/chondrogenic phenotype.

Aims

According to the findings in the literature, we formulated the following hypotheses:

Hypothesis 1: High glucose facilitates osteo-/chondrogenic transdifferentiation of human LECs by activating HIF-1 signalling.

Specific aims:

1. To establish a cellular model for studying the osteo-/chondrogenic transdifferentiation of human LECs.
2. To study the effect of high glucose levels on HIF-1 activation in LECs.
3. To explore the role of HIF-1 activation in osteo-/chondrogenic transdifferentiation of human LECs.
4. To provide insights into the molecular mechanism involved in the development of cataracts in diabetic individuals.

Hypothesis 2: CKD triggers heart calcification associated with increased osteogenic and hypoxia markers expressions in the heart.

Specific aims:

1. To establish a mouse model of CKD with heart tissue calcification.
2. To explore whether heart calcification is associated with increased expression of osteogenic and hypoxia markers.

Materials and Methods

- Animal experiments were performed per the University of Debrecen institutional ethics committee guidelines and the Directive of the European Parliament on the protection of animals used for scientific purposes and ARRIVE guidelines. 10 male C57BL/6 mice (8–10-week-old, n=5/group) were randomly divided into control (Ctrl) and CKD. CKD was induced with a diet containing 0.2% adenine and 0.7% phosphate for six weeks, followed by 0.2% adenine and 1.8% phosphate for another four weeks.
- Renal function and haematology parameters were determined from citrate anti-coagulated murine whole blood samples. A kinetic assay evaluated plasma urea, phosphate, and creatinine levels spectrophotometrically.
- Heart calcification was visualised with OsteoSense^{TM} dye. The dye was injected through the retro-orbital venous sinus. Imaging was performed 24 hours post-injection.
- Human lens epithelial cells (HuLECs) were cultured in DMEM at 37 °C in a humidified atmosphere with 5% CO_2. All experiments were performed on HuLECs between passages 4 and 10. HuLECs were treated with cell culture medium with either normal glucose (NG, 1 g/L) or high glucose (HG, 4.5 g/L). Osteogenic stimulus (OM) was provided by

supplementing the growth medium with inorganic phosphate (Pi, 1.0-3.0 mmol/L, pH 7.4) and Ca ($CaCl_2$, 0.3-0.9 mmol/L). In some experiments, the hypoxia mimetic drugs desferrioxamine (DFO, 20 μmol/L) and $CoCl_2$ (200 μmol/L) were used.

- Calcification was detected by Alizarin Red Staining. HuLECs were fixed and stained with 2% Alizarin Red solution (pH 4.2). To quantify AR staining, 100 μL of hexadecyl-pyridinium chloride solution (100 mmol/L) was added to each well, and the optical density was measured at 560 nm.
- To quantify Ca deposition, HuLECs were washed and decalcified with HCl (0.6 mol/L) for 30 minutes at room temperature. The Ca content of the HCl supernatant was determined by the QuantiChrome Calcium Assay Kit. Then the Ca content of the cells was normalized to the protein content and expressed as μg/mg protein.
- Alkaline phosphatase detection was performed using an alkaline phosphatase detection kit, samples were fixed with 4% PFA for 2 minutes, and then a ratio of 2:1:1 of Fast Red Violet solution: Naphthol phosphate solution: deionized water was added to each well for 15 minutes at room temperature. Then the cells were washed with Tris-buffered saline and 0.1% Tween (TBS-T).

- Cell viability was determined with MTT assay. After cell treatment, the cells were washed, and then 100 µL of 3-[4,5-dimethylthiazol-2-yl]-2,5- diphenyltetrazolium bromide (MTT, 0.5 mg/mL) solution was added. After 3 hours of incubation at 37 °C, the MTT solution was removed. The formazan crystals were dissolved in 100 µL of DMSO, and the optical density was measured at 570 nm.
- To evaluate protein expression western blot was performed, and lysate was electrophoresed on SDS-PAGE (7.5 or 10%) and blotted onto a nitrocellulose membrane. Membranes were blocked in ono-fat dry milk, then the membranes were incubated with primary antibody. The next day, the membranes were washed and incubated with HRP-labeled anti-rabbit or anti-mouse IgG secondary antibodies. Next, the antigen-antibody complexes were detected by enhanced chemiluminescence. Signals were detected by X-ray film or digitally by using a C-Digit Blot Scanner.
- Immunofluorescence Staining on HuLECs were done by cultured on coverslips in 12-well plates. After treatment, the cells were washed and fixed with 4% PFA then permeabilized with 0.1% Triton X-100 solution for 15 minutes. The slides were then blocked with 1% BSA for 45 minutes, incubated with either a crystalline alpha B chain antibody anti-Runx2 antibody,

anti-Sox9 antibody or anti-HIF1α antibody for 2 hours, and then incubated with goat anti-mouse IgG-CFL antibody for 1 hour. Slides were counterstained with 4’,6-diamidino-2-phenylindole (DAPI) to stain the nucleus.

- To knock down Runx2, HIF-1α, and HIF-2α gene expressions we used Lipofectamine RNAiMAX to transfect HuLECs with siRNA according to the manufacturer’s protocol.
- To detect osteocalcin HuLECs were washed with PBS, and then 100 µL of EDTA was added to each well. OCN was determined from the EDTA-solubilized supernatant by an enzyme-linked immunosorbent assay. OCN content was normalized to protein content and expressed as ng OCN/mg protein.
- Total RNA was isolated with Tri reagent, and cDNA was obtained using a High-Capacity cDNA Reverse Transcription Kit qPCR was performed using iTaq^{TM} Universal SYBR® Green Supermix. The comparative Ct method was used to calculate the expression level of the transcripts, and HPRT was used for normalization as an internal control.
- The results are presented as mean ± SD. For all in vitro studies, at least three independent experiments were carried out. GraphPad Prism software was used for statistical analysis. The Shapiro-Wilk test was used to determine the distribution's normality. Because all of the data passed the normality and

equal variance tests, parametric tests were used to calculate p values. A two-tailed Student's t-test was used to determine whether there were statistically significant differences between the two groups. One-way ANOVA was used to compare more than two groups, followed by Tukey's multiple comparisons test. We used a one-way ANOVA followed by Dunnett's post hoc test to compare each of the treatment groups to a single control group. A $p < 0.05$ value was considered significant.

Results

In vitro model for human LECs (HuLECs) calcification.

We developed a calcification model for HuLECs osteo-/chondrogenic transdifferentiation. HuLECs were cultured in either control (Ctrl) or osteogenic medium for up to 6 days. ECM calcification started on the third day and increased progressively until day 6 in OM-treated HuLECs, while no calcification was observed in Ctrl conditions. Calcium levels were quantified from HCl-solubilized ECM samples, revealing a more than 16-fold increase in ECM calcium content in osteogenic medium-treated HuLECs compared to controls, confirming AR staining results. Additionally, OCN levels, indicative of bone-specific calcium-binding protein, significantly increased by 8-fold in response to osteogenic stimuli compared to Ctrl, as measured in EDTA-solubilized ECM of HuLECs.

High glucose promotes calcification of HuLECs.

LECs play crucial roles in cataractogenesis, contributing to processes such as proliferation, migration, extracellular matrix accumulation, epithelial-mesenchymal transition (EMT), and osteo-/chondrogenic transdifferentiation, all relevant to cataract formation. To investigate the impact of elevated glucose levels on LECs osteogenic transdifferentiation, we used HuLECs as an experimental model. HuLECs exhibited typical epithelial morphology and expressed alpha crystalline, a major lens crystalline type. Previous research has shown HuLECs' ability to transdifferentiate into osteoblast-like cells, producing an extracellular matrix rich in hydroxyapatite, a prevalent component in cataractous lenses. In culture, confluent HuLECs were exposed to an osteogenic medium (OM) with excess phosphate (Pi) and calcium (Ca) under normal glucose (NG) and high glucose (HG) conditions. Alizarin red staining revealed earlier calcification onset in the HG group at lower Pi and Ca concentrations than NG, indicating that glucose facilitates OM-induced calcification. Further investigation showed accelerated and intensified calcification in HG compared to NG over time. Quantification of ECM calcium content corroborated these findings, demonstrating dose- and time-dependent calcification in HuLECs, with HG promoting OM-induced calcification. Assessment of cell viability after seven days of treatment revealed a moderate decrease in viability under NG conditions induced by OM, while such effect was not observed under HG conditions, suggesting that HG-induced promotion of calcification in HuLECs is not associated with cell death.

High glucose promotes the calcification of HuLECs in a Runx2-dependent manner.

Runx2, a key transcription factor in osteo-/chondrogenic transdifferentiation, was investigated for its role in HG-induced calcification of HuLECs. Treatment with OM (2.5 mmol/L Pi and 0.3 mmol/L Ca) for 48 hours modestly increased Runx2 expression under NG conditions, whereas HG triggered a robust elevation in Runx2 expression under Ctrl conditions, further intensified in OM-treated cells. HG-induced nuclear translocation of Runx2. Downregulation of Runx2 using siRNA almost completely inhibited OM-induced calcification under HG conditions, highlighting its critical involvement in HG-induced HuLEC calcification.

Further investigation into additional markers of osteo-/chondrogenic transdifferentiation revealed moderate increases in Sox9 and ALP protein expression under OM treatment, evaluated by Western blot analysis. Under Ctrl conditions, HG upregulated both Sox9 and ALP expression compared to NG Ctrl. OM stimulation under HG conditions intensified Sox9 but not ALP expression relative to HG Ctrl. HG-induced nuclear translocation of Sox9, as shown by immunofluorescence staining. ALP activity staining revealed increased activity in HuLECs under OM treatment, further heightened under HG conditions. OCN concentration in HuLECs significantly increased due to OM under HG conditions compared to NG conditions, quantified by ELISA.

High glucose induces HIF signalling and promotes calcification of HuLECs in a HIF-1α- and HIF-2α- dependent manner.

The potential role of HIF-1 pathway activation in HG-induced calcification in HuLECs was investigated. HG significantly upregulated HIF-1α and HIF-2α expression under Ctrl and OM conditions. Under HG conditions, OM elevated HIF-2α expression compared to Ctrl, whereas OM did not further upregulate HIF-1α expression, additionally, HG-induced nuclear translocation of HIF-1α under OM conditions. Furthermore, OM treatment increased the mRNA expression of HIF-1 target genes, including Glut-1, VEGFA, LDHA, and PDK4, under NG conditions, which was further increased in HG conditions. To investigate the HIF-1α and HIF-2α role in HuLEC calcification, their expression was knocked down under HG conditions. Successful knockdown was confirmed by western blot analysis. Subsequent analysis revealed that knockdown of either HIF-1α or HIF-2α resulted in partial inhibition of calcification, as shown by alizarin red staining, suggesting that both HIF-1α and HIF-2α play vital roles in HG-induced promotion of HuLEC calcification.

Hypoxia mimetics promote calcification of HuLECs.

To elucidate the involvement of HIF alpha subunit stabilisation in HuLECs calcification, hypoxia mimetic agents $CoCl_2$ and desferrioxamine (DFO) were used. Both $CoCl_2$ and DFO were able to stabilise both HIF-1α and HIF-2α under NG conditions. An investigation into whether $CoCl_2$ and DFO promote OM-induced HuLECs calcification under NG conditions was conducted. HuLECs were treated with Ctrl or OM in the presence or absence of $CoCl_2$ or DFO for seven days. The results showed that both hypoxia mimetic agents further intensified OM-induced calcification.

High phosphate activates osteogenic and hypoxia pathways in the hearts of CKD mice.

CKD was induced in C57BL/6 mice via a two-phase diet: adenine (0.2%) and moderately elevated phosphate (0.7%) for six weeks, followed by adenine (0.2%) and high phosphate (1.8%) for another four weeks. The Ctrl group received a standard mouse diet containing 0.3% phosphate. Significant reductions in body weight and elevated plasma levels of urea, creatinine, and phosphate indicated disease progression. Analysis of mRNA levels of osteo-/chondrogenic transcription markers Runx2 and Sox9, as well as hypoxia markers HIF-1α and HIF-2α in the hearts of Ctrl and CKD mice, revealed substantial upregulation in the heart tissue of CKD mice compared to Ctrl. Additionally, OsteoSense^{TM} staining showed significantly higher fluorescence intensity in cardiac tissue of CKD mice compared to Ctrl mice, indicating increased osteogenic activity.

Discussion

Osteo-/chondrogenic transdifferentiation is essential in physiological bone and cartilage growth, repair, and remodelling. However, pathological osteo-/chondrogenic transdifferentiation is involved in ectopic calcification. VC is the most studied form of ectopic calcification, mostly in VSMCs and VICs; thus, the results of my dissertation will be discussed in comparison to these cell lines. Cataracts, the opacification of the lens, is a major health issue and the leading cause of blindness globally. Diabetic patients have a fivefold increased risk of cataract formation.

Several mechanisms have been attributed to the pathophysiology of diabetes-associated cataract formation, including polyol pathway upregulation, non-enzymatic glycation, and subsequent lens crystallin aggregation.

Composition analysis of senile and congenital cataracts revealed the presence of hydroxyapatite in the cataractous lenses. The current proposed mechanism for cataract formation could not explain this finding. Thus, our group investigated the possibility that HuLECs can undergo transdifferentiation into osteoblast-like cells. That work provided evidence that HuLECs upregulate the expression of osteo-/chondrogenic markers in response to osteogenic stimuli. Additionally, they found that the OCN levels and calcium content were significantly elevated in human cataractous lenses compared to control lenses. This suggests a potential role for osteo-/chondrogenic differentiation of HuLECs in lens calcification, similar to the process observed in VC.

VC and cataract formation are strongly associated with aging, diabetes, and CKD. Moreover, several studies have revealed that ectopic calcification and cataracts may share common etiological factors.

In T2D patients, calcification of the peripheral arteries is considered an independent predictor of cardiovascular mortality, stroke, and coronary heart disease. Hyperglycemia is commonly associated with diabetes; in addition, it promotes VC by inducing inflammation, endothelial dysfunction, and oxidative stress. High glucose enhances osteo-/chondrogenic marker expression, thereby enhancing VSMC calcification. In agreement with these previous, our results show that HG is a potent inducer of calcification in HuLECs.

Runx2 and Sox9 play key roles in VC development. Our research revealed that high glucose-induced apoptosis does not affect HuLEC calcification. Instead, the expression and nuclear translocation of transcription factors Runx2 and Sox9 were found to be involved in this process.

Hypoxia plays a critical role in the pathogenesis of T2D. In diabetic patients, hypoxia can affect several organs/tissues. It is common in cardiovascular disease, as blood flow to the affected tissue is reduced.

Our study found that hypoxia accelerated the development of VC, and HIF-1 has been identified as a potential target against it.

CKD was induced in C57BL/6 mice using adenine and a high-phosphate-containing diet. Adenine induces CKD by damaging the interstitial layer of tubules and leading to renal dysfunction. In addition, an adenine diet can cause severe renal failure in mice, which is characterised by elevated blood urea nitrogen levels and irreversible kidney damage. We measured serum urea, creatinine, and phosphate levels to evaluate the kidney function of mice fed with adenine and a high-phosphate-containing diet. We found that these values were elevated, confirming that CKD induction was successful. Using OsteoSense^{TM} staining, we were able to assess heart tissue calcification. In addition, we found elevated expression of hypoxic markers HIF-1α and HIF-2α as well as the osteo-/chondrogenic markers Sox9 and Runx2 in the heart derived from mice with CKD. In conclusion, our studies highlighted the role of HIF-1 pathway activation in soft tissue calcification, which can be relevant in diabetes-induced cataract formation and CKD-associated valve calcification.

Summary

- Ectopic calcification, like vascular calcification, is linked to ageing, diabetes, and chronic kidney disease (CKD).
- Type 2 diabetes increases cataract risk, particularly with prolonged hyperglycemia.
- Osteo-/chondrogenic transdifferentiation contributes to vascular calcification and cataract formation.
- High glucose levels induce osteo-/chondrogenic markers in lens cells (HuLECs) and stabilize hypoxia-inducible factors.
- In HuLECs, high glucose triggers calcification, mitigated by Runx2 deficiency and HIF-1α or HIF-2α silencing.
- A CKD mouse model exhibits cardiac calcification, elevated HIF-1α/HIF-2α, and osteo-/chondrogenic marker expression.
- HIF-1 pathway activation may play a role in ectopic calcification, relevant to diabetic cataracts and CKD-associated valve calcification.

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Subject: PhD Publication List

Candidate: Haneen Muntasir Ababneh
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List of publications related to the dissertation

1. **Ababneh, H. M.**, Balogh, E., Csiki, D. M., Lente, G., Fenyvesi, F., Tóth, A., Jeney, V.: High glucose promotes osteogenic differentiation of human lens epithelial cells through hypoxia-inducible factor (HIF) activation.
   J. Cell. Physiol. [Epub ahead of print], 2024.
   DOI: http://dx.doi.org/10.1002/jcp.31211
   IF: 5.6 (2022)
2. Csiki, D. M.*, **Ababneh, H. M.***, Tóth, A., Lente, G., Szöőr, Á., Tóth, A., Fillér, C., Juhász, T., Nagy, B. J., Balogh, E., Jeney, V.: Hypoxia-inducible factor activation promotes osteogenic transition of valve interstitial cells and accelerates aortic valve calcification in a mice model of chronic kidney disease.
   Front. Cardiovasc. Med. 10, 1-15, 2023.
   DOI: http://dx.doi.org/10.3389/fcvm.2023.1168339
   * These authors contributed equally to this work.
   IF: 3.6 (2022)
3. Chowdhury, A., Balogh, E., **Ababneh, H. M.**, Tóth, A., Jeney, V.: Activation of Nrf2/HO-1 Antioxidant Pathway by Heme Attenuates Calcification of Human Lens Epithelial Cells.
   Pharmaceuticals (Basel). 15 (5), 1-13, 2022.
   DOI: http://dx.doi.org/10.3390/ph15050493
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List of other publications

4. Tóth, A., Csiki, D. M., Nagy, B. J., Balogh, E., Lente, G., **Ababneh, H. M.**, Szöőr, Á., Jeney, V.: Daprodustat Accelerates High Phosphate-Induced Calcification Through the Activation of HIF-1 Signaling.
   Front. Pharmacol. 13, 1-12, 2022.
   DOI: http://dx.doi.org/10.3389/fphar.2022.798053
   IF: 5.6

5. Balogh, E., Chowdhury, A., **Ababneh, H. M.**, Csiki, D. M., Tóth, A., Jeney, V.: Heme-Mediated Activation of the Nrf2/HO-1 Axis Attenuates Calcification of Valve Interstitial Cells.
   Biomedicines. 9 (4), 1-17, 2021.
   DOI: http://dx.doi.org/10.3390/biomedicines9040427
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