GRANT CLOSING REPORT

1. Background and aims

Calcification of the cardiovascular system, especially the aorta and heart valves is linked to an increased risk of heart disease, stroke and atherosclerotic plaque rupture. Calcification is an actively regulated process that develops as a consequence of unbalanced activity of calcification activators and inhibitors. Pro-calcifying milieu triggers osteochondrogenic transdifferentiation of vascular smooth muscle cells (VSMCs) and valve interstitial cells (VICs). Calcified vessels and heart valves are hypoxic, and in this project we investigated the possibility that hypoxia is not only present, but plays an etiopathogenic role in the calcification process.

Our aim was to understand the role of hypoxia-inducible factor 1 (HIF1) activation, and the importance of glycolytic switch in the osteochondrogenic transdifferentiation of VSMCs and VICs. We also aimed to investigate whether metabolic changes are important to fuel metabolite requirement of epigenetic reprogramming during osteochondrogenic transdifferentiation of VSMCs. Finally, we wanted to understand the effect of HIF1 activation on vascular and valve calcification *in vivo*.

2. Major published results

2.1. Daprodustat accelerates high phosphate-induced calcification through the activation of HIF-1 signaling

CKD is a public health problem worldwide affecting about 10% of the general population in high- and middle-income countries. CKD patients have five- to ten-fold higher risk of premature death than the general population, which is largely attributed to death from cardiovascular diseases. Additionally, CKD is frequently associated with other chronic diseases, such as anemia. CKD-associated anemia is a considerable burden because it significantly worsens the quality of life of CKD patients, increases hospitalization, causes cognitive impairment, propagates the progression of CKD, and increases the risk of cardiovascular events and mortality.

Daprodustat (DPD) is a new generation drug to treat CKD-associated anemia that relies on the activation of the HIF pathway, leading to transcriptional activation of numerous genes, including erythropoietin, and a subsequent increase in erythropoiesis.

In this project we showed that DPD activates the HIF-1 pathway and increases high phosphate-induced calcification in VSMCs *in vitro* and in mouse aorta rings *ex vivo*. Our data revealed that DPD-induced calcification is HIF-1 and ROS-dependent. Using a CKD mice model, we found that DPD

administration improves anemia, but it increases aorta calcification. Based on this work we urge clinical studies with a long follow-up period to evaluate the possible risk of sustained activation of HIF-1 by DPD in accelerating medial calcification in CKD patients with hyperphosphatemia.

This work has been published: Tóth A, Csiki DM, Nagy B Jr, Balogh E, Lente G, Ababneh H, Szöőr Á, **Jeney V**. Daprodustat Accelerates High Phosphate-Induced Calcification Through the Activation of HIF-1 Signaling. Front Pharmacol. 2022 Feb 7;13:798053. doi: 10.3389/fphar.2022.798053. eCollection 2022. (IF: 5.81)

2.2. Hypoxia-inducible factor activation promotes osteogenic transition of valve interstitial cells and accelerates aortic valve calcification in a mice model of chronic kidney disease

Valve calcification is a widespread complication in CKD patients. Valve calcification is an active process with the involvement of in situ osteogenic transition of VICs. Valve calcification is accompanied by the activation of the HIF pathway, but the role of HIF activation in the calcification process remained undiscovered.

In this project using *in vitro* and *in vivo* approaches we addressed the role of HIF activation in osteogenic transition of VICs and CKD-associated valve calcification. We showed elevation of osteogenic (Runx2, Sox9) and HIF activation markers (HIF-1α and HIF-2α) in the kidney of adenine-induced CDK mice. We found that high phosphate (Pi) induced upregulation of osteogenic (Runx2, alkaline-phosphatase, Sox9, osteocalcin) and hypoxia markers (HIF-1α, HIF-2α, Glut-1), and calcification in VICs. Down-regulation of HIF-1α and HIF-2α inhibited, whereas further activation of HIF pathway by hypoxic exposure (1% O_2) or hypoxia mimetics [desferrioxamine, CoCl2, Daprodustat (DPD)] promoted Pi-induced calcification of VICs. Pi augmented the formation of reactive oxygen species (ROS) and decreased viability of VICs, whose effects were further exacerbated by hypoxia. N-acetyl cysteine inhibited Pi-induced ROS production, cell death and calcification under both normoxic and hypoxic conditions. Importantly, we showed that DPD treatment corrected anemia but promoted aortic valve calcification in the adenine-induced CKD mice model. We concluded that HIF activation plays a fundamental role in Pi-induced osteogenic transition of VICs and CKD-induced valve calcification. The cellular mechanism involves stabilization of HIF-1α and HIF-2α, increased ROS production and cell death. Targeting the HIF pathways may thus be investigated as a therapeutic approach to attenuate aortic valve calcification.

This work has been published: Csiki DM, Ababneh H, Tóth A, Lente G, Szöőr Á, Tóth A, Fillér C, Juhász T, Nagy B Jr, 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. 2023 Jun 2;10:1168339. doi: 10.3389/fcvm.2023.1168339. eCollection 2023. (IF: 5.846)

2.3. The role of ROS in vascular calcification

ROS are byproducts of aerobic metabolism. Physiological levels of ROS play a central role in redox signaling, whereas excessive ROS production causes oxidative stress, which is implicated in the initiation and progression of numerous diseases. Accumulating evidence suggests that (i) vascular calcification is associated with elevated ROS production, and (ii) excess ROS play a pathophysiological role in the process of vascular calcification. We wrote a review in which we summarized our current knowledge about the involvement of ROS in the development of vascular calcification.

This work has been published: Tóth A, Balogh E, **Jeney V**. Regulation of Vascular Calcification by Reactive Oxygen Species. Antioxidants (Basel). 2020 Oct 8;9(10):963. doi: 10.3390/antiox9100963. (IF: 6.313)

2.4. Activation of the Nrf2/HO-1 axis attenuates calcification of VICs

The elevation of reactive oxygen species (ROS) formation plays a role in both vascular and valve calcification. Normally, ROS formation is counterbalanced by a complex antioxidant defense system. Nuclear factor erythroid 2-related factor 2 (Nrf2) is an important element of this antioxidant network. Under homeostasis, Nrf2 binds to its negative regulator, Kelch-like ECH-associated protein 1 (Keap1) in the cytosol. This interaction initiates the polyubiquitinylation and proteasomal degradation of Nrf2. Different oxidants and electrophiles induce the modification of cysteine residues of Keap1, leading to the disruption of the Nrf2–Keap1 interaction, Nrf2 stabilization, and nuclear translocation. Once in the nucleus, Nrf2 binds to the antioxidant response elements located at the promoter regions of a variety of antioxidant genes. Under this project we investigated whether upregulation of the Nrf2 system attenuates VICs calcification.

We used heme to activate the Nrf2 system in VICs. We found that heme inhibited VICs calcification and phosphate-induced increase in alkaline phosphatase and osteocalcin (OCN) expression. Heme induced Nrf2 and HO-1 expression in VICs. We showed that heme lost its anti-calcification potential when we blocked transcriptional activity Nrf2 or enzyme activity of HO-1. Our data revealed that the heme catabolism products bilirubin, carbon monoxide, and iron, and also ferritin inhibited VICs calcification. We concluded that heme-mediated activation of the Nrf2/HO-1 pathway inhibits the calcification of VICs. The anti-calcification effect of heme is attributed to the end products of HO-1-catalyzed heme degradation and ferritin.

This work has been published: Balogh E, Chowdhury A, Ababneh H, Csiki DM, Tóth A, **Jeney V**.

Heme-Mediated Activation of the Nrf2/HO-1 Axis Attenuates Calcification of Valve Interstitial Cells. Biomedicines. 2021 Apr 15;9(4):427. doi: 10.3390/biomedicines9040427. (IF: 6.081)

3. Major results not yet published

The following works are closely associated with the original proposal and these results will likely be published within a year in two papers.

3.1. High glucose promotes VSMCs calcification in a glucose transporter-1 (Glut-1)-dependent manner

To study the role of the glycolytic pathway activation in vascular calcification, first we exposed VSMCs to calcification medium with normal (1 g/L) or high glucose content (4.5 g/L). We found that high glucose promotes VSMCs calcification as revealed by a positive Alizarin red staining (**Figure 1A**). High glucose induced the expression of Glut-1 (**Figure 1B**). To investigate whether Glut-1 played a critical role in high glucose-induced VSMCs calcification, we knock-down Glut-1 expression with Glut-1-specific siRNA (**Figure 1C**). We did not observe calcification in Glut-1 knock-down cells which suggests that Glut-1, or Glut-1-mediated glucose uptake is critically involved in the calcification process of VSMCs (**Figure 1D**).

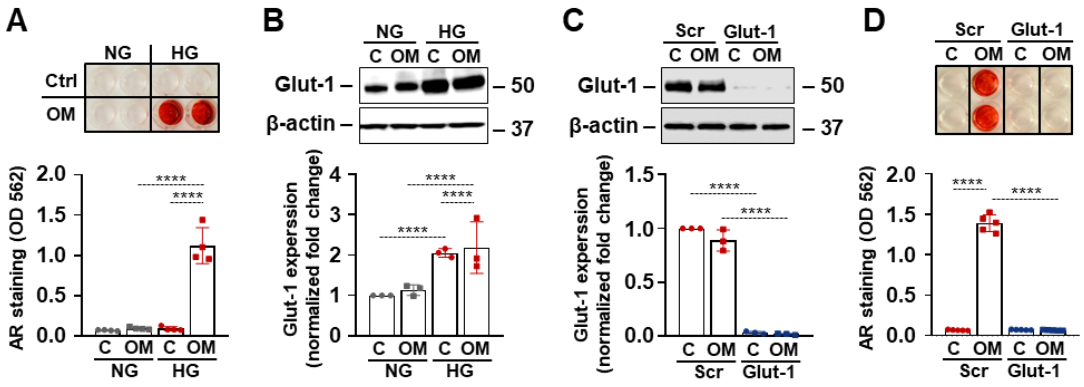

Figure 1. Confluent human aorta smooth muscle cells (VSMCs) were cultured in control (Ctrl) or osteogenic medium (OM: 2.5 mmol/L Pi, 0.6 mmol/L Ca) with normal glucose (NG, 1 g/L) and high glucose (HG, 4.5 g/L) conditions. (A) Representative Alizarin red staining and quantification (day 5). (B) Glut-1 expression (48 h). (C) VSMCs were kept in Ctrl or OM in HG conditions in the presence of Glut-1 or scrambled siRNA. (C) Protein expression of Glut-1 was detected by Western blot in whole cell lysates (48 h). Membranes were reprobed for β-actin. Representative Western blots and relative expression of Glut-1 normalized to β-actin from 3 independent experiments. (D) Representative Alizarin red staining (day 5) and quantification. Data are expressed as the mean ± SD. Each panel shows the result of a representative experiment. Ordinary one-way ANOVA followed by Tukey's multiple comparison test was used to calculate *p* values. ****$p<0.001$

3.2. Glycolysis is essential for HG-induced VSMCs calcification

To further investigate the role of glycolytic reprogramming in vascular calcification we applied specific inhibitors of the glycolytic enzymes and investigated their effect on OM-induced VSMCs calcification. We used 2-Deoxy-D-glucose (2-DG) to inhibit hexokinase (HK), 3-(3-Pyridinyl)-1-(4-pyridinyl)-2-

propen-1-one (3-PO) to inhibit 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3), and oxamate (Oxa) to inhibit lactate dehydrogenase A (LDHA) (**Figure 2A**). We found that inhibition of any of these key glycolytic enzymes is associated with attenuation of OM-induced VSMCs calcification as revealed by alizarin red staining (**Figure 2B**).

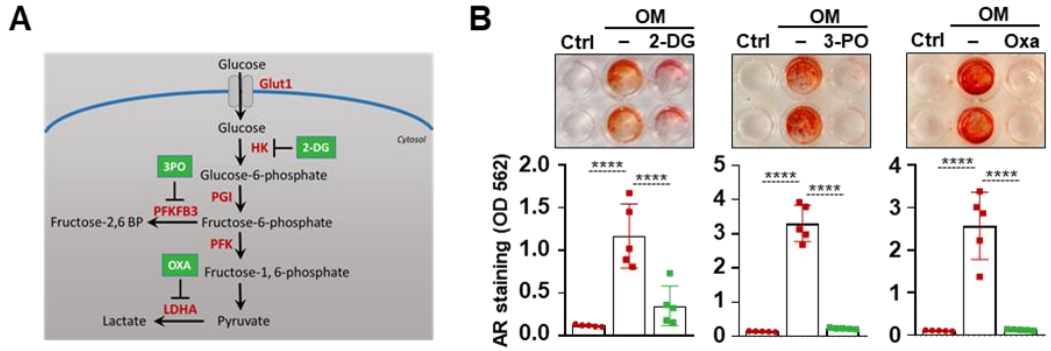

Figure 2. (A) Scheme of the glycolytic pathway and the applied inhibitors in green boxes. (B) Confluent VSMCs were cultured in high glucose control (Ctrl) or osteogenic medium (OM: 2.5 mmol/L Pi, 0.6 mmol/L Ca) with or without inhibitors of glycolytic enzymes 2-DG (1 mmol/L), 3-PO (5 µmol/L) and Oxa (10 mmol/L). (A) Representative Alizarin red staining and quantification (day 5). Data are expressed as the mean ± SD. Each panel shows the result of a representative experiment. Ordinary one-way ANOVA followed by Tukey's multiple comparison test was used to calculate *p* values. ****$p<0.001$

3.3. Modification of the pyruvate-acetyl-CoA conversion effects HG-induced VSMCs calcification

Then we looked at whether pyruvate to acetyl-CoA conversion play a regulatory role in the calcification process. First we used CPI-613, an inhibitor of pyruvate dehydrogenase (PDH), then we applied dichloroacetate (DCA), an inhibitor of pyruvate dehydrogenase kinase (PDK), an enzyme that inhibits PDH activity via phosphorylation (**Figure 3A**). CPI increased, whereas DCA inhibited HG-induced VSMCs calcification (**Figure 3B**). CPI is considered as a glycolysis enhancer, because inhibition of PDH reduces entry of pyruvate into mitochondria thereby attenuates oxidative phosphorylation. Therefore this result is an agreement with our previous result that enhancing glycolysis increases VSMCs calcification.

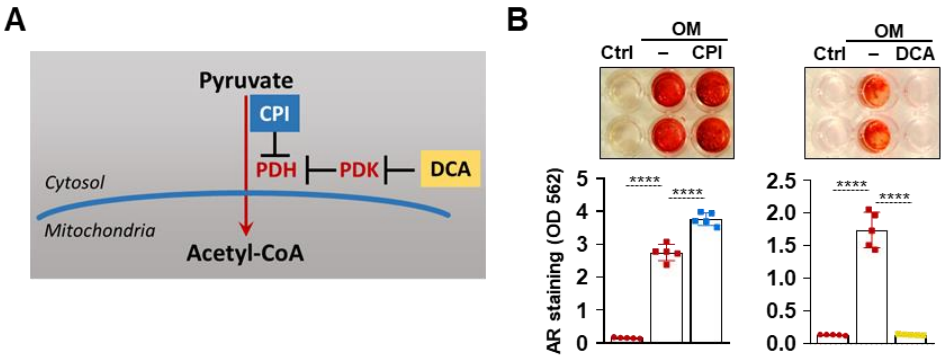

Figure 3. (A) Scheme of the pyruvate to acetyl-CoA conversion and the applied inhibitors. (B) Confluent VSMCs were cultured in high glucose control (Ctrl) or osteogenic medium (OM: 2.5 mmol/L Pi, 0.6 mmol/L Ca) with or without inhibitor of pyruvate dehydrogenase (PDH) CPI-613 and inhibitor of pyruvate dehydrogenase kinase dichloroacetate (DCA, 10 mmol/L). (A) Representative Alizarin red staining and quantification (day 5). Data are expressed as the mean ± SD. Each panel shows the result of a representative experiment. Ordinary one-way ANOVA followed by Tukey's multiple comparison test was used to calculate *p* values. ****$p<0.001$

3.4. Interplay between ER stress signalling a HIF-1 pathway in the regulation of calcification

As shown previously (2.1) we demonstrated that Daprodustat (DPD), a prolyl hydroxylase inhibitor accelerates Pi-induced calcification of VSMCs via HIF-1 pathway activation. Furthermore, our results showed that DPD increases CKD-associated aortic calcification in CKD mice. Emerging evidence suggest that ER stress and unfolded protein response (UPR) play an important role in the pathomechanism of vascular calcification.

Based on this evidence we aimed to investigate the involvement of ER stress in DPD-induced soft tissue calcification in a murine model of CKD, as previously described. Mice (C57BL/6, 8-12 weeks old, male) were fed with a diet containing adenine (0.2%) and elevated phosphate (0.7%) for 6 weeks, then the phosphate content was further increased up to 1.8% and mice received this diet for an additional 3 weeks. DPD was administered orally at a dose of 25 mg/kg/day in the last 3 weeks of the experiment (**Figure 4A**).

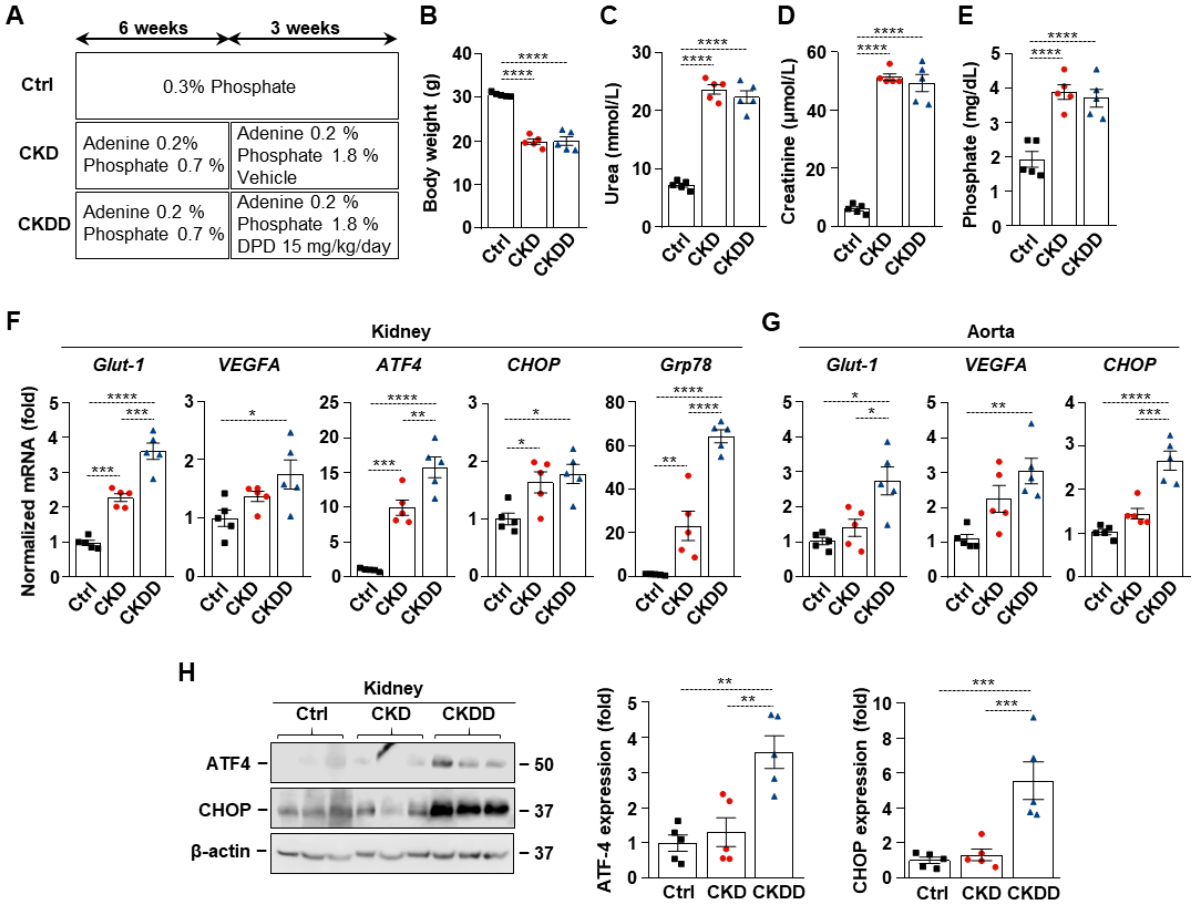

Figure 4. DPD promotes HIF pathway activation and ER stress in kidney and aorta in C57BL/6 mice fed with adenine+high Pi diet. (A) Scheme of the experimental protocol. (B) Body weight, (C) serum urea, (D) serum creatinine, (E) serum phosphorus level. (F) Normalized mRNA expressions of mice kidney samples. (G) Protein expressions of ATF-4 and CHOP in mice kidney lysates. Membranes were reprobed for β-actin. Representative Western blots and analyses (n=3). (H) Normalized mRNA expression of mice aorta samples. Data are expressed as mean±SD, n=5. Ordinary one-way ANOVA followed by Tukey's multiple comparison test were used to calculate p values. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001

CKD was developed in all mice fed with the adenine+high phosphate diet based on elevated serum urea and creatinine levels regardless the DPD treatment (**Figure 4B-E**). We addressed mRNA expression of HIF-related markers (VEGF, Glut-1) and ER stress-related markers (ATF-4, CHOP, Grp78) in kidney (**Figure 4F**). Next, we also addressed the protein expression of ATF-4 and CHOP in DPD treated CKD mice (Figure 4G). Parallel with this, we investigated the mRNA expression of HIF and ER stress markers in aorta of DPD treated CKD mice (**Figure 4H**).

Induction of HIF pathway and ER stress was associated with increased aortic and kidney calcification as revealed by Osteosense staining and aortic calcium measurement (**Figure 5A-E**). The mechanism of vascular calcification shares similarities with physiological bone formation, therefore, we investigated osteo/chondrogenic markers in kidney and aorta of DPD treated CKD mice, and found upregulation of these markers in the kidney and aorta of DPD-treated CKD mice (**Figure 5C-D**).

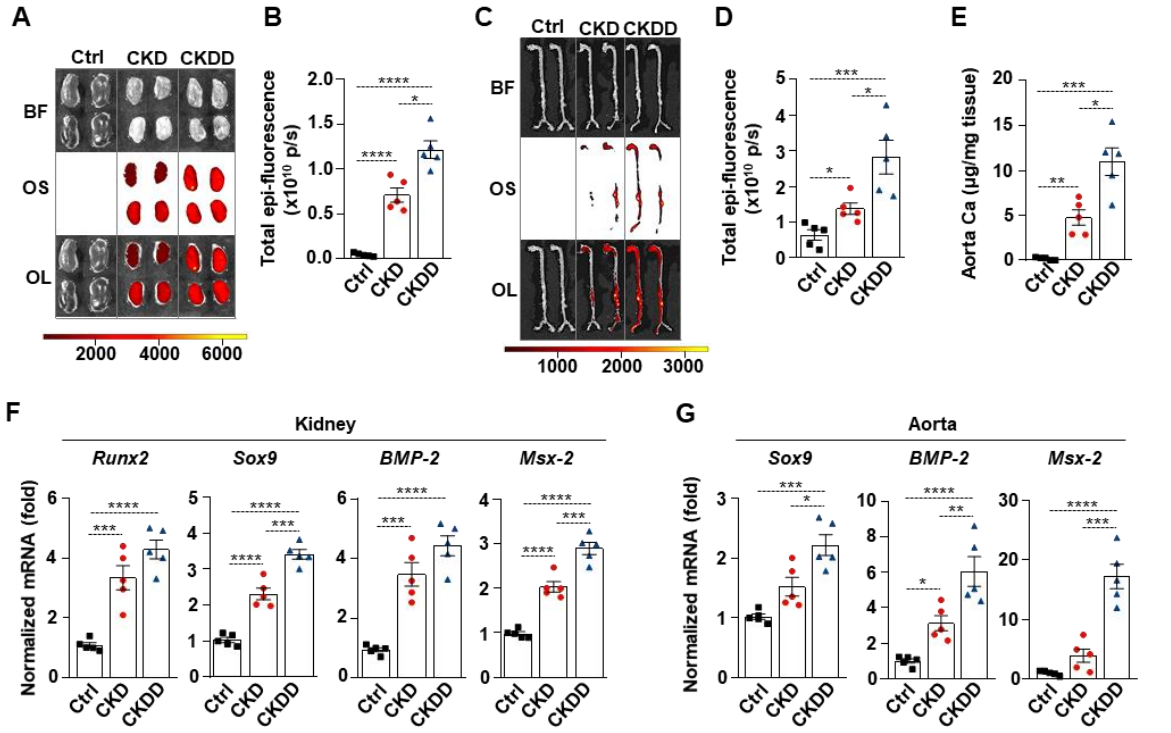

Figure 5. DPD increases calcification in kidney and aorta of CKD mice. Mice were treated as described previously on Figure 3A. (A-B) Daprodustat increases CKD-associated kidney and aorta calcification in CKD-mice. Brightfield and macroscopic reflectance imaging of (A) kidney and (B) aorta, quantification and Ca content of aortas normalized to protein level. (C) Normalized mRNA expressions of kidney and (D) aorta samples. Data are expressed as mean±SD, n=5. Ordinary one-way ANOVA followed by Tukey's multiple comparison test were used to calculate p values. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001

To further investigate the role of DPD-induced ER stress in the calcification process, we performed *in vitro* experiments on VSMCs. DPD is a hypoxia mimetic drug that increases the expression of HIF-1α and Glut-1 in a dose-dependent manner (**Figure 6A**). Hypoxia is a pathophysiological condition that induces ER stress through sensor protein kinase RNA-like ER kinase (PERK), therefore, next we investigated PERK activation in VSMCs in response to Pi and Pi+DPD. Pi-induced PERK phosphorylation was further exacerbated by DPD (**Figure 6B**).

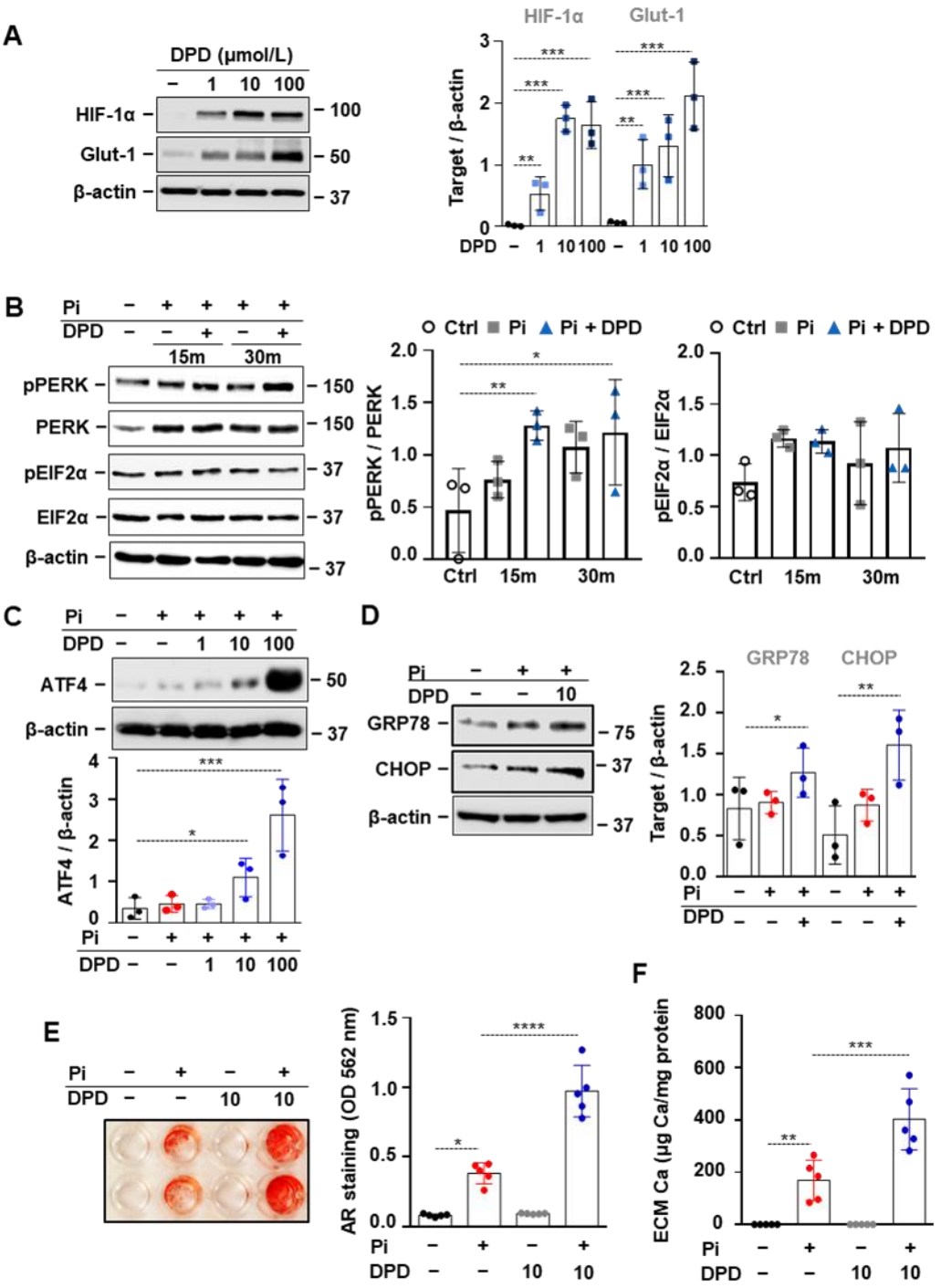

Figure 6. DPD induces hypoxia signaling and endoplasmic reticulum stress and promotes Pi-induced calcification of VSMCs. VSMCs were cultured in the presence of DPD (1-100 μmol/L). (A) Protein expression of HIF-1α, Glut-1 in whole cell lysates was evaluated after 24 hours of treatment. Membranes were reprobed for β-actin. Representative Western blots and densitometry analyses on the relative expression of HIF-1α and Glut-1 (n=3). (B) Protein expression of phospho-PERK (pPERK), PERK, phospho-EIF2α (pEIF2α) and EIF2α in whole cell lysates (15 min, 30 min). Membranes were reprobed for β-actin. Representative Western blots and relative expression of pPERK normalized to PERK and pEIF2α normalized to EIF2α (n=3). (C-D) Protein expression of ATF-4, CHOP and Grp78 in whole cell lyasates (6 h). Membranes were reprobed for β-actin. (C) Representative Western blots and (D) densitometry analyses on the relative expression of ATF-4, CHOP and Grp78 (n=3). (E-F) VSMCs were cultured in osteogenic medium supplemented by elevated phosphate (2 mmol/L Pi) in the presence or absence of DPD (10 μmol/L). (E) Representative Alizarin Red staining (day 4) and quantification (n=5), and (F) Ca content of HCl-solubilized ECM samples. Data are expressed in as mean ± SD. Ordinary one-way ANOVA followed by Tukey's multiple comparison test were used to calculate p values. *p<0.05, **p<0.01, ***p<0.005, ****p<0.001

Additionally, Pi and Pi+DPD slightly increased the level of phosphorylated initiation factor eukaryotic translation initiator factor 2α (pEIF2α) over control (**Figure 6B**). The activation of the PERK pathway by Pi+DPD induced a massive upregulation of the expression of activating transcription factor 4 (ATF4) and the pro-apoptotic transcription factor C/EBP-homologous protein (CHOP) and Grp78 (**Figure 6C-D**). Then we addressed the pro-calcifying effect of DPD in VSMCs. We treated VSMCs in osteogenic medium supplemented with 2.5 mmol/L Pi in the presence or absence of DPD. Alizarin red staining revealed that DPD increased high Pi-induced calcification and ECM Ca content of VSMCs (**Figure 6E-F**).

After establishing that DPD accelerates high Pi-induced calcification, we investigated whether ER stress plays a role in VSMCs calcification triggered by Pi+DPD. First, we tested the effect of ER stress inhibitor 4-phenylbutyrate (4-PBA) on VSMCs calcification. AR staining revealed that 4-PBA inhibited Pi+DPD-induced calcification of VSMCs (**Figure 7A**). Additionally, 4-PBA inhibited Pi+DPD-triggered accumulation of ECM Ca and OCN of VSMCs and x vivo aorta calcification (**Figure 7B-D**). Furthermore, knockdown of ATF4 by siRNA decreased Pi+DPD-induced calcification of VSMCs as evaluated by AR staining, Ca and OCN measurements from the ECM (**Figure 7E-H**).

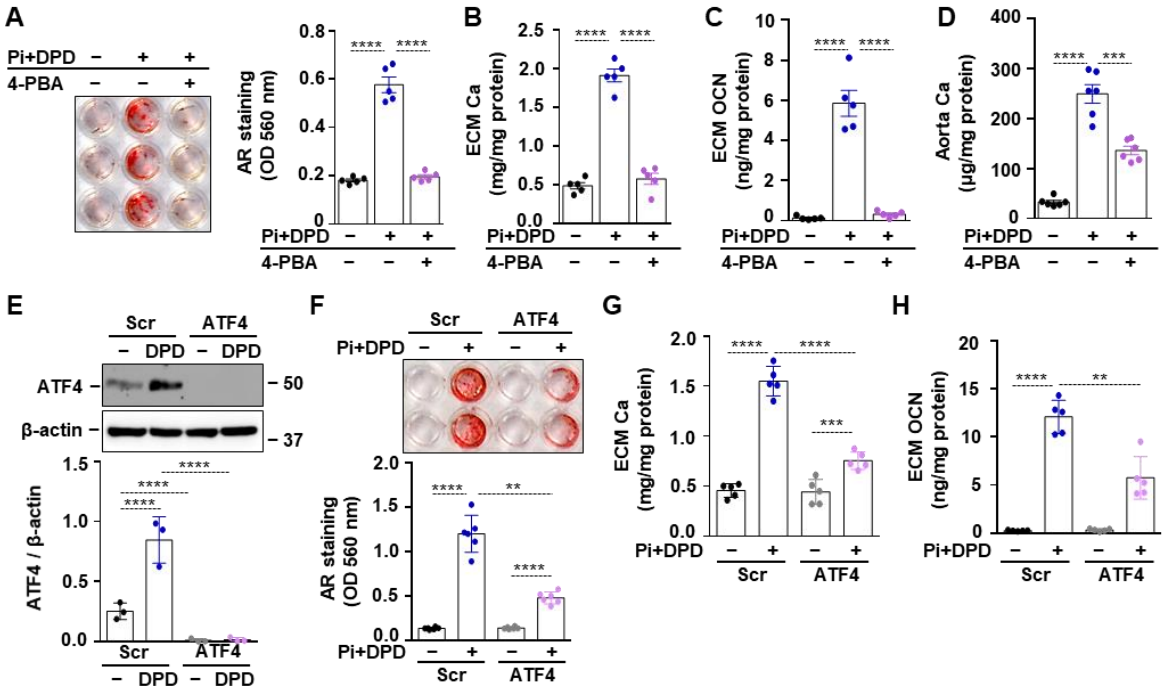

Figure 7. DPD increases calcification of VSMCs through ER stress and ATF-4 activation. (A-C) VSMCs were exposed to high Pi (2 mmol/L) and DPD (10 µmol/L) in the presence or absence of 4-PBA (250 µmol/L). (A) Representative AR staining (day 4) and quantification. (B) Ca content of HCl-solubilized ECM (day 4). (C) OCN level in EDTA-solubilized ECM samples (day 10). (D) Aortic rings of C57BL/6 mice were cultured in control, high Pi+DPD (25 µmol/L) and high Pi+DPD+4-PBA conditions. Ca content of aorta rings normalized to protein level (day 7). (E-H) VSMCs were exposed to Pi (2 mmol/L) and DPD (10 µmol/L) in the presence of ATF4 or scrambled siRNA. (E) Protein expression of ATF4 in whole cell lysates (6 h). Membranes were reprobed for β-actin. Representative Western blots and relative expression of ATF4 normalized to β-actin. (F) Representative AR staining (day 4) and quantification. (G) Ca content of HCl-solubilized ECM (day 4). (H) OCN level in EDTA-solubilized ECM samples (day 8). Data are expressed as mean ± SD, n = 3-6. Ordinary one-way ANOVA followed by Tukey's multiple comparison test were used to calculate p values. **p<0.01, ***p<0.005, ****p<0.001

Recent studies highlighted the causative role of hypoxia and HIF-1 activation in vascular calcification and several studies demonstrated that ER stress can exacerbate calcification. Furthermore, SMC-specific ATF-4 knock-out mice have reduced calcification, in contrast ATF-4 overexpression led to increased calcification in both normal and CKD conditions, suggest the important role of ATF-4 in the calcification process.

Therefore, we investigated the cross-communication of HIF-1 and ATF-4 in the presence of Pi and DPD in VSMCs. First, we applied targeted siHIF-1α and examined the protein expression of HIF-1α and ATF-4 (**Figure 8A-C**). Then in a separate experiment we applied targeted siATF-4 and evaluated the protein level of ATF-4 and HIF-1α (**Figure 8D-F**). Our results revealed that siHIF-1α knock-down significantly reduces the DPD and Pi+DPD induced expression of ATF-4, whereas the knock-down of ATF-4 have not changed the DPD and Pi+DPD induced expression of HIF-1α in VSMCs.

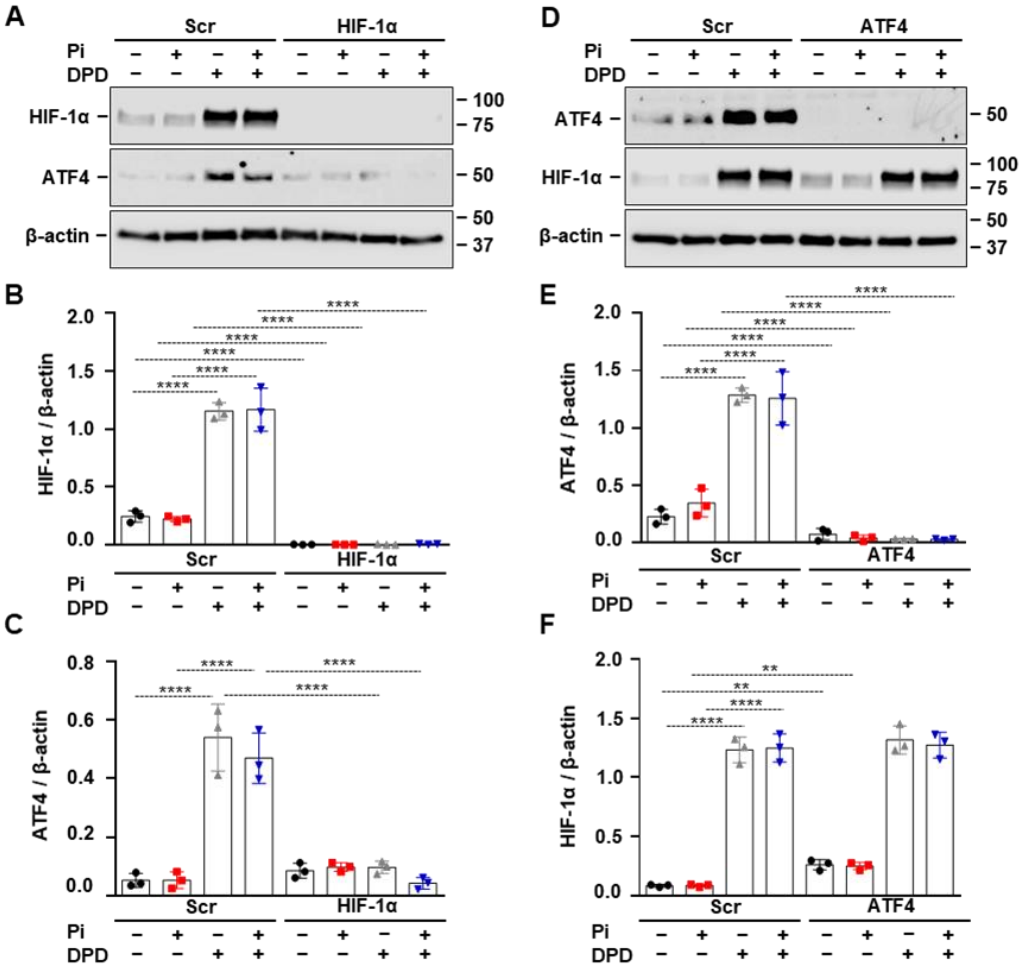

Figure 8. Crosstalk between hypoxia signaling and ER stress in Pi+DPD-induced HIF-1α and ATF-4 response in VSMCs. VSMCs were exposed to Pi (2 mmol/L) and DPD (10 μmol/L) in the presence of HIF-1α, ATF-4 and scrambled siRNA. (A-C) Protein expression of HIF-1α and ATF-4 in whole cell lysates (24 h). Membranes were reprobed for β-actin. (A) Representative Western blots and relative expression of (B) HIF-1α and (C) ATF4 normalized to β-actin. (D-F) Protein expression of HIF-1α and ATF-4 in whole cell lysates (24 h). Membranes were reprobed for β-actin. (D) Representative Western blots and relative expression of (E) ATF-4 and (F) HIF-1α normalized to β-actin. Data are expressed as mean ± SD, n=3. Ordinary one-way ANOVA followed by Tukey's multiple comparison test were used to calculate p values. **p<0.01, ****p<0.001

These results indicates that the pro-calcifying effect of DPD is regulated by the HIF-1α-ATF4 axis. We concluded that administration of DPD efficiently corrects anemia, but further increases the CKD-associated vascular calcification via HIF-1 activation and ER stress response.

4. Results not strictly associated with the proposal

Besides of the above mentioned articles and the ongoing manuscripts we have published several articles in the time-frame of the proposal which topics were not closely associated with the original proposal. The OTKA grant has been acknowledged in them, because – besides other grants – we used resources (human and/or infrastructural) supported by this grant to accomplish those projects. We would appreciate if these publications would also be considered as a result of this grant.

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