CKJ REVIEW

Role of prolyl hydroxylase/HIF-1 signaling in vascular calcification

Armando Luis Negri [ID]

Instituto de Investigaciones Metabólicas, Universidad del Salvador, Buenos Aires, Argentina

Correspondence to: Armando Luis Negri; E-mail: armando.negri@gmail.com

ABSTRACT

Morbidity and mortality of chronic kidney disease (CKD) patients are largely associated with vascular calcification, an actively regulated process in which vascular smooth muscle cells (VSMCs) change into cells similar to osteocytes/chondrocytes, known as trans-differentiation. Cellular and systemic response to low oxygen (hypoxia) is regulated by the prolyl hydroxylase/hypoxia-inducible factor (HIF)-1 pathway. Recent studies highlighted that hypoxia-mediated activation of HIF-1 induces trans-differentiation of VSMCs into bone-forming type through an increase in osteo-/chondrogenic genes. Inhibition of the HIF-1 pathway abolished osteochondrogenic differentiation of VSMCs. Hypoxia strongly enhanced elevated phosphate-induced VSMC osteogenic trans-differentiation and calcification. HIF-1 was shown to be essential for phosphate enhanced VSMC calcification. O_2-dependent degradation HIF-1 is triggered by the prolyl hydroxylase domain proteins (PHD). Prolyl hydroxylase inhibitors, daprodustat and roxadustat, increase high phosphate–induced VC in VSMCs, stabilizing HIF-1α and activating the HIF-1 pathway in these cells. Whether the use of these PHD inhibitors to treat anemia in CKD patients will favor the development and progression of vascular calcification remains to be explored.

Keywords: HIF-1, hypoxia, prolyl hydroxylase, reactive oxygen species, vascular calcification

INTRODUCTION

Vascular calcification (VC) of the arterial media was considered for decades as a passive degenerative process in which calcium and phosphate deposit in the form of hydroxyapatite in the vascular wall. This paradigm has changed recently, and it has become widely accepted that VC is a highly regulated process sharing many features with embryonic bone formation [1].

Arterial media calcification is a cell-mediated pathological process, predominantly driven by vascular smooth muscle cells (VSMCs) and pericytes [2]. Osteochondrogenic trans-differentiation of these cells in the vascular wall is now considered a major cellular mechanism leading to VC [3–5]. Several pathological stimuli *in vitro* have shown to induce a transition of VSMCs into cells with an osteo-/chondrogenic phenotype: high calcium and phosphate levels [6], uremic toxins [7] and inflammation/oxidative stress [8]. Bone formation at the vessel wall in patients with arterial media calcification can follow the two classical forms of bone formation: intramembranous (without a cartilage intermediate) or endochondral (where a cartilage intermediate is transformed into bone matrix) [9]. These transdifferentiated VSMCs like osteoblasts release exosome vesicles loaded with calcium-phosphate crystals into the extracellular matrix leading to VC [10, 11]. The trans-differentiation of VSMCs to cells with a bone-forming phenotype is preceded by an upregulation of osteo-/chondrogenic marker genes, including runt-related transcription factor 2 (Runx2 or Cbfa-1), non-specific alkaline phosphatase (TNAP), osteopontin (OPN or secreted phosphoprotein 1), Phospho1 and bone gamma-carboxyglutamate protein 2 (Bglap2) [2].

HYPOXIA SIGNALING AND BONE FORMATION

Cellular and systemic homeostatic response to hypoxia is regulated by the prolyl hydroxylase/hypoxia-inducible factor (HIF)-1 pathway. HIF-1 is an obligate heterodimer transcription factor, consisting of an oxygen-labile α subunit (HIF-1α) and a stable constitutive β subunit [12]. HIF-1α is expressed ubiquitously in all cells while HIF-1β is selectively expressed in certain tissues. Under normoxia, HIF-1α is constantly synthesized and degraded, while under hypoxia, HIF-1α becomes stable and dimerizes with the β subunit [12]. The heterodimer translocates into the nucleus, binds to hypoxia response elements in HIF-1 target genes, recruits coactivator molecules, as p300 and CREB (cAMP response element-binding protein)-binding protein, and the complex activates transcription [12].

The majority of HIF-1α-interacting proteins that have been identified thus far regulate the stability of HIF-1α in either an O_2-dependent or O_2-independent manner. O_2-dependent degradation is triggered by the prolyl hydroxylase domain proteins PHD1–3 [13]. Hydroxylation of HIF-1α at proline residue 402 or 564 facilitates binding of the von Hippel-Lindau protein (VHL), which recruits an E3 ubiquitin–protein ligase complex that catalyzes the covalent linkage of ubiquitin to lysine residues in HIF-1α, which serves as a signal for proteasomal degradation [14]. The HIF hydroxylases are dependent on ascorbate, and the activation of PHDs in ascorbic acid treated cells results in ubiquitin-mediated proteasomal degradation of prolyl hydroxylated HIF-1α.

Many systemic and local growth factors regulate bone formation processes. At the molecular level, hypoxia signaling is identified as a key signaling pathway that plays a central role in the regulation of bone formation both during normal physiology and under disease conditions [15]. Among the prolyl hydroxylase domain proteins, PHD2 has been found to be expressed in high abundance in bone cells and disruption of *Phd2* gene in bone cells result in severe skeletal phenotype [16, 17]. Analyses of mouse models carrying a gain-of-function or a loss-of-function mutation of osteoblastic HIF-1 have established that HIF-1 is a positive regulator of bone formation, as well as osteoblast number and activity *in vivo*, at least in part by stimulating non-oxidative glycolysis [18, 19]. Inversely, HIF-2α is a negative regulator of osteoblastogenesis and bone mass accrual [20].

The effect of hypoxia on osteogenic differentiation has been studied in diverse osseous and pluripotent mesenchymal cells. Hypoxia promotes osteogenesis of multipotent human mesenchymal stromal cells in a HIF-1 and RUNX2-dependent way, and induces osteogenesis of periosteal cells [21, 22].

Bone is formed primarily via two routes known as intramembranous and endochondral bone ossification [23]. Both osteogenic pathways begin with a mesenchymal tissue precursor, but how cells transform into bone differs. During intramembranous ossification that occurs in the flat bones of the skull, clavicle and most of the cranial bones, mesenchymal stem cells differentiate into osteoblasts to form bone. Endochondral ossification, occurring in the axial skeleton and the long bones, involves a more complex route with mesenchymal tissue transforming into a cartilage which is subsequently replaced by bone. During intramembranous and endochondral bone formation mechanisms, mesenchymal stem cells differentiate into osteoblasts and chondrocytes, respectively, by processes that are controlled by a complex transcriptional network that involves HIFs [15].

HYPOXIA SIGNALING IN VASCULAR CALCIFICATION: INTERACTION HIF-1A–RUNX2

Patients with pulmonary arterial hypertension (PAH) show calcified lesions within the distal pulmonary arteries (DPAs). Ruffenach *et al.* assessed calcification lesions and RUNX2 expression in harvested human lung tissues from patients with PAH [24]. Pulmonary artery smooth muscle cells (PASMC) were also isolated from these tissues. They showed that RUNX2 was upregulated in lungs DPAs. RUNX2 expression histologically correlated with vascular remodeling and calcification. Sustained RUNX2 expression activated HIF-1α, which produced abnormal proliferation, resistance to apoptosis and subsequent trans-differentiation of these cells into osteoblast-like cells. HIF-1α promoted while adenovirus expressing a dominant negative form of HIF-1α inhibited calcification of PASMC [24]. In VSMCs, Mokas *et al.* analyzed the role of hypoxic signaling during elevated inorganic phosphate-induced calcification using *in vivo* and *in vitro* rodent models [25]. Cell mineralization studies showed that elevated inorganic phosphate rapidly induced VSMC calcification. Hypoxia strongly enhanced elevated inorganic phosphate-induced VSMC calcification and osteogenic trans-differentiation, as seen by osteogenic marker expression. HIF-1 was essential for enhanced VSMC calcification. Balogh *et al.* [26] also found that hypoxia induced stabilization of HIF-1α and elevated the expressions of hypoxia response genes (*VEGFA* and *GLUT1*) in VSMCs and triggered an osteochondrogenic differentiation program characterized by upregulation of osteochondrogenic transcription factors and bone-specific proteins. The hypoxia-mediated switch to the osteochondrogenic phenotype eventually led to ECM calcification *in vitro*. They also showed that inhibition of the HIF-1 pathway abolished hypoxia-mediated osteochondrogenic differentiation of VSMCs, suggesting that HIF-1 activation is critically involved in the osteochondrogenic effect of hypoxia [27]. Their results are in strong agreement with the work of targeting HIF-1 expression in murine VSMC-blocked calcification in a model with hypoxia and elevated inorganic phosphate, while HIF-1 activators, including roxadustat (a prolyl hydroxylase inhibitor), recreated a pro-calcifying environment. Elevated inorganic phosphate rapidly activated HIF-1, even in normal oxygenation, an effect mediated by HIF-1α subunit stabilization. Thus, hypoxia synergizes with elevated inorganic phosphate to enhance VSMC osteogenic trans-differentiation. Tóth *et al.* [28] investigated the effect of daprodustat, another prolyl hydroxylase inhibitor, on high phosphate–induced VC in primary human aortic VSMCs, in mouse aorta rings, and in an adenine and high phosphate–induced chronic kidney disease (CKD) murine model. Daprodustat stabilized HIF-1α and HIF-2α and activated the HIF-1 pathway in VSMCs. Treatment with daprodustat increased phosphate-induced calcification in cultured VSMCs and murine aorta rings. Oral administration of daprodustat to adenine and high phosphate-induced CKD mice corrected anemia but increased aortic calcification. The inhibition of the transcriptional activity of HIF-1 by chetomin or silencing of HIF-1α attenuated the effect of daprodustat on VSMC calcification.

These findings are also supported by the previous clinical observation that HIF-1α plasma levels significantly and independently predict the presence of coronary artery calcification in patients with type 2 diabetes mellitus [29]. Using the data from the Multi-ethnic study of atherosclerosis, Luo *et al.* [30, 31] found that chili consumption was negatively correlated with coronary artery calcification and confer a smaller progression burden during follow-up, suggesting a role for capsaicin in

the attenuation of vascular calcification. They used CKD mice and cultured-VSMC under high phosphate concentration as calcification model to investigate the anti-calcification effect of capsaicin. Capsaicin reduced calcium deposition and osteogenic trans-differentiation both *in vivo* and *in vitro*. They showed that capsaicin promoted SIRT6-mediated deacetylation and degradation of HIF-1α.

HYPOXIA/ROS/HIF-1 AXIS

It is generally accepted that intracellular reactive oxygen species (ROS) levels change during hypoxia. It remains controversial the direction of the change and the origin of the ROS, either from mitochondrial origin or from NADPH. Although it sounds paradoxical, hypoxia stimulates ROS production in diverse mammalian cells [32]. Studies showed that mitochondrial electron transport chain complexes I and III and NADPH oxidases are involved in hypoxia-mediated increase in ROS production [27, 32–34]. Moreover, the finding that mitochondria-targeted antioxidants abolish the hypoxia response proved that mitochondrial ROS production is essential for propagation of the hypoxic signal toward the activation of the HIF pathway [35]. Balogh *et al*. [26] have shown that hypoxia induces elevation of mitochondrial ROS production in VSMCs. Inhibition of ROS production with NAC and the mitochondrial complex I inhibitor rotenone abolished hypoxia-induced HIF-1α stabilization and inhibited hypoxia-induced upregulations of RUNX2, SOX9 and OCN, and attenuated calcification. Previous studies already suggested a causative role of excessive ROS production in cardiovascular pathophysiology including VC [31, 36, 37]. Osteogenic differentiation of VSMCs has been linked to enhanced ROS production, and vascular cells expressing osteogenic markers were identified as sources of excess ROS around calcifying foci [31]. Importantly, inhibition of mitochondrial ROS generation attenuates phosphate-mediated osteogenic differentiation of VSMCs [31, 38]. Byon *et al*. [39] showed that hydrogen peroxide promotes osteogenic differentiation of VSMCs through the induction of RUNX2. In agreement with those findings, Balogh *et al*. [26] showed that hypoxia-mediated production of hydrogen peroxide plays an essential role in the stabilization of HIF-1α and in the promotion of osteochondrogenic response in VSMCs. In agreement with previous observations, they found that hydrogen peroxide was sufficient to stabilize HIF-1α under normoxic condition in VSMCs [40, 41]. Inhibition of HIF-1 stabilization by ROS scavengers under hypoxic conditions suggests that ROS generation is upstream of HIF-1 stabilization in VSMCs under hypoxia, as has been described in many other experimental conditions and recognized as hypoxia/ROS/HIF-1 axis [41]. Interestingly, Balogh *et al*. [26] found that inhibition of HIF-1 activity abolished hypoxia-mediated ROS production, suggesting a more complex interplay between ROS generation and HIF-1 activity. A similar phenomenon was described in a previous study, in which decreased ROS production was observed in heterozygous HIF-1α-deficient mice upon hypoxia exposure [42]. Thus, once HIF-1 is activated, it may function to maintain increased ROS levels and a positive feed-forward loop between ROS and HIF-1 activity has been proposed [43] (Fig. 1).

In a recent study, Wu *et al*. [38] tested the hypothesis that estrogen inhibits coronary artery calcification via the HIF-1α pathway. Expression of HIF-1α mRNA and protein were significantly increased in vascular cells with calcification as compared with those without calcification. Estrogen treatment decreased vascular cell calcification and lowered HIF-1α mRNA and protein. Also, oral administration of HIF-1α inhibitor dimethyllox-
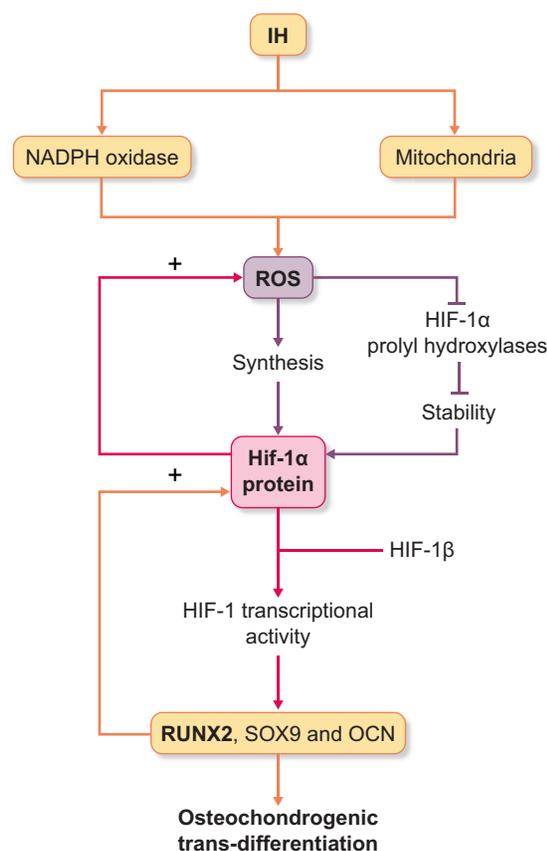

Figure 1: Intermittent Hypoxia (IH) triggers osteochondrogenic trans-differentiation of VSMC through ROS/Hif-1 axis.

etane to ovariectomized rats decreased vascular calcifications and expression of osteogenic related transcription factors such as RUNX2 and BMP2.

CONCLUSION

Collectively, pre-clinical *in vitro* and *in vivo* studies suggest that hypoxia, through the activation of HIF-1α pathway, may play an important role on vascular calcification. EZN-2968 is an antisense oligodeoxynucleotide that specifically targets HIF-1α [44], and could be a therapeutic alternative to treat vascular calcifications in the future. At the present moment it is not clear whether the use of PHD inhibitors to treat anemia in CKD patients will favor the development and progression of vascular calcification. Prolyl hydroxylase inhibitors differ in their capacity to inhibit different PHD isoforms: roxadustat is a pan inhibitor of PHD isoforms; daprodustat, vadadustat and molidustat inhibits PDH3 > PDH1 > PDH2 [45]. As different tissues have different abundance of PDH isoforms, the differing selectivity of these compounds could have a different effect on the risk of vascular calcifications. All these points remain to be explored in the future.

DATA AVAILABILITY STATEMENT

No new data were generated or analyzed in support of this research.

CONFLICT OF INTEREST STATEMENT

None declared.

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