Hypoxia-inducible factor-1 plays a role in phosphate-induced vascular smooth muscle cell calcification

Sophie Mokas[1], Richard Larivière[2], Laurent Lamalice[1], Stéphane Gobeil[3], David N. Cornfield[4], Mohsen Agharazii[2] and Darren E. Richard[1]

[1]*Department of Molecular Biology, Medical Biochemistry, and Pathology, Centre de recherche du CHU de Québec, Université Laval, Québec, Québec, Canada;* [2]*Department of Medicine, Centre de recherche du CHU de Québec, Université Laval, Québec, Québec, Canada;* [3]*Department of Molecular Medicine, Centre de recherche du CHU de Québec, Université Laval, Québec, Québec, Canada; and* [4]*Center for Excellence in Pulmonary Biology, Stanford University, Stanford, California, USA*

Medial vascular calcification is a common complication of chronic kidney disease (CKD). Although elevated inorganic phosphate stimulates vascular smooth muscle cell (VSMC) osteogenic transdifferentiation and calcification, the mechanisms involved in their calcification during CKD are not fully defined. Because hypoxic gene activation is linked to CKD and stimulates bone cell osteogenic differentiation, we used *in vivo* and *in vitro* rodent models to define the role of hypoxic signaling during elevated inorganic phosphate-induced VSMC calcification. Cell mineralization studies showed that elevated inorganic phosphate rapidly induced VSMC calcification. Hypoxia strongly enhanced elevated inorganic phosphate-induced VSMC calcification and osteogenic transdifferentiation, as seen by osteogenic marker expression. Hypoxia-inducible factor-1 (HIF-1), the key hypoxic transcription factor, was essential for enhanced VSMC calcification. Targeting HIF-1 expression in murine VSMC blocked calcification in hypoxia with elevated inorganic phosphate while HIF-1 activators, including clinically used FG-4592/Roxadustat, recreated a procalcifying 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 transdifferentiation. Our work identifies HIF-1 as an early CKD-related pathological event, prospective marker, and potential target against vascular calcification in CKD-relevant conditions.

Kidney International (2016) ■, ■–■; http://dx.doi.org/10.1016/j.kint.2016.05.020

KEYWORDS: chronic kidney disease; HIF-1; hypoxia; mineral imbalance; vascular calcification

Correspondence: *Darren E. Richard, Centre de recherche du CHU de Québec, L'Hôtel-Dieu de Québec, 9 Rue McMahon, Québec, Quebec G1R 3S3, Canada. E-mail: darren.richard@crchudequebec.ulaval.ca*

Received 30 October 2015; revised 12 May 2016; accepted 19 May 2016

Medial vascular calcification (VC), a hallmark of chronic kidney disease (CKD) and associated with calcium-phosphate deposits within arteries, exacerbates vessel stiffness and cardiovascular disorders.[1] This highly regulated process shares many similarities with bone development and mineralization.[2] A key event that drives VC is vascular smooth muscle cell (VSMC) transdifferentiation toward a bone-like cell phenotype.[3] Elevated inorganic phosphate ($HiPO_4$), a consequence of CKD, is a causal factor for VSMC osteoblastic transdifferentiation and predisposes to VC.[4] $HiPO_4$-treated VSMC up-regulate osteogenic activators, but lose VSMC lineage markers, such as smooth muscle actin alpha-2 (ACTA2) and transgelin (TAGLN/*SM22*).[5] Among VSMC osteogenic activators, bone morphogenetic protein-2 (BMP-2) is important for bone formation and VC.[6–8] BMP-2 signaling increases the expression of calcification promoters, such as runt-related transcription factor 2 (RUNX2).[9] RUNX2 is essential for bone cell differentiation and a key mediator driving VSMC osteogenic transdifferentiation and calcification.[2,10] RUNX2 is up-regulated in $HiPO_4$-treated VSMC as well as calcifying vascular tissues.[11,12]

Pathophysiological conditions observed in CKD, such as abnormal mineral metabolism, inflammation, and oxidative stress, are associated with VC.[13,14] Decreased oxygen delivery (hypoxia), caused by low blood perfusion and anemia, is also associated with CKD.[15,16] Hypoxia is known to influence the osteogenic potential of bone cells.[17,18] Although a direct link between hypoxia and VC has not been described, studies have associated hypoxia-inducible factor-1 (HIF-1) with VC.[19,20] Therefore, hypoxic signaling may indeed be implicated in VSMC osteogenic transdifferentiation leading to VC. Hypoxic signaling is predominantly mediated through HIF-1, the main hypoxia-inducible transcription factor in VMSC.[21] HIF-1 regulates the expression of adaptive genes involved in cellular oxygen (O_2) control and responses.[22] HIF-1 is a heterodimer comprising the O_2-sensitive HIF-1α and constitutively expressed HIF-1β.[23] HIF-1 binds to hypoxic response elements (HRE) found on target genes, such as vascular endothelial growth factor A (*VEGFA*) and glucose transporter type 1 (*GLUT-1*).[24] HIF-1α protein stability is controlled by its oxygen-dependent degradation domain (ODDD). The ODDD contains key proline residues that are

hydroxylated by specific HIF-prolyl hydroxylases (PHD) in the presence of O_2.[25,26] PHD hydroxylation allows the recognition of HIF-1α by VHL, the product of the von Hippel-Lindau tumor suppressor gene.[27,28] VHL directs ubiquitination and proteasome-dependent HIF-1α degradation.[29] During hypoxia, HIF-1α hydroxylation is blocked, halting HIF-1α degradation and forming the active HIF-1 complex.[30]

Because hypoxic signaling is important for bone cell differentiation and similarities exist between bone and VC, we sought to investigate the role of hypoxia in VSMC calcification. Using a cell model of conditions related to CKD, we demonstrate that hypoxia strongly enhances $HiPO_4$-induced VSMC osteogenic transdifferentiation and calcification. Our results indicate that this effect is mediated through HIF-1. We also show that HIF-1 is rapidly induced and activated by $HiPO_4$ in VSMC, even in the presence of O_2. Taken together, we identify HIF-1 as an important intermediate for $HiPO_4$-induced VSMC calcification.

RESULTS

Hypoxia/HIF-1-responsive gene expression in the calcifying aorta

Previous studies of CKD rats undergoing mineral imbalance ([MI], elevated inorganic phosphate, calcitriol) revealed enhanced media remodeling and VSMC osteogenic transdifferentiation correlated with increased arterial stiffness and medial aortic calcification.[31,32] To gain further insight into the underlying mechanisms involved in the etiology of VC during CKD, we used thoracic aorta sections from our previous CKD rat model for immunohistochemistry assays using a specific HIF-1α antibody. Strikingly, aortic sections from CKD animals undergoing MI showed increased nuclear staining for HIF-1α as compared to CKD animals without MI (Figure 1). Interestingly, elevated HIF-1α protein levels were accompanied by increased HIF-1 target gene expression. Two important HIF-1-regulated genes, *VEGFA* and *GLUT-1*, were significantly increased in CKD animals undergoing MI (Figure 1c). As expected, aortic tissue samples from CKD animals undergoing MI also showed reduced mRNA expression of VSMC markers *ACTA2* (Figure 1c), *CNN1*, *SM22*, and *MYH11* (Supplementary Figure S1). It is important to note that CKD animals treated with calcitriol alone showed no significant changes in calcification, HIF-1α, or target gene expression (results not shown). Our results suggest that hypoxic signaling and HIF-1 are implicated in the development of VC during CKD.

Hypoxia enhances VSMC calcification

We then investigated whether hypoxic signaling played a role in the early events of VC. Because our studies using calcitriol showed no significant effect on VMSC calcification, we

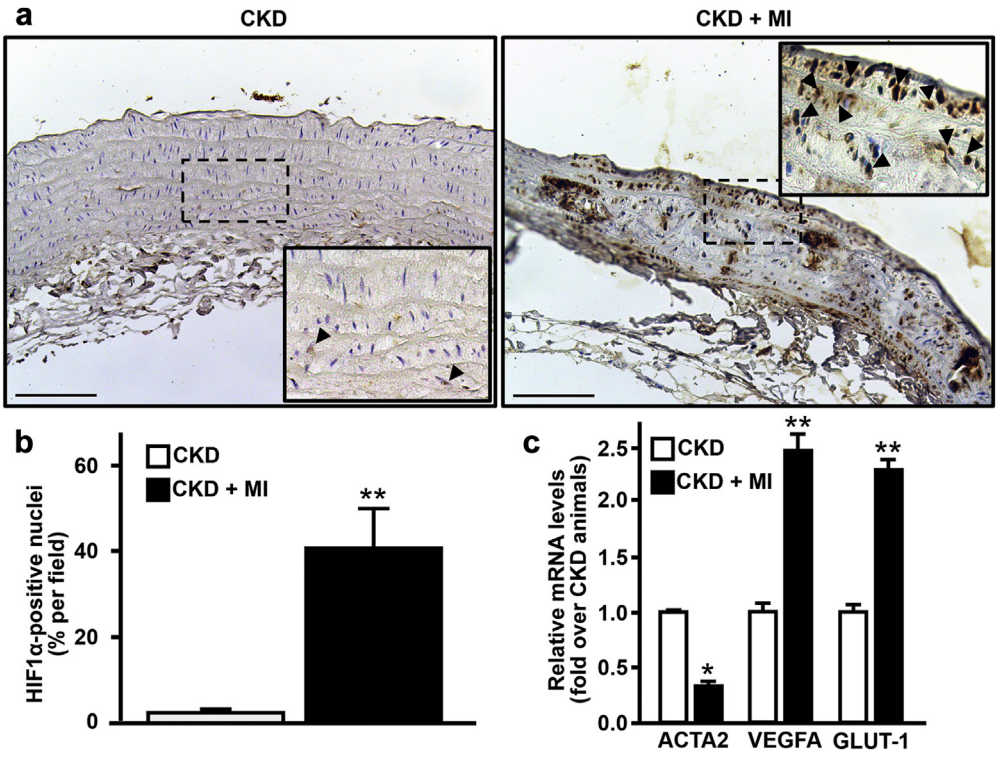

Figure 1 | Hypoxia-inducible factor-1 (HIF-1) activation in calcifying aortas from a chronic kidney disease (CKD) rat model. Aortas from nephrectomized rats receiving a normal diet (CKD) or undergoing mineral imbalance (CKD+MI) were surgically isolated. (**a**) Representative images of immunohistochemical analysis performed using a HIF-1α antibody. Arrowheads indicate HIF-1α positive nuclear staining. Bar = 100 μm. (**b**) Quantification of HIF-1α positive nuclei from immunohistochemical analyses (arrowheads in **a**). (**c**) RNA was extracted and real-time quantitative reverse transcriptase–polymerase chain reaction was performed to determine mRNA expression levels for smooth muscle actin alpha-2 (*ACTA2*), vascular endothelial growth factor A (*VEGFA*), glucose transporter type 1 (*GLUT-1*), and *HPRT1* (reference gene). Results are an average ± SEM. CKD, $n = 6$; CKD+MI, $n = 6$. $^{*}P < 0.05$; $^{**}P < 0.01$ as compared to CKD animals.