Inorganic Phosphate Stimulates Fibronectin Expression in Renal Fibroblasts

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ABSTRACT

Elevated plasma phosphate levels are signifcantly associated with progression of chronic kidney disease (CKD). Interstitial fibrosis is an important factor in the progression of CKD. In this study we investigate the role of inorganic phosphate in stimulating fibronectin (FN) synthesis in a kidney fibroblast cell line (NRK-49F). We find that phosphate increases FN abundance and message in a dose–dependent fashion and that both ERK1/2 and AKT are important signaling pathways that mediate phosphate-dependent FN expression in NRK-49F cells. Moreover phosphate srimulates the expression of the transcription factors osterix and NFATc1, which form complexes and mediate FN synthesis. Another transcription factor involved in phosphate-dependent FN synthesis is the AP1 family member c-Fos. In summary we show that even mildly elevated serum phosphate levels can induce synthesis of the interstitial matrix protein fibronectin through activation of ERK1/2 and AKT signaling pathways in kidney fibroblasts and that the synthesis of fibronectin is mediated by a transcriptional complex consisting of NFATc1, osterix and c-Fos.

BACKGROUND

Chronic kidney disease (CKD) is a highly prevalent health problem with a rising incidence and a strong tendency for progression [1]. Increase in interstitial fibrosis of the kidneys is significantly associated with progression of CKD [2]. Elevated plasma phosphate levels have been shown to be an independent risk factor for accelerated decline in renal function in patients with chronic kidney disease (CKD) [3]. The benefit of a low phosphate diet in slowing progression of CKD has been shown in humans [4], [5].However, the cellular and molecular mechanisms that mediate phosphate-dependent renal interstitial fibrosis have not been elucidated to date.The bulk of plasma inorganic phosphate (Pi) is filtered at the glomerulus and reabsorbed in the kidney proximal tubule [6]. Inorganic phosphate has been shown to be an important mediator capable of enhancing expression of pivotal genes that regulate complex cellular processes such as gene transcription, signal transduction and cell cycle regulation [7]. However, the signaling pathways induced by Pi in kidney fibroblasts and their role in stimulating fibrogenic molecules in kidney fibroblasts are unknown.

Osterix is a novel zinc finger-containing transcription factor of the SP gene family, and high Pi levels have been shown to induce Osterix in other non-skeletal tissue, such as vascular smooth muscle cells in mice [8]. We hypothesized that high phosphate concentrations induce Osterix expression in kidney fibroblasts and stimulate the synthesis of the matrix protein fibronectin. One of the NFAT transcription factors, NFATc1 has been shown to form a transcriptional complex with osterix thus mediating the synthesis of bone matrix formation in osteoblasts [9]. A recent study showed that NFATc1 stimulates fibronectin synthesis through transcriptional activation in mesangial cells [10]. We therefore hypothesized that osterix and NFATc1 act synergistically to mediate phosphate-dependent synthesis of fibronectin in kidney fibroblasts.

AIMS

In this study, we asked whether elevated culture medium phosphate levels stimulate the synthesis of interstitial matrix proteins such as fibronectin (FN) in kidney fibroblasts and which transcription factors and signaling pathways are activated by high phosphate treatment. Our results show that phosphate exposure activates ERK1/2 and Akt1 signaling pathways and stimulates the abundance and expression of the transcription factors NFATc1, c-fos and Osterix, which mediate fibronectin expression in a renal fibroblast cell line (NRK-49F).

METHODS

NRK-49F cells were treated with 1.25, 2.5 and 5.0 mM of phosphate (NaH2PO4 and Na2HPO4) for 30 or 60 minutes in signaling experiments and for 48 hours in all other experiments. *RT-PCR:* Total RNA was isolated using an RNA extraction kit (Rneasy, Qiagen). First-strand cDNA synthesis was accomplished using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed using Power SYBR Green PCR. PCR amplifications were carried out using ABI 7300 real-time cycler. Relative gene expression level (the amount of target, normalized to endogenous control gene) was calculated using the comparative Ct method formula 2-ΔΔCt. *Western blot analysis:* Lysate protein was separated by SDS-polyacrylamide gels and then electrophoretically transferred onto a PVDF membrane. The blots were visualized using ECL-Plus detection kit (Perkin Elmer). The blot images were quantitated by densitometry using the Image J analysis. The protein concentration was determined by Bradford method. *Luciferase Reporter Assay:* Cells were co-transfected with a fibronectin promoter driven luciferase construct and firefly luciferase reporter vector, using Lipofectamine 2000. Luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega) and FarCyte Tecan Ultra Plate Reader (Pharmacia). Relative luciferase activity was calculated by normalizing the firefly luminescence as to the renilla luminescence. Cell extracts were treated with IgG or Anti-NFATc1 antibody and the immune precipitates were collected using a Protein A agarose column. Total cell extract or immune precipitates were fractionated by electrophoresis on a 10% SDS-polyacrylamide gel, blotted onto nitro cellular membranes (Bio-Rad), probed with anti-Osterix antibody (Abcam), and visualized with secondary antibody and chemiluminescence.

RESULTS

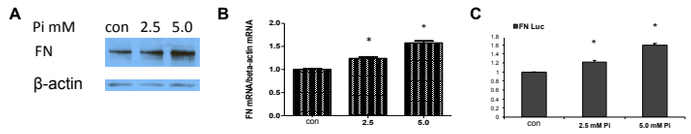


Figure 1. (**A**) Fibronectin abundance was increased in renal fibroblast (NRK-49F cells) following exposure to 2.5 and 5.0 mM phosphate medium concentration. (**B**)Fibronectin mRNA increased by 25% at 2.5 mM and 60% at 5 mM phosphate treatment. (**C**) Transfection of NRK-49F cells with a fibronectin promoter construct showed dose-dependent increase in FN promoter activity by phosphate treatment. *, p<0.05 compared to control medium.

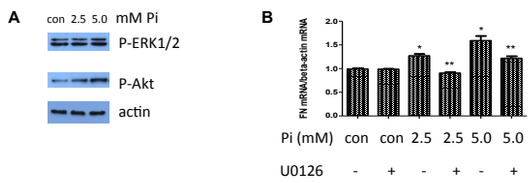


Figure 2. (**A**) Treatment of renal fibroblast with 2.5 and 5.0 mM phosphate stimulated ERK1/2 and AKT1 phosphorylation. (**B**) Pretreatment of NRK 49f cells with ERK1/2 inhibitor UO126 inhibited the phosphate-dependent increase in fibronectin mRNA by 110% in the 2.5 mM Pi group and by 45% in the 5.0 mM Pi group. *, p<0.05 compared to control medium. **, p<0.05 compared to same medium phosphate concentration but without UO126.

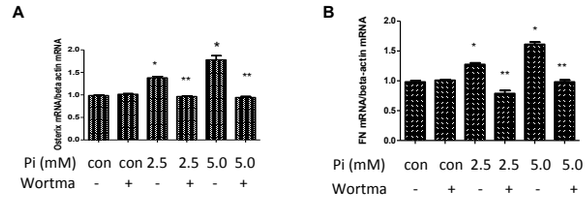


Figure 3. (**A**) The addition of phosphate to the culture medium increased osterix mRNA levels by 40% at 2.5 mM and by 75% at 5 mM phosphate concentration. Treatment of NRK-49F cells with PI3K-Inhibitor Wortmannin (10μM) inhibited phosphate-dependent osterix mRNA increase completely at both the 2.5 and 5.0 mM phosphate medium concentrations. (**B**) Wortmannin treatment also completely inhibited phosphate-dependent fibronectin mRNA increases at both 2.5 and 5.0 mM phosphate medium concentrations. *, p<0.05 compared to control medium. **, p<0.05 compared to same medium phosphate concentration but without Wortmannin.

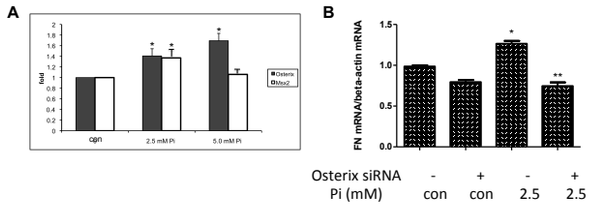


Figure 4. (**A**) Exposure to 2.5 and 5.0 mM phosphate in cell culture medium increased the mRNA levels of osterix, a zink finger transcription factor, by 40% and 75% respectively in kidney fibroblasts. The mRNA levels of the transcription factor Msx2 increased by 39% at 2.5 mM phosphate, but only 10% at 5.0 mM phosphate medium concentration. (**B**) Transfection of the kidney fibroblast cell line with osterix siRNA completely inhibited the increase in fibronectin mRNA seen with 2.5 mM phosphate treatment. *, p<0.05 compared to control medium. **, p<0.05 compared to same medium phosphate concentration but without Osterix siRNA transfection.

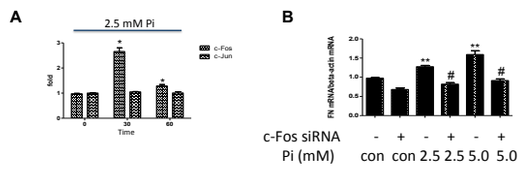


Figure 5. (**A**) Phosphate concentration of 2.5 mM in culture medium increased c-fos mRNA by 175% within 30 minutes, but had no effect on c-Jun mRNA levels. After 60 minutes of phosphate treatment, c-fos levels had returned back to control levels. (**B**) Exposure to 2.5 and 5.0 mM phosphate increased c-fos mRNA levels by 25% and 60% respectively. Transfection of NRK-49F cells with c-fos siRNA decreased the phosphate–induced increase of fibronectin mRNA completely. *, p<0.05 compared to 0 time point. **, p<0.05 compared to control medium. #, p<0.05 compared to same medium phosphate concentration but without c-fos siRNA transfection.

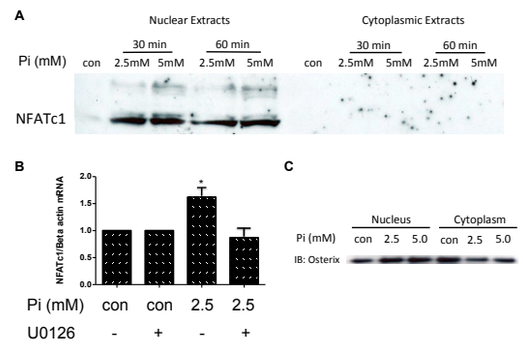
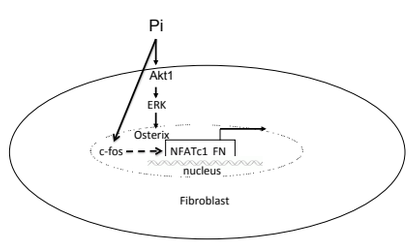


Figure 6. (**A**) Western blot shows an increase in NFATc1 abundance in nuclear extracts of kidney fibroblasts treated with 2.5 mM phosphate for 30 minutes, compared to controls. Exposure to 5 mM phosphate for 30 minutes increased NFATc1 abundance even further. No further increase of nuclear NFATc1 abundance was seen after 60 minutes of phosphate exposure. Cytoplasmic extracts did not show significant NFATc1 abundance over the same time period. (**B**) NFATc1 mRNA levels increased by 60% in the 2.5 mM phosphate group compared to controls. The ERK-inhibitor UO126 decreased the NFATc1 mRNA stimulation completely. *, p<0.05 compared to control medium. (**C**) Immunoprecipitation of osterix with an anti-NFATc1 antibody from nuclear extracts showed increased abundance after 2.5 and 5.0 mM phosphate treatment. The cytoplasmic fraction showed decreased osterix abundance following the same treatment. *, p<0.05 compared to control medium.

Figure 7. Signaling pathways and transcription factors involved in phosphate-dependent fibronectin expression in NRK-49F fibroblasts. Arrows demonstrate stimulation.



CONCLUSIONS

In summary our results show that phosphate stimulates ERK1/2 and Akt1 signaling pathways, which activate the downstream transcription factors osterix and cfos, which through complex formation with NFATc1 mediate fibronectin synthesis in kidney fibroblasts. **Figure 7** depicts the hypothetical model of phosphate-dependent stimulation of fibronectin synthesis. Understanding the cell mechanisms of phosphate-dependent fibronectin synthesis in kidney fibroblasts will not only enhance our knowledge of progression of interstitial fibrosis, but will also lead to the development of future drugs to ameliorate this process.

REFERENCES

1. Coresh J, Astor BC, Greene T, Eknoyan G, Levey AS: Prevalence of chronic kidney disease and decreased kidney function in the adult us population: Third national health and nutrition examination survey. *Am J Kidney Dis* 2003;41:1-12.
2. Bohle A, Strutz F, Muller GA: On the pathogenesis of chronic renal failure in primary glomerulopathies: A view from the interstitium. *Exp Nephrol* 1994;2:205-210.
3. Voormolen N, Noordzij M, Grootendorst DC, Beetz I, Sijpkens YW, van Manen JG, Boeschoten EW, Huisman RM, Krediet RT, Dekker FW: High plasma phosphate as a risk factor for decline in renal function and mortality in pre-dialysis patients. *Nephrol Dial Transplant* 2007;22:2909-2916.
4. Delmez JA, Slatopolsky E: Hyperphosphatemia: Its consequences and treatment in patients with chronic renal disease. *Am J Kidney Dis* 1992;19:303-317.
5. Barsotti G, Morelli E, Cupisti A, Meola M, Dani L, Giovannetti S: A low-nitrogen low-phosphorus vegan diet for patients with chronic renal failure. *Nephron* 1996;74:390-394.
6. Takeda E, Taketani Y, Morita K, Tatsumi S, Katai K, Nii T, Yamamoto H, Miyamoto K: Molecular mechanisms of mammalian inorganic phosphate homeostasis. *Adv Enzyme Regul* 2000;40:285-302.
7. Beck GR, Jr., Moran E, Knecht N: Inorganic phosphate regulates multiple genes during osteoblast differentiation, including nrf2. *Exp Cell Res* 2003;288:288-300.
8. Mathew S, Tustison KS, Sugatani T, Chaudhary LR, Rifas L, Hruska KA: The mechanism of phosphorus as a cardiovascular risk factor in ckd. *J Am Soc Nephrol* 2008;19:1092-1105.
9. Koga T, Matsui Y, Asagiri M, Kodama T, de Crombrugghe B, Nakashima K, Takayanagi H: Nfat and osterix cooperatively regulate bone formation. *Nat Med* 2005;11:880-885.
10. Cobbs SL, Gooch JL: Nfatc is required for tgfbeta-mediated transcriptional regulation of fibronectin. *Biochem Biophys Res Commun* 2007;362:288-294.