Inorganic Phosphate Stimulates Fibronectin Expression in Renal Fibroblasts

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ABSTRACT

Elevated plasma phosphate levels are significantly 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 stimulates the expression of the transcription factor NFATc1, which complexes with and mediates FN synthesis. Another transcription factor involved in phosphate-dependent FN synthesis is the API family member c-Fos. In NRK-49F cells, even mildly elevated serum phosphate levels can induce synthesis of the interstitial matrix 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, c-Fos 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) [2]. The benefit of a low phosphate diet in slowing progression of CKD has been shown in humans [4]. However, the cellular and molecular mechanisms that mediate phosphate-dependent renal interstitial matrix proteins such as FN have not been elucidated to date. To the best of our knowledge, the role of phosphate in stimulating FN synthesis in renal fibroblasts has not been investigated.

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 AKT, signaling pathways and stimulates the abundance and expression of the transcription factors NFATc1, c-Fos and c-Fos, 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 (Na2HPO4 and NaH2PO4) for 30 minutes in signaling experiments and for 48 hours in all other experiments. RT-PCR. Total RNA was isolated using a RNA extraction kit (RNeasy, Qiagen). First-strand cDNA synthesis was accomplished using the Reverse-Transcription System for RT-PCR reaction (Invitrogen). Real-time PCR was performed using 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 gene expression and expressed as fold change) was calculated using the comparative Ct method formula 2^-ΔΔCt. Western blot analysis: Lysing protocol was expanded to 0.5% SDS/phenylmethylsulfonyl fluoride and then precipitated to obtain a protein pellet. Western blot images were quantified using the Gel-Pro software (Media Cybernetics, Silver Springs, MD, USA). Western blots were semi-quantified for densitometric analysis. The gel images were analyzed and quantified using ImageJ software. The protein concentration was determined by Bradford method. Luciferase Reporter Assay Cells were co-transfected with a Renilla luciferase driven promoter construct and firefly luciferase reporter vector, using the FuGENE HD transfection reagent (Roche). Nuclear extracts were prepared using the NE-PER nuclear extraction kit (Thermo Scientific). The cytosolic extracts were prepared using the Total Protein Extraction Kit (Bio-Rad). Extracts were subjected to SDS-PAGE and Western Blot analysis was performed using specific antibodies. The firefly luciferase activity was measured using a VICTOR 1420 multilabel counter (Perkin Elmer). The firefly luciferase activity was normalized to the Renilla luciferase activity for each sample, and reported as fold increase over control. All experiments were repeated at least three times. RT-PCR and Western Blot images were analyzed using density analysis software (AIDA software, Raytest detection, Germany). RESULTS

Figure 1. (A) Fibronectin abundance was increased in renal fibroblast (NRK-49F cells) following treatment with 1.25, 2.5 and 5.0 mM phosphate for 48 hours. (B) Treatment of renal fibroblasts with 2.5 and 5.0 mM phosphate-stimulated ERK1/2 and AKT phosphorylation in a dose-dependent manner. (C) Transfection of NRK-49F cells with a Wt promoter driven luciferase construct increased FN promoter activity by 73% within 30 minutes but had no effect on c-Jun mRNA levels. After 60 minutes of phosphate treatment, Fn mRNA increased by 25% at 2.5 mM and 40% at 5.0 mM phosphate treatment. (#, p<0.05 compared to control medium.

Figure 2. (A) Western blot shows an increase in FN abundance in renal fibroblasts treated with 1.25, 2.5 and 5.0 mM phosphate for 48 hours. (B) Normalization of Western blot results to the control medium. (C) Western blot shows an increase in FN abundance in renal fibroblasts treated with phosphate for 48 hours. (D) Western blot shows an increase in FN abundance in renal fibroblasts treated with phosphate for 48 hours. (E) Western blot shows an increase in FN abundance in renal fibroblasts treated with phosphate for 48 hours.

Figure 3. (A) The addition of phosphate to the culture medium increased FN mRNA levels. (B) Treatment of NRK-49F fibroblasts with 2.5 and 5.0 mM phosphate significantly promoted osteogenesis. (C) Western blot analysis showed increased osteogenesis in renal fibroblasts treated with phosphate for 48 hours. (D) Western blot analysis showed increased osteogenesis in renal fibroblasts treated with phosphate for 48 hours.

CONCLUSIONS

In summary our results show that phosphate stimulates ERK1/2 and AKT signaling pathways, which activate the downstream transcription factors c-Fos and c-Fos. Increased expression of c-Fos results in increased FN expression through complex formation with NFATc1 mediates fibronectin synthesis in kidney fibroblasts. Figure 7 depicts the hypothetical model of phosphate-dependent fibronectin synthesis. Understanding the cell mechanisms of phosphate-dependent fibronectin synthesis in kidney fibroblasts will not only enhance our knowledge of fibronectin synthesis, but also lead to the development of future drugs to ameliorate this process.

REFERENCES