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High-level of Inorganic Phosphate Induces Angiogenesis Through Activation of Protein Translation in Human Lung Cells

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ABSTRACT

Inorganic phosphate (Pi) is an essential material in cellular metabolism. However, the exact mechanism in translation by high Pi has not been elucidated. To address this issue, we examined mRNA microarray analysis to compare mRNA levels by high Pi treatment in NHBE cells. Our results clearly showed that high dose (20 mM) of Pi induced the mRNA expression levels of Eif4enif1, Fgf2, Mmp23, and Amotl2. Furthermore, cap-dependent protein translation, was induced in cells treated with high Pi. High dose of Pi induced angiogenesis by transcriptional up-regulation. The analysis of the data presented here sheds new light on the important roles of Pi, and such research would be invaluable to future studies on lung cancer prevention.

Key words : Inorganic phosphate, Cap-dependent translation, Angiogenesis

Introduction

Inorganic phosphate (Pi) is present in fungus, bacteria, plant, and animal cells. It plays a key role in diverse cellular functions involving mineral metabolism, intermediary metabolism, and energy-transfer. Pi is a vital component of membrane phospholipids, nucleotides that provide energy, RNA and DNA, and is necessary for phosphorylated intermediates in cellular signaling [1].

Eukaryotic translation initiation factor 4E nuclear import factor 1 (Eif4enif1) is a nucleo-cytoplasmic shuttle protein for eIF4E transport. This protein interacts with the importin alpha-beta complex to mediate nuclear import of eIF4E. It is predominantly present in cytoplasm and its own nuclear import is regulated by a nuclear localization signal and nuclear export signals [2]. In previous study, we reported that Pi affected cell growth and facilitated Mnk1 translocation from cytosol into nucleus through activating PI3K/Akt as well as Raf/MEK/ERK pathways [3].

Additionally, previous studies demonstrated that high level of Pi enhanced cap-dependent translation and increased tumorigenesis through Akt signaling pathway in brain, lung, and liver of mice [4-7]. However, the effect of Pi on nontumorigenic human bronchial epithelial (NHBE) cells has not yet investigated. Therefore, this study was undertaken to elucidate the effects of Pi on normal lung cells by mRNA microarray.

Bronchial epithelial cells are often exposed to diverse xenobiotics including toxicants. Many investigators elucidated that the precise mechanisms of pulmonary toxicants-induced nontumorigenic bronchial epithelial cell damage and the response of bronchial epithelium to chemical and physical injury has

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been associated with the induction of hyperplasia [8]. The altered response of normal bronchial epithelial cells to external/internal stimuli causes a disturbance in the homeostasis between normal cell survival and growth. Therefore, the elucidation of such disturbed homeostatic process could provide a critical clue to cope with pulmonary diseases such as cancer.

Materials and Methods

1. Cell culture

NHBE (ATCC number: CRL-2503) cells were incubated in RPMI 1640 medium supplemented with 10% FBS and 100 μL/mL penicillin/streptomycin and subsequently cultured normal media (5.63 mM $NaPO_4$) or high phosphate media (20 mM $NaPO_4$, pH 7.4).

2. Materials

Penicillin and streptomycin (P/S) were purchased from Invitrogen (Carlsbad, CA, USA) and sodium phosphate was from Sigma-Aldrich (St. Louis, MO, USA). The bicistronic construct, pcDNA-fLUC-polIRES-rLUC, was kind gifts from Dr. Gram (Novartis Pharma AG, Switzerland). Anti-FGF-2 and anti-VEGF antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against GAPDH was purchased from AbFrontier (Seoul, Korea).

3. Luciferase assay

Luciferase activities in the tissue extracts were measured by EG&G Berthold luminometer (Bundoora, Australia). Briefly, the lungs were homogenized in passive lysis buffer (Promega, Madison, WI, USA). The homogenates were centrifuged for 20 min at 5,400 × g at 4°C and the supernatant was centrifuged for an additional 15 min at 15,700 × g at 4°C. LucF and LucR activities were measured using a dual luciferase assay kit (Promega). For *in vitro* assay, cells were grown on six-well plates and were transfected with bicistronic reporter gene using FuGENE 6 Transfection Reagent (Roche, Basel, Switzerland). After 48 h incubation, cells were treated normal or high Pi for 0, 4, 8, 24 hours. The cells were washed twice in ice-cold PBS, extracted in passive lysis buffer and assayed for firefly and *renilla* luciferase activities, according to the manufacturer's instruction.

4. Microarray

Oligo arrays (22,272), print Mm-FCRF-CGEN1extv4p4_082703, were printed by the Laboratory of Molecular Technology (Frederick, MD, USA) and described at nciarray.nci.nih.gov/cgi-bin/gipo. Total RNA from normal- and high phosphate-treated samples was labeled with either Cy3 or Cy5 Mono-Reactive Dye (Amersham Biosciences, Piscataway, NJ, USA) using Superscript Indirect cDNA Labeling System (Invitrogen) following the manufacturer's protocol. Arrays were scanned using a GenePix microarray scanner (Axon Instruments, Union City, CA, USA), and data were analyzed by GenePix Pro 4.0.

5. RT-PCR

NHBE cells were cultured in a normal (5.63 mM) or high Pi (20 mM) RPMI-1640 media for 24 h. Total RNA was isolated and subjected to RT-PCR (ONE-STEP RT-PCR PreMix kit, iNtRON Biotechnology, Korea) following the manufacturer's protocol. Two μg of RNA were amplified with specific primers at the following cycling conditions: RT step-reverse transcription reaction at 45°C for 30 min, denaturation of RNA : cDNA hybrid at 94°C for 5 min, PCR step-denaturing at 94°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min, 35 cycles. As an internal control, we amplified GAPDH. The sequence of the primers used for RT-PCR were as follows; for *Eif4enif1*, forward 5′-TTC AGG AGA GAG TTT GGA G -3′ and reverse 5′-GGT CTT CCA ATG GTA TAG G-3′ (592 bp); for *GAPDH*, forward 5′-GAA GGA CTC ATG ACC ACA G-3′ and reverse 5′-CTT CAC CAC CTT CTT GAT G-3′ (276 bp). Products were analyzed by electrophoresis on 2% agarose gels.

6. Western blot analysis

Twenty-five μg of cell lysate and 50 μg of homogenized lysate were separated using 10-15% SDS-PAGE and transferred to nitrocellulose membranes. After membranes were blocked in TTBS containing 5% skim milk for 1 hours, immunoblotting was performed by incubating overnight at 4°C with the corresponding primary antibodies in 5% skim milk and then with second antibodies conjugated to horseradish peroxidase (HRP) for 1 hour at room temperature. After washing, the bands-of-interests were visualized by luminescent image analyzer LAS-3000 (Fujifilm, Japan). Results were quantified using a measure program of LAS-3000.

7. Statistical analysis

All results are given as means±S.E. Results were analyzed by unpaired Student's t test (GraphPad Software, San Diego, CA, USA). *$p<0.05$ was considered significant and **$p<0.01$ and ***$p<0.001$ highly significant compared to corresponding control.

Results and Discussion

In this study, to investigate the effect of high Pi, NHBE cells were treated with 20 mM $NaPO_4$ for 24 hours, the relative change in response was measured by mRNA microarray analysis. The results showed that high dose of inorganic phosphate increased the mRNA levels of eukaryotic translation initiation factor 4E nuclear import factor 1 (Eif4enif1), fibroblast growth factor 2 (Fgf2), matrix metalloproteinase 23 (Mmp23), and angiomotin like 2 (Amotl2) in the cells treated with high Pi. Genes that displayed at least a 1.50-fold change when averaged are included in Table 1.

Eif4enif1 is a transporter protein for the translation initiation factor eIF4E. It interacts with the importin alpha-beta complex to mediate nuclear import of eIF4E. Eif4enif1 is predominantly presented in the cytoplasm and its own nuclear import is regulated by a nuclear localization signal and nuclear export signals. To confirm the result of mRNA microarray, RT-PCR and Western blot analyses were carried out in high Pi treated NHBE cells. The results showed increased Eif4enif1 expression levels in the cells treated with high Pi (Fig. 1A). Densitometric

Table 1. Genes with change in expression (>1.50-fold) by high Pi

Gene	Description	Avg.
Eif4enif1	eukaryotic translation initiation factor 4E nuclear import factor 1	1.89
Fgf2	fibroblast growth factor 2	2.03
Mmp23	matrix metalloproteinase 23	1.73
Amotl2	angiomotin like 2	1.51

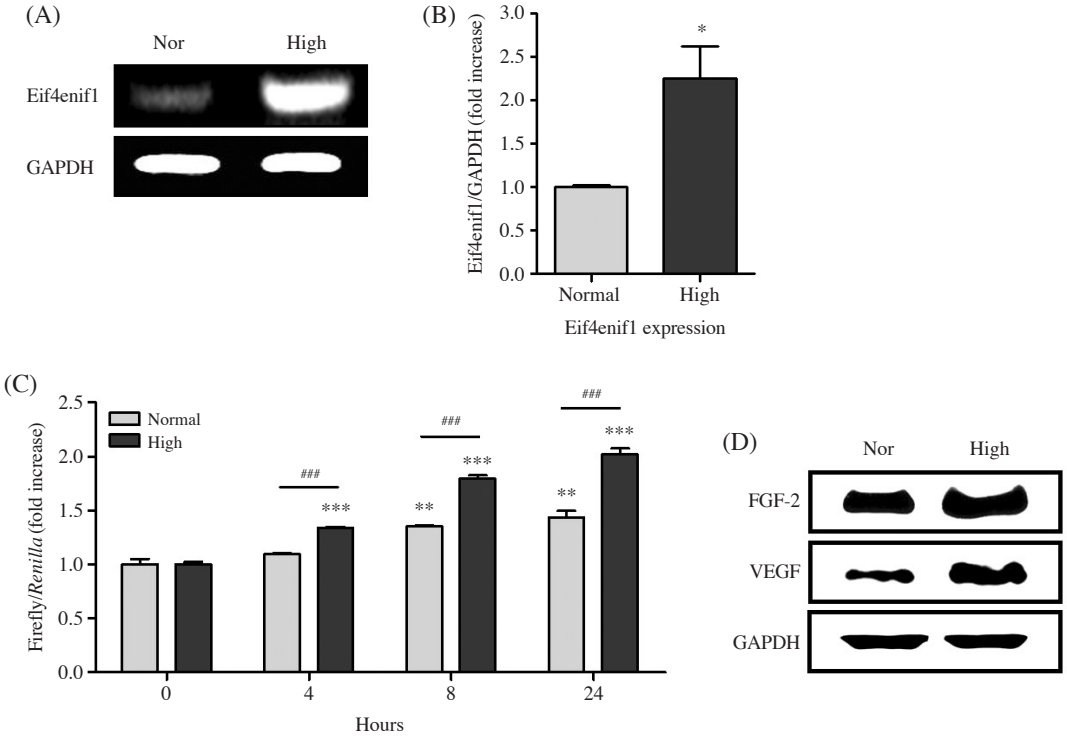

Fig. 1. RT-PCR, luciferase activities, and Western blot anlaysis in cells treated with Pi. (A) RT-PCR analysis for Eif4enif1 gene in NHBE cells. NHBE cells were cultured in a normal (5.63 mM) or high Pi (20 mM) RPMI-1640 media for 24 hours. Cells were harvested after 24 hours, and total RNA was isolated and subjected to RT-PCR analysis. (B) Densitometric analysis for Eif4enif1 gene in NHBE cells. Values are the means± S.E. of 3 independent experiments. *$p<0.05$. (C) Time course luciferase activity in NHBE cells. Cells were transfected with bicistronic reporter construct, pcDNAfLUC-polIRES-rLUC for 24 hours. After additional incubation for 24 hours in a normal (5.63 mM) or high Pi (20 mM), cells were lysed and assayed for firefly and *renilla* luciferase activities. The ratios of cap-dependent (Firefly) to IRES dependent (*Renilla*) are shown. **$p<0.01$, ***$p<0.001$ compared to zero time and $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to normal group (mean±S.E., n=3, 2 times). (D) Western blot analysis for FGF-2 and VEGF. NHBE cells were cultured in a normal (5.63 mM) or high Pi (20 mM) RPMI-1640 media for 48 hours. Cells were harvested after 48 hours, and lysates were subjected to Western blot analysis. Blots were probed with antibody of FGF-2 or VEGF.

analysis confirmed the RT-PCR result (Fig. 1B).

To determine the precise role of high Pi on protein translation, transfection with bicistronic reporter gene into the cells was performed. As mentioned previously, firefly luciferase represents cap-dependent while *renilla* luciferase activity represents cap-independent protein translation [3]. When the cells were treated with high Pi, cap-dependent protein translation was increased significantly at 4, 8, and 24 hours (Fig. 1C). The results clearly demonstrated that high Pi increased cap-dependent protein translation in NHBE cells.

It has been reported that elevated eIF4E greatly facilitates translation of a selected spectrum of mRNA coding for proteins critical to angiogenesis and growth such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in several tumors [9-11]. One of the markers for angiogenesis is VEGF, which induces endothelial cell proliferation and migration.

To examination whether increased cap-dependent protein translation can induce angiogenesis in NHBE cells, Western blot analysis was performed to quantify protein expression levels. The levels of both FGF-2 and VEGF were noticeably increased in the cells treated with high Pi compared to the cells treated with normal Pi. The present observation demonstrated that high Pi may regulate angiogenesis and endothelial cell proliferation (Fig. 1D).

In addition, the results obtained from microarray MMP23 (MMP23B), a member of the matrix metalloproteinase (MMP) family, is involved in disease processes, such as arthritis and metastasis [12], and Amotl2 also plays important roles in regulating multiple behaviors of endothelial cells during angiogenesis [13]. Together, our results clearly demonstrated that high Pi induced angiogenesis in NHBE cells.

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