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Extracellular regulated protein kinases play a key role via bone morphogenetic protein 4 in high phosphate-induced endothelial cell apoptosis

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ABSTRACT

Aims: Hyperphosphatemia is an independent risk factor of cardiovascular events in the patients with chronic kidney disease. High phosphate can induce endothelial cell apoptosis, but the exact mechanism is not clear. This study fills this knowledge gap.
Materials and methods: Microarray analysis was used to identify differentially expressed gene profiles in human umbilical vein endothelial cells (HUVECs) in high phosphate (3.0 mM) and normal phosphate (1.0 mM) medium. Microarray informatics analysis was used to explore key pathways and genes. High phosphate-induced apoptosis is marked by annexin V-FITC/PI staining and cleavage of caspase-3. Immunoblotting and quantitative real-time PCR were performed to identify the microarray analysis.
Key findings: Our microarray informatics analysis reveals that the mitogen-activated protein kinase (MAPK) plays a key role. As suggested by gene coexpression network analysis, bone morphogenetic protein 4 (BMP4) gene is a potential key regulatory gene in high phosphate environment. Both the expressions of BMP4 protein and mRNA are decreased. Extracellular regulated protein kinases (ERKs) are activated, while the inhibition of ERK by U0126 increases the expression of BMP4. Both recombinant BMP4 protein pretreatment and U0126 pretreatment reduce the apoptosis of endothelial cells in simulated hyperphosphatemia. However, BMP4 protein pretreatment had no effect on the activation of ERK MAPK pathway.
Significance: Our results indicate that the inhibition of ERK MAPK pathway protects endothelial cells from apoptosis by upregulating bone morphogenetic protein 4 in endothelial cells exposed to hyperphosphatemia. Our study provides potential molecular targets for developing new strategies to reduce the endothelial cell apoptosis induced by high phosphate.

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1. Introduction

Phosphate is an important element that is essential in multiple physiological functions, including cellular metabolism and mineral metabolism. Chronic kidney disease (CKD) can cause complicated disorders in calcium and phosphorus metabolism. Clinical evidence reveals that high serum phosphate is positively related to increased risk of cardiovascular and all-cause death in CKD patients [1,2]. The risk of death increased 18% for every 1 mg/dL elevation in serum phosphate in CKD patients [3]. Moreover, even relatively small increase in serum phosphate in normal range is positively correlated with the risk of death and cardiovascular events in the population without CKD [2,4].

Hyperphosphatemia induced acute endothelial dysfunction (ED) in vivo and endothelial cell apoptosis by increasing reactive oxygen species production in vitro [5,6]. More importantly, endothelial cell apoptosis is the first step in the development of atherosclerotic lesions and other diverse cardiovascular diseases [7]. Our previous study indicated that the mitogen-activated protein kinase (MAPK) pathway was involved in human umbilical vein endothelial cell (HUVEC) apoptosis induced by simulated hyperphosphatemia [8]. In order to understand how hyperphosphatemia impacts endothelial cells, we carried out a microarray analysis and identified differentially expressed gene profiles in HUVECs exposed to normal or high phosphate medium. Our microarray data reveals that MAPK pathway plays a key role, while bone morphogenetic protein 4 (BMP4) is a potential regulatory gene in a high phosphate environment. The mechanism by which high phosphate levels induce the apoptosis of endothelial cells and the exact roles that MAPK and BMP4 play in this process are poorly understood. Thus, we designed a study to ascertain how BMP4 and MAPK influence the apoptosis of endothelial cells when exposed to high inorganic phosphate concentration. In the present study, we found that the inhibition of ERK MAPK pathway protected endothelial cells from apoptosis by

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upregulating bone morphogenetic protein 4 when exposed to simulated hyperphosphatemia.

2. Materials and methods

2.1. Cell culture and treatment

HUVECs were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Endothelial Cell Growth Medium (EGM) (Lonza, Walkersville, MD) in an incubator (37 °C and 5% CO_2). When the cell confluence reached 90%, the cells were subcultured in tissue culture dishes (BD Biosciences, Franklin Lakes, USA) and incubated for 48 h to reach 85–90% confluence. The cells were then incubated with media containing different phosphate concentrations. The physiologic range of serum phosphate concentration is approximately 0.87–1.45 mM. The EGM medium contains 0.5 mM phosphate. It was added to appropriate amounts of sodium phosphate buffer (1 M Na_2HPO_4/NaH_2PO_4, pH 7.4) to achieve different phosphorus concentrations (1.0 and 3.0 mM) as previously described [8]. Treatments with recombinant BMP4 (100 ng/mL, 24 h, Santa Cruz, USA) and ERK inhibitors (U0126, 100 ng/mL, 24 h, Santa Cruz, USA) were performed on cells seeded in EGM.

2.2. Microarray analysis

Differential gene expression in HUVECs seeded in normal phosphate (1.0 mM) and high phosphate (3.0 mM) medium was identified using Affymetrix GeneChip Human Gene 1.0 ST arrays. Three chips were used to minimize the random error in each group. Random variance model (RVM) t-test was used to effectively increase the degree of freedom for small sample sizes. After a significant analysis and a false discovery rate (FDR) analysis, p values and FDRs of less than 0.05 were considered statistically significant. Pathway-net analysis, an interaction network of significant pathways bearing differentially expressed genes, was built based on the interaction among the pathways, as classified by Kyoto Encyclopedia of Genes and Genomes (KEGG) database [9]. Each pathway in the network was shown as indegree, outdegree or total degree according to its number of upstream and downstream interacting pathways. A higher total "degree" of a pathway suggested that it regulated or was regulated by several other pathways, implying a more important role in the signaling network [10]. GeneRelNet (gene coexpression networks) were built by the normalized signal intensity of specific expression genes. To study the properties of the networks, k-cores in graph theory were introduced [11]. Genes with the highest k-core values were regarded as crucial genes in simulated hyperphosphatemia. The microarray data in this paper have been deposited in NCBI's Gene Expression Omnibus (GEO: GSE60937).

2.3. Quantitative real-time PCR

Quantitative real-time PCR was performed using sequence-specific primer pairs for BMP4: 5′-TTCACTGCAACCGTTCAGAGGTC-3′ (forward); 5′-AACAAACTTGCTGGAAAGGCTCA-3′ (reverse). After extracting total RNA from HUVECs, cDNA was synthesized using AMV reverse transcriptase. Real-time PCR was performed using ABI SybrGreen PCR Master Mix and ABI Step One Plus Real-Time PCR System (CA, USA). The quantification of PCR products was performed using the ABI 7300 software system.

2.4. **Western blot** analysis

Briefly, the protein content of the treated HUVECs lysate was measured using the BCA Protein Assay Kit (Beyotime, China) and 20 μg total protein was loaded and separated on 4–12% Tris–glycine gel (BD Biosciences) at 100 V for 2 h. After electrophoresis, the protein was transferred to nitrocellulose membrane (Hybond-ECL, Amersham, Buckinghamshire, UK). The following primary antibodies were used: cleaved caspase-3 antibody (1:500, Cell Signaling, USA), phospho-p38MAPK antibody (1:1000, Cell Signaling, USA), p38MAPK antibody (1:1000, Cell Signaling, USA), phospho-SAPK/JNK (1:1000, Cell Signaling, USA), SAPK/JNK antibody (1:1000, Cell Signaling, USA), phospho-p44/42MAPK (Erk1/2) antibody (1:800, Cell Signaling, USA), p44/42MAPK antibody (1:1000, Cell Signaling, USA), and anti-BMP4 (1:500, Abcam, Cambridge, UK). The membranes were incubated for 1 h with blocking buffer plus secondary antibody. Specific proteins were detected by the Odyssey Scanning System (Li-Cor, Lincoln, NE, USA). The band intensities were analyzed using Scion Image (WinB403).β-Actin was used as an internal control.

2.5. Apoptosis assay

The apoptosis rate of cells was detected using the fluorescent dye Annexin V-FITC/propidium iodide (PI) apoptosis detection kit (BD Biosciences Pharmingen) according to the manufacturer's instructions. Briefly, cells were washed with cold PBS and resuspended in 250 μL of binding buffer. 5 μL of Annexin V was supplemented and the cells were incubated at 4 °C for 15 min. Finally, 10 μL of PI was added just 5 min before analysis using a flow cytometer (FACSCanto™ II; BD Biosciences). The ModFitLT software (Verity Software House) was used to calculate the percentage of apoptotic cells. Ten thousand events were analyzed in each sample.

3. Statistical analyses

Data are expressed as mean ± SEM of three independent experiments, each done in triplicate (n = 3 dishes). One-way analysis of variance (ANOVA) and Student's t-test were used to compare between groups. p-Values less than or equal to 0.05 were considered significant.

4. Results

4.1. Microarray data analysis

460 genes were identified as being significantly (RVM t-test $p < 0.05$ and FDR $p < 0.05$) differentially regulated between the two groups (triplicates were examined per group; Supplementary Tables 1, 2). Of these, 127 genes were upregulated and 333 genes were downregulated in simulated hyperphosphatemia. The pathway net analysis indicated that the most prominent two pathways were the MAPK signaling pathway and apoptosis (Table 1). The MAPK pathway had the highest degree of representation, implying a critical role for this pathway (Fig. 1). Next, we examined if the differentially expressed genes were interconnected to each other by constructing gene coexpression networks. Among these genes, the BMP4 gene exhibited the highest connectivity degree and k-core value (Table 2), indicating that BMP4

Table 1
The top ten pathways ranked by total degree according to the path-net analysis of significantly altered genes in endothelial cells in simulated hyperphosphatemia.

Pathway name	Outdegree	Indegree	Total degree
MAPK signaling pathway	5	27	32
Apoptosis	3	21	24
Pathways in cancer	23	0	23
Cell cycle	3	17	20
Focal adhesion	8	7	15
p53 signaling pathway	2	13	15
Calcium signaling pathway	4	9	13
Wnt signaling pathway	7	5	12
ErbB signaling pathway	7	4	11
Glycolysis/gluconeogenesis	3	8	11

Indegree represents the number of upstream pathways and outdegree represents the number of downstream pathways. Total degree represents the summation of both indegree and outdegree.

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