Hyperphosphatemia, Phosphoprotein Phosphatases, and Microparticle Release in Vascular Endothelial Cells

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ABSTRACT

Hyperphosphatemia in patients with advanced CKD is thought to be an important contributor to cardiovascular risk, in part because of endothelial cell (EC) dysfunction induced by inorganic phosphate (Pi). Such patients also have an elevated circulating concentration of procoagulant endothelial microparticles (MPs), leading to a prothrombotic state, which may contribute to acute occlusive events. We hypothesized that hyperphosphatemia leads to MP formation from ECs through an elevation of intracellular Pi concentration, which directly inhibits phosphoprotein phosphatases, triggering a global increase in phosphorylation and cytoskeletal changes. In cultured human ECs (EAhy926), incubation with elevated extracellular Pi (2.5 mM) led to a rise in intracellular Pi concentration within 90 minutes. This was mediated by PiT1/slc20a1 Pi transporters and led to global accumulation of tyrosine- and serine/threonine-phosphorylated proteins, a marked increase in cellular Tropomyosin-3, plasma membrane blebbing, and release of 0.1- to 1-μm-diameter MPs. The effect of Pi was independent of oxidative stress or apoptosis. Similarly, global inhibition of phosphoprotein phosphatases with orthovanadate or fluoride yielded a global protein phosphorylation response and rapid release of MPs. The Pi-induced MPs expressed VE-cadherin and superficial phosphatidylserine, and in a thrombin generation assay, they displayed significantly more procoagulant activity than particles derived from cells incubated in medium with a physiologic level of Pi (1 mM). These data show a mechanism of Pi-induced cellular stress and signaling, which may be widely applicable in mammalian cells, and in ECs, it provides a novel pathologic link between hyperphosphatemia, generation of MPs, and thrombotic risk.


Cardiovascular disease is the principal cause of death in advanced CKD, and in humans, elevated plasma concentrations of inorganic phosphate (Pi) are regarded as an important contributor to cardiovascular risk, particularly in hyperphosphatemic patients with advanced CKD. Although the cardiovascular effects of hyperphosphatemia partly occur through soft tissue calcification and indirect effects mediated by hormones regulating calcium and phosphate metabolism, it is also thought that Pi itself exerts direct signaling or activating effects on cells of the vasculature. Membrane-derived microparticles (MPs) are submicrometer vesicles shed from plasma membranes of several cell types (notably platelets, endothelial...
cells [ECs], and leukocytes) in response to apoptosis or cellular activation. There is now considerable evidence that CKD leads to activation of ECs, culminating in MP generation. MPs occurring in the plasma of healthy subjects are mainly of platelet origin, but endothelial MPs are increasingly recognized as powerful markers for vascular dysfunction in CKD and potential causes of thrombosis and cardiovascular disease. Recent evidence from our laboratory has shown that endothelial and platelet MPs increase significantly in patients on hemodialysis or peritoneal dialysis compared with matched controls. Plasma containing these MPs is prothrombotic, because it can generate significantly higher levels of thrombin.

Figure 1. Hyperphosphatemia acutely induces MP release from EAhy926 ECs. Acute release of MPs from EAhy926 ECs incubated for 90 minutes with control (1 mM) and high (2.5 mM) [Pi] medium. (A) Scanning electron micrographs showing MPs budding off the cell surface with (left panel) 1 mM Pi or (right panel) high phosphate milieu (2.5 mM Pi; right panel). Original magnification, ×4000. (B) Negatively stained transmission electron micrograph of the MP fraction (fraction 2 in Table 1) from the medium showing a Pi-derived MP of approximately 100–200 nm in diameter with an intact membrane. Original magnification, ×100,000. (C) NTA performed on uncentrifuged medium showing (left panel) particle concentration expressed as millions (E6) per milliliter and (right panel) average particle size. n=35. **P<0.01; ***P<0.001. (D) Flow cytometry data showing the number of particles (obtained after incubation of cells with medium for t=90 minutes at the specified Pi concentration) that were (left panel) dual labeled with anti-CD144-PE antibody and Annexin V-FITC and (right panel) labeled with Annexin V-FITC only. Using medium from a 75-cm² culture flask, particles were prepared (fraction 2 in Table 1) and suspended in 500 µl MP-Buffer (145 mM NaCl, 2.7 mM KCl, and 10 mM Hepes, pH 7.4), and 38 µl suspension was subjected to FACS analysis as described in Concise Methods. n=3. *P<0.05 versus 1 mM Pi control. (E and F) Blunting of the Pi-induced MP release by loading the medium with (E) fructose or (F) the Pi analog PFA (an inhibitor of sodium-dependent PiT1/2 Pi transporters). n=3. *P<0.05. (G) Continued particle release after the extracellular Pi concentration had been raised to 2.5 mM for 1.5 hours and then adjusted back to the control level of 1 mM for additional 1.5 hours (an indication that Pi-derived particles are MPs rather than Ca/Pi-nanocrystals forming in the medium as a direct result of the high Pi concentration). (Left panel) Particle concentration measured by NTA in uncentrifuged medium. (Right panel) Total protein determined in particles sedimented from the medium at 18,000×g (fraction 3 in Table 1). n=3. *P<0.05; **P<0.01.
in patients’ plasma than in controls. This effect is attributable to MPs, because it is abolished by filtration to remove MPs. MP are, therefore, associated with a prothrombotic state and may contribute to acute occlusive events in CKD.

It has recently been reported that Pi can increase the generation of MPs from cultured ECs, but the molecular basis of Pi’s action is obscure. In this study, we test the hypothesis that hyperphosphatemia elevates intracellular Pi concentration in ECs, resulting in direct global inhibition by Pi ions of phosphoprotein phosphatases. We show that the resulting changes in protein phosphorylation lead to release of strongly procoagulant MPs, which may be important contributors to the increased procoagulant MP population and cardiovascular risk that are observed in hyperphosphatemic patients with advanced CKD in vivo.

RESULTS

Hyperphosphatemia Acutely Induces MP Release from EAhy926 Cells

Perturbing extracellular Pi concentration typically leads to changes in intracellular Pi in mammalian cells within 90 minutes. After 90 minutes of exposure to an elevated extracellular Pi concentration of 2.5 mM, significant membrane blebbing was detected on the surface of EAhy926 cells by scanning electron microscopy (Figure 1A). Examination of the culture medium by transmission electron microscopy (Figure 1B), nanoparticle tracking analysis (NTA) (Figure 1C), and flow cytometry (Figure 1D) showed that this was accompanied by a marked increase in the release of particles resembling in vivo endothelial MPs (i.e., membrane-limited vesicles of 0.1- to 1.0-μm diameter [Figure 1B] expressing CD144 [VE-cadherin] and phosphatidylserine on their surface [Figure 1D]).

Enhanced release of particles persisted even after the extracellular Pi concentration had been returned to the control value of 1 mM (Figure 1G) and was not attributable to artifactual formation of calcium phosphate nanocrystals by the high Pi concentration (Supplemental Figure 1). Enhanced release of MP containing protein was confirmed by centrifugation of the medium and analysis of the sedimentable protein pellet (Figure 1G). Such enhanced release of sedimentable protein persisted even at 24 hours (Supplemental Figure 2).

Hyperphosphatemia Raises Intracellular Pi Concentration by Transport through Active Na⁺–Linked PIT1 (slc20a1) Pi Transporters

To confirm that a rise in intracellular Pi concentration was the signal triggering the acute increase in MP release, Pi was directly measured in the cell layer and shown to increase significantly within 90 minutes of exposure to medium with 2.5 mM Pi, achieving particularly high levels after 48 hours (Figure 2A). This Pi was shown to be intracellular and dependent on inwardly directed active Na⁺–dependent Pi transporters, because it could be blocked by (1) collapsing the plasma membrane Na⁺ gradient with ouabain (which removes the thermodynamic driving force for these Pi pumps) (Figure 2B), (2) blockade of the Pi transporters with PFA (Figure 2C), or (3) metabolic trapping of intracellular phosphate with fructose (Figure 2D).

Additional characterization of Pi transport into these cells using 3²Pi confirmed that transport depended on extracellular Na⁺ (Figure 3A) and was efficiently blocked by PFA (Figure 3A) or selective siRNA silencing of expression of slc20a1 (PIT1) Pi transporters, leading to depletion of intracellular Pi (Figure 3B) despite compensatory upregulation of slc20a2 (PIT2) transporters (Figure 3C). Depletion of intracellular Pi by metabolic trapping with the slowly metabolized sugar fructose (Figure 1E) or blocking Pi...
transporters with the Pi analog PFA (Figure 1F) significantly blunted release of MPs from the cells when they were exposed to elevated extracellular Pi concentration (2.5 mM), suggesting that elevated intracellular Pi concentration was the primary cause of the increase in MP output.

**Pi Induces Global Changes in Protein Phosphorylation**

Elevated Pi concentration has previously been reported to increase endothelial production of reactive oxygen species, a potential cause of apoptosis and consequent MP production. However, no significant oxidative stress or apoptosis was detected in response to 2.5 mM Pi in this study (Supplemental Figures 3–5), suggesting that Pi-induced MP output arose from some alternative cell stress or signaling induced by Pi. At physiologic concentrations, Pi is a potent inhibitor of a wide range of phosphotyrosine protein phosphatases (PTPases) and phosphoserine/threonine protein phosphatases (PSPases) in mammalian cells—an inhibitory effect confirmed here with as little as 100 μM Pi by assaying catalytic activity in lysates from EAhy926 cells (Figure 4, A and B).

If this inhibition is functionally important in eliciting the MP output observed with 2.5 mM Pi, applying other inhibitors capable of affecting a wide range of these phosphatases should exert a similar effect (i.e., with orthovanadate as a PTPase inhibitor and fluoride as a PSPase inhibitor). PTPase inhibition was found to mimic the acute (90-minute) effect of Pi on MP output (Figure 4C), whereas PSPase inhibition mimicked the chronic (24-hour) Pi effect on particle output (Figure 4D).

If intracellular Pi accumulation inhibits phosphoprotein phosphatases in intact cells, global increases in protein phosphorylation should be observed in Pi-loaded cells. On probing cell lysates with pan-specific antiphospho-Tyrosine or antiphospho-Serine/Threonine antibodies, such global increases in phosphorylation were detectable within 90 minutes of exposure to 2.5 mM Pi (Figure 5, A–D, Supplemental Figure 11, A–D) and reversed by siRNA silencing of PiT-1/slc20a1 gene expression (Figure 5, E–H). The increased phosphorylation induced by 2.5 mM extracellular Pi was sustained for at least 48 hours (Figure 5, A and C) despite the compensatory upregulation of at least one major cellular protein phosphatase (the low molecular weight PTPase) (Figure 5, I and J). Similar global phosphorylation increases were observed on treating cells with orthovanadate and fluoride (data not shown).

**Proteomic Analysis Shows Pi-Induced Changes in Tropomyosin Expression**

The cytoskeletal regulatory protein Tropomyosin and its phosphorylation have been implicated in regulation of endothelial membrane blebbing and MP formation. Analysis of Pi-treated EAhy926 cells by two-dimensional gel electrophoresis

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**Figure 3.** Pi transport in ECs is mainly through active Na⁺-Linked PiT1 (slc20a1) Pi transporters. (A) Effect of replacing Na in the HEPES-buffered saline (HBS) medium with choline or blocking Pi transporters with 1 mM PFA on transport of 32Pi. Cells were incubated to steady state for 90 minutes in HBS with 1 mM Pi at 37°C in air followed immediately by assay of 32Pi transport by incubating for exactly 5 minutes at 20°C in medium with 0.1 mM 32Pi at 2 μCi/ml. n=3. ****P<0.001. (B) Effect of siRNA silencing of PiT1 and/or PiT2 and/or PiT-1/2 dual siRNA silencing on total cell layer Pi. After removal of the transfection medium and allowing an additional 24-hour recovery period in Growth Medium, cells were incubated in HBS with 1 mM Pi for 90 minutes at 37°C in air. n=3. *P<0.05; **P<0.003; ***P<0.001. (C) Relative mRNA levels of PiT-1 and PiT-2 in EAhy926 cells transfected with scrambled/nontargeting siRNA, PiT-1 siRNA, and PiT-2 siRNA for 24 hours. After removal of the transfection medium and allowing an additional 24-hour recovery period in Growth Medium, RNA was extracted from the cells, reverse transcribed, and subjected to quantitative RT-PCR. n=5. ****P<0.001.
(2-DE) revealed a prominent approximately 30-kD protein accumulating within 90 minutes of exposure to 2.5 mM Pi (Figure 4A) and immunostaining with antiphospho-Serine/Threonine antibody (Figure 4I). Excision and mass spectrometry of the protein spot identified it as Tropomyosin-3 (TM-3) (Figure 4, B and C), and its rapid upregulation was subsequently confirmed by immunoblotting with antibody specific for TM-3 (Figure 4, D–H, Supplemental Figure 11, E and F). Despite marked upregulation of this protein, the intensity of its anti-phospho-Serine/Threonine antibody (Figure 4I). Excision and mass spectrometry of the protein spot identified it as Tropomyosin-3 (TM-3) (Figure 4, B and C), and its rapid upregulation was subsequently confirmed by immunoblotting with antibody specific for TM-3 (Figure 4, D–H, Supplemental Figure 11, E and F). Despite marked upregulation of this protein, the intensity of its anti-phospho-Serine/Threonine immunostaining decreased in Pi-treated cells (Figure 6, I and J), indicating that it was profoundly hypophosphorylated.

Pi-Derived MPs Are Strongly Procoagulant

Because the phosphatidylserine expressed on the surface of the MPs in Figure 1D has been shown to be procoagulant,23 the possible procoagulant effect of the MP fraction derived from the Pi-stimulated cultures was assayed in a Thrombin generation assay (Figure 7, A–D). In assays on the 18,000 × g MP fraction from the medium (after removal of apoptotic bodies, detached cells, and other large fragments by serial centrifugation) (Table 1), MPs derived from the Pi-treated cultures were found to be significantly more procoagulant than controls from cultures maintained at 1 mM Pi (Figure 7, A–D), although the total protein content of this particle fraction was similar at 1 and 2.5 mM Pi (Figure 7, E and F). This procoagulant effect was completely abolished when MPs were removed by ultrafiltration (Figure 7A).

DISCUSSION

Rapid Pi Stimulation of MP Output Implies a Direct Pi Signal within Endothelial Cells

There has been only one previous report that elevated extracellular Pi can induce MP production from cultured ECs.12 We have now made the important observation that intracellular Pi is the crucial signal generating potential pathologic events in ECs during hyperphosphatemia. Using a selective and well characterized assay for intracellular Pi,24 we have shown that, unlike other cell types,14,25 human vascular ECs experience an acute increase in intracellular Pi concentration when extracellular Pi is elevated as in hyperphosphatemia.

The concept of a powerful effect of intracellular Pi signaling on cytoskeletal and MP biology is strongly supported by the demonstration that the intracellular Pi can be depleted by silencing of slc20 Pi transporters (Figure 3), collapse of the transmembrane Na⁺ gradient with ouabain (Figure 2B), the Pi transport inhibitor PFA (Figure 2C), and phosphate trapping with fructose (Figure 2D); Pi depletion effects which blunt the subsequent release of MPs in response to elevated extracellular Pi (Figure 1, E and F).
Figure 5. Pi induces global changes in protein phosphorylation. Net global effects of hyperphosphatemia on protein phosphorylation and/or dephosphorylation in EAhy926 ECs. (A–D) Representative immunoblots and quantitative analyses by densitometry of (A and B) protein tyrosine phosphorylation probed with pan-specific antiphosphotyrosine antibody and (C and D) protein serine/threonine phosphorylation probed with pan-specific antiphosphoserine/threonine antibody. Densitometry is shown for cells incubated for 1.5 hours.
The predicted increases in protein phosphorylation on exposure to Pi were observed for some but not all of the major protein bands that stained with pan-specific antiphosphoantibodies (Figure 5, A–H), resulting in total cellular increases in protein phosphorylation of the order of 30%–50%. There are several reasons for the failure of Pi to affect all phosphoproteins. First, global inhibition of phosphatases by Pi may result in hyperphosphorylation of some protein kinases at inhibitory phosphorylation sites, thus leading to hypophosphorylation of that kinase’s substrates. (The hypophosphorylation of TM-3 in Figure 6, I and J is presumably an example of this, possibly through the previously reported inhibitory Ser-308 phosphorylation of death-associated protein kinase-1, inhibiting the reported ability of death-associated protein kinase-1 to phosphorylate Tropomyosin on Ser-283.) Second, although most phosphoprotein phosphorylations with Pi sensitivity that has been reported are inhibited by Pi, at least one such enzyme is activated by Pi. Third, compensatory upregulation of phosphoprotein phosphatase expression may occur in response to prolonged inhibition with Pi or orthovanadate (Figure 5, I and J). Fourth, although exposure of cells to 2.5 mM Pi was shown to increase total Pi in the cell layer (probably initially in the cytosol), the Pi concentration may not increase immediately in all subcellular compartments, resulting in negligible protein phosphorylation changes in any unaffected compartments.

**Phosphate Stress Is Associated with Rapid Changes in the Cytoskeletal Regulator Tropomyosin**

A proteomic screen of EAhy926 cells showed that, after initial Pi-induced phosphorylation events that were seen in these cells (Figure 5, A–H), a reproducible and rapid (90 minutes onward) accumulation of TM-3 was readily visible on two-dimensional gels and confirmed by immunoblotting cell lysates with a specific antibody against TM-3. Despite the approximately 4-fold increase in total concentration of TM-3, probing of two-dimensional gels with antiphosphoSer/Thr antibody indicated that, in Pi-treated cells, this protein was hypophosphorylated (Figure 6, I and J). It has been reported that, at least in ECs subjected to oxidative stress, hypophosphorylation of Tropomyosin leads to membrane blebbing and hence, MP formation, suggesting that the rapid changes affecting TM-3 in this study are functionally important in the MP response to Pi.

The mechanism of the rapid (possibly compensatory) 90-minute increase in total TM-3 (Figure 6, D and E) is, at present, unknown but apparently too rapid for de novo synthesis of the protein. We note, however, that the Pi analog orthovanadate, which closely mimicked the acute MP effect of Pi in this study (Figure 4C), is an inhibitor of proteasomal protein degradation, a mechanism that may contribute to not only the increase in TM-3 but also, the reproducible accumulation of previously absorbed albumin that was observed in the same Pi-loaded EAhy926 cells (Figure 6A).

**Clinical Implications of These Effects of Pi**

We have presented here evidence linking three important areas of research relevant to cardiovascular risk in CKD: hyperphosphatemia, MPs, and thrombotic risk. It should be emphasized that the MPs mediate this link (rather than the elevated Pi concentration itself through Pi being carried over into the thrombin generation assay). This was clearly shown by the observation that the procoagulant effect of the cell-derived particles in Figure 7, A–D was abolished by removal of MPs by ultrafiltration. The occurrence of phosphatidylserine on the surface of MPs is regarded as an important contributor to their procoagulant effect. However, whether additional biochemical features of specifically Pi-derived endothelial MPs also promote coagulation remains to be determined.

Finally, it should be emphasized that, in view of the ubiquitous role of protein phosphorylation in regulating mammalian cells and its dependence on phosphoprotein phosphatases, which are almost universally responsive to Pi (Figures 4, A and B and 5), the intracellular effects reported here should be widely applicable in understanding the pathologic effects of hyperphosphatemia or indeed, any factor that stimulates Pi uptake into cells. This should be true in not only hyperphosphatemia in CKD but also, other disorders, such as hypoxia and ischemia, which are associated with an elevated intracellular Pi concentration arising from impaired energy metabolism with resulting large-scale Pi generation from cytosolic organophosphorus metabolites, such as ATP and phosphocreatine. Clearly, such effects would be expected to be particularly severe during cardiovascular events in CKD in which hyperphosphatemia and ischemia/hypoxia coexist.

**CONCISE METHODS**

**Cell Culture and Incubations**

Immortalized human EC line EAhy926 was used for all experiments between passages 5 and 20. Cells were maintained in DMEM (11880; Life Technologies) with 10% (vol/vol) heat-inactivated FBS, 2 mM

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**in medium with 1 or 2.5 mM Pi. For tyrosine phosphorylation, n=4. For serine/threonine phosphorylation, n=4. MW, molecular mass.**

*P<0.05; **P<0.01. (E–H) Effect of siRNA silencing of PiT-1 transporter expression during 1.5-hour incubations of cells with 1 or 2.5 mM Pi. Representative immunoblots and quantitative analyses by densitometry of (E and F) protein tyrosine phosphorylation and (G and H) protein serine/threonine phosphorylation. Control denotes cultures treated with transfection agent only. In F, the densitometry analysis was performed on all bands in the 60- to 220-kD region of the blots. n=3. In H, densitometry was performed at 70–220 kD. n=4. *P<0.05; **P<0.01; ***P<0.001. (I and J) Effect of 24 or 48 hours of hyperphosphatemia on expression of low molecular weight protein tyrosine phosphatase (LMP-PTP) determined by immunoblotting and densitometry. n=3. *P<0.05.
L-glutamine, penicillin (10^2 IU•mL\(^{-1}\)), and streptomycin (100 \(\mu\)g•mL\(^{-1}\)) at 37°C in a humidified 5% CO\(_2\) atmosphere. Unless otherwise stated, cells were seeded for experiments in 35-mm six-well plates at 3\(\times\)10^5 cells/cm\(^2\) and used at 70% confluence. Experimental incubations were performed in MEM (21090; Life Technologies) with 2 mM L-glutamine, penicillin (10^2 IU•mL\(^{-1}\)), and streptomycin (100 \(\mu\)g•mL\(^{-1}\)) at pH 7.4 with 1.8 mM [Ca\(^{2+}\)] and 1 mM [Pi]. To model hyperphosphatemia, NaH\(_2\)PO\(_4\) was added to raise the [Pi] to 2.5 mM – a concentration that has been used extensively elsewhere.\(^{4,5,12}\) Medium harvested from experimental incubations was subjected to centrifugation as shown in Table 1.

Figure 6. Hyperphosphatemia induces changes in Tropomyosin-3 in ECs. (A) 2-DE (representative of three independent experiments) showing the effect on cell proteins of incubation of EAhy926 cells in medium with 1 or 2.5 mM Pi for 1.5 hours. Gels were stained with RAPIDstain Reagent followed by MALDI-TOF MS analysis of two prominent protein spots at approximately 75 (spot 1 on each gel) and approximately 30 kD (spot 2 on each gel). (B) Typical MALDI-TOF MS peptide fragment pattern of spot 2 taken from gel 1 (1 mM Pi-treated cells). (C) Peptide sequence homology with TM-3 (shown in bold) identified by MALDI-TOF MS and Mascot database search. The sequence coverage of TM-3 reached 38%. Similar results were obtained from spot 2 on gel 2 (2.5 mM Pi-treated cells; data not shown). (Similar analysis of spot 1 from both gels identified BSA [data not shown].) (D) Tropomyosin immunoblots (representative of three independent experiments) obtained from cells incubated as in A and probed using anti-TM-3 antibody to confirm the accumulation of TM-3 in cells treated with 2.5 mM Pi over a time course from 90 minutes to 48 hours. (E–H) Corresponding densitometry analyses on TM-3 immunoblots at (E) 90 minutes, (F) 8 hours, (G) 24 hours, and (H) 48 hours. n=3. *P<0.05. (I and J) Tropomyosin phosphorylation. Immunoblotting and densitometry analyses of 2-DE gels blotted on nitrocellulose membranes and probed with pan-specific anti–P-Ser/Thr antibody. (Spot 2-designated P–TM-3 denotes phosphorylated Tropomyosin). MW, molecular mass.

L-glutamine, penicillin (10^2 IU•mL\(^{-1}\)), and streptomycin (100 \(\mu\)g•mL\(^{-1}\)) at 37°C in a humidified 5% CO\(_2\) atmosphere. Unless otherwise stated, cells were seeded for experiments in 35-mm six-well plates at 3\(\times\)10^5 cells/cm\(^2\) and used at 70% confluence. Experimental incubations were performed in MEM (21090; Life Technologies) with 2 mM L-glutamine, penicillin (10^2 IU•mL\(^{-1}\)), and streptomycin (100 \(\mu\)g•mL\(^{-1}\)) at pH 7.4 with 1.8 mM [Ca\(^{2+}\)] and 1 mM [Pi]. To model hyperphosphatemia, NaH\(_2\)PO\(_4\) was added to raise the [Pi] to 2.5 mM — a concentration that has been used extensively elsewhere.\(^{4,5,12}\) Medium harvested from experimental incubations was subjected to centrifugation as shown in Table 1.

MP Isolation

MPs were isolated from the culture medium as described previously\(^7\) with slight modification (Table 1).

Briefly, medium from cultures was centrifuged (step 1) at 1500\(\times\)g at 20°C for 20 minutes to remove detached cells and large particles/apoptotic bodies. The top 90% of the supernatant from step 1 was centrifuged (step 2) at 18,000\(\times\)g at 20°C for 30 minutes to pellet MPs. The top 90% of the supernatant from this step was aspirated, and the pellet was resuspended in the following 0.2-\(\mu\)m-filtered MP-Buffer (145 mM NaCl, 2.7 mM KCl, and 10 mM Hepes, pH 7.4) and recentrifuged (step 3) as before to wash MPs before
resuspending again in MP buffer and storing at −80°C for additional analysis.

**NTA**  
The number and size of the particles in fractions isolated as in Table 1 were analyzed by NTA using a NanoSight LM10 with NTA software v2.2 (NanoSight Ltd., Amesbury, UK) and 90-second video capture as previously described.7

**Flow Cytometry Analysis of MPs**  
Samples (38 μl; fraction 2) (Table 1) were thawed and mixed with 32 μl × Annexin V-Binding buffer with calcium (556454; BD Pharmingen); 5 μl each anti-human CD144 (VE-cadherin)-PE (eBioscience) and Annexin V-FITC (BD Pharmingen) were added, and samples were stored in the dark for 25 minutes followed by dilution to a final volume of 500 μl with Annexin V-Binding buffer before analysis on a Flow Cytometer (BD FACSaria II; BD Bioscience, San Jose, CA). Samples incubated with mouse IgG1-PE (BioCytex) were used as isotype control. Samples incubated with Annexin V-FITC in binding buffer without calcium were used as a negative control for Annexin V binding. Single labeling with either CD144-PE or Annexin V-FITC was carried out to perform gating using Megamix (BioCytex) beads of 0.5, 0.9, and 3 μm in diameter. Events were gated according to size in a forward/side scatter dot plot.

**Transmission Electron Microscopy**  
Particles from 1 and 2.5 mM Pi-treated cultures (fraction 2) (Table 1) were fixed in 25% Glutaraldehyde followed by two 1% uranyl acetate washes and air drying, and they were viewed using a JEOL JEM1400 transmission electron microscope with an accelerating voltage of 80 kV. Images were captured by a Megaview III digital camera with iTEM software.

**Scanning Electron Microscopy**  
Subconfluent cells on 13-mm round coverslips were treated with 1 or 2.5 mM [Pi] medium for 90 minutes and then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate with 2 mM calcium chloride (pH 7.4) followed by analysis on a Hitachi S3000H scanning electron microscope with an accelerating voltage of 5 kV.

**Transfection with siRNA**  
Transient silencing of PiT-1/2 (slc20a1/a2) phosphate transporter expression in cells was performed by siRNA targeting the specific genes using Silencer Select Validated siRNAs (Ambion) with a scrambled nontarget Silencer Select Negative Control siRNA (Ambion) as a negative control. siRNA oligonucleotides were incubated for 4 hours with the cultures at 10 nM final concentration as described in the manufacturer’s instructions using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). Uptake of siRNA was confirmed using BLOCK-iT Fluorescent Oligonucleotides (Invitrogen) followed by

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**Figure 7.** Pi-derived MPs are strongly procoagulant. Effect in a thrombin generation assay of MPs sedimented at 18,000 × g from medium (with 1 or 2.5 mM Pi) cultured for 24 hours with EAhy926 cells. Particle centrifugation was performed as described in Table 1. Sedimented particles (fraction 2 in Table 1) were resuspended in pooled filtered plasma (PFP) before the assay. Control curves are also shown for PFP alone and particle preparations from which particles had been removed by ultrafiltration. (A) Representative thrombin generation curves (showing definitions of the Thrombogram parameters). (B–D) Analyses of peak thrombin, endogenous thrombin potential (ETP), and lag time of control and Pi-derived MPs showing significantly increased peak thrombin and ETP with MPs from Pi-treated cells, although the time at which thrombin burst commenced (lag time) was not different between the two MP preparations. t=24 hours. n=3. *P<0.05. (E) Total protein concentration of the 18,000×g sedimented MP pellet (fraction 2 in Table 1) from the control (1 mM Pi) and Pi-loaded (2.5 mM Pi) culture medium showing similar MP content. t=24 hours. n=3. (F) Analysis of thrombin generated per microgram of protein indicating release of more procoagulant MP from high Pi medium. t=24 hours. n=3. *P<0.05.
quantification by flow cytometry. Gene silencing was confirmed by quantitative RT-PCR using primers specific for PIT-1/2.

RNA Extraction and Quantitative RT-PCR
Total RNA was extracted using TRIzol reagent (Invitrogen). Using 1 μg total RNA, cDNA was synthesized using an AMV Reverse Transcription System (Promega) according to the manufacturer’s instructions. Forward and reverse primer sequences for SLC20A1, SLC20A2, and GAPDH are listed in Table 2. Real-time PCR was performed using Applied Biosystems 7500 Fast Real-Time PCR System (Life Technologies) using SYBR green PCR reagent (Life Technologies). Relative amounts of mRNA were normalized to the corresponding GAPDH signal for each sample, and relative expression is presented as (2^ΔΔCt).30

Immunoblotting and Gel Staining
Cell lysates were subjected to SDS-PAGE (20 μg protein per lane) followed by either immunoblotting or gel staining by Silver Stain Plus (Bio-Rad) or RAPIDstain Reagent (Calbiochem) as described in the manufacturer’s instructions. Immunoblotting was performed onto nitrocellulose membranes (Amersham) followed by probing with primary antibodies against TM-3 (Cell Signaling Technology), global P-Tyr (Santa Cruz Biotechnology), global P-Ser/Thr (Antibodies-Online; Becton Dickinson), low molecular weight protein tyrosine phosphatase (Thermo Fisher Scientific), and β-actin (Abcam, Inc.). Polyclonal rabbit anti-mouse and goat anti-rabbit Igs/HRP (DakoCytomation) were used as secondary antibodies as appropriate, and HRP-labeled proteins were detected by chemiluminescence (ECL-Amersham). Band intensities were quantified by Image Studio Software v 4.0.21 (LI-COR Biosciences, Lincoln, NE), and data are presented as the ratio of the intensity for the protein of interest/housekeeping protein expressed as a percentage of the corresponding ratio under control conditions.

2-DE
Cell lysates were desalted using Protein Desalting Spin Columns (Thermo Fisher Scientific). 2-DE was performed using a PROTEAN IEF Cell (Bio-Rad) followed by 12% SDS-PAGE. The 2-DE gels were stained by Silver Stain Plus (Bio-Rad) or RAPIDstain Reagent (Calbiochem; if 2-DE was to be followed by mass spectrometry). In some experiments, proteins from 2-DE gels were blotted onto nitrocellulose membranes (Amersham) followed by probing with primary pan-specific antibody against P-Ser/Thr (Antibodies-Online; Beckton Dickinson).

Mass Spectrometry
Protein spots on 2-DE gels stained with RAPIDstain Reagent (Calbiochem) were excised and subjected to proteomics analysis by trypsin digestion followed by Orbitrap (LC MS/MS) or MALDI-TOF mass spectrometry (Voyager DE-STR). The resulting LC MS/MS data were analyzed by Scaffold-3 software, and MALDI-TOF MS data were analyzed by the Mascot search engine.

Measurement of Cell Layer Pi
Pi was determined in neutralized deproteinized cell extracts as previously described.24

32Pi Transport Assays
Pi transport into intact cell monolayers was assayed from the rate of uptake of 32Pi as described in ref. 14, but the cells were incubated in Hepes-buffered saline (140 mM NaCl, 20 mM Hepes, 2.5 mM MgSO4.7H2O, 5 mM KCl, 1 mM CaCl2.2H2O, and 10 mg/L Phenol Red) with 32Pi at room temperature for only 5 minutes; the total Pi concentration in the assay was 0.1 mM. To show the Na dependence of Pi transport, NaCl in the Hepes-buffered saline was replaced with 140 mM choline chloride.

Determination of the Inhibitory Effect of Pi on PTPase Activity
PTPase activity in EAhy926 cell lysates was determined using a Tyrosine Phosphatase Assay System (V2471; Promega) as described in the manufacturer’s instructions.

Thrombin Generation Assay Using Calibrated Automated Thrombography
The ability of Pi-derived MPs to enhance thrombin generation was determined7 using calibrated automated thrombography with PRP Reagent (Diagnostica Stago) containing 1 pM tissue factor.

Statistical Analyses
Data are presented as the means±SEM and were analyzed using GraphPad Prism 6.0. Two group data comparisons were analyzed by t test (for normally distributed data) or Wilcoxon matched pairs signed rank test (for nonparametric data). One-way ANOVA (combined with Tukey’s post hoc test for normally distributed data or Dunn’s nonparametric post hoc test) was applied for multiple comparison tests as appropriate. P values <0.05 were considered statistically significant.

Table 1. Centrifugation steps applied to conditioned medium from EAhy926 cells

<table>
<thead>
<tr>
<th>Step Number</th>
<th>Starting Material</th>
<th>Centrifugation Applied</th>
<th>Fraction Analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Medium from cultures</td>
<td>1500×g at 20°C for 20 min</td>
<td>Fraction 1 (resuspended pellet)</td>
</tr>
<tr>
<td>2</td>
<td>Supernatant from step 1</td>
<td>18,000×g at 20°C for 30 min</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>Resuspended pellet from step 2</td>
<td>18,000×g at 20°C for 30 min</td>
<td>Fraction 2 (MPs; resuspended pellet from step 3)</td>
</tr>
<tr>
<td>4</td>
<td>Medium from cultures</td>
<td>18,000×g at 20°C for 30 min</td>
<td>Fraction 3 (assay total protein in pellet)</td>
</tr>
</tbody>
</table>

Table 2. Primers used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>SLC20A1</td>
<td>PIT-1</td>
<td>Forward: CCAACTGTGCAAGCCTAGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TTCTTCGTGTCGTGCTATT</td>
</tr>
<tr>
<td>SLC20A2</td>
<td>PIT-2</td>
<td>Forward: AGGATCTCAGGTCGCAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: AGGCCAGACATGAACACAGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td>Forward: CATGATGTGAGGTCAAGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CGAGCCACATCGCTCAG</td>
</tr>
</tbody>
</table>

ACKNOWLEDGMENTS

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DISCLOSURES

None.

REFERENCES


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