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.7 MPs 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,12 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.13,14 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 nanocrystals15 by the high Pi concentration (Supplemental Figure 1). Enhanced release of MPs 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 fructose16 (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,5,8 a potential cause of apoptosis and consequent MP production.4 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 cells17,19—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 inhibitor20 and fluoride as a PSPase inhibitor21). 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 tropomysin expression
The cytoskeletal regulatory protein Tropomyosin and its phosphorylation have been implicated in regulation of endothelial membrane blebbing and MP formation.22 Analysis of Pi-treated EAhy926 cells by two-dimensional gel electrophoresis

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.
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 rapid upregulation of this protein, the intensity of its anti-phospho-Serine/Threonine antibody (Figure 6I). Excision and mass spectrometry of the protein spot identified it as Tropomyosin-3 (TM-3) (Figure 6, B and C), and its rapid upregulation was subsequently confirmed by immunoblotting with antibody specific for TM-3 (Figure 6, 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

Figure 4. Pi inhibits phosphoprotein phosphatases. (A and B) Direct inhibition by Pi of tyrosine protein phosphatase catalytic activity in lysates of EAhy926 cells assayed in vitro in the presence of exogenous Pi at the stated concentration using two different tyrosine phosphatase substrates (A, V2471 substrate-1; B, V2471 substrate-2; Promega). n=3. *P<0.05. (C) Mimicry by broad spectrum PTPase inhibitor (Vanadate) of the acute (90-minute) Pi-induced increase in particle output detected by NTA in uncentrifuged medium from EAhy926 cells. n=3. Particle concentration is expressed as millions (E6) per milliliter. *P<0.05; ***P<0.001. (D) Mimicry by broad spectrum PSpase inhibitor (sodium fluoride) of the chronic (24-hour) Pi-induced increase in particle output detected by measuring total sedimentable protein after centrifugation at 18,000×g (fraction 3 in Table 1). n=3. **P<0.01; ***P<0.001; ****P<0.0001.

(2-DE) revealed a prominent approximately 30-kD protein accumulating within 90 minutes of exposure to 2.5 mM Pi (Figure 6A) and immunostaining with antiphospho-Serine/Threonine antibody (Figure 6I). Excision and mass spectrometry of the protein spot identified it as Tropomyosin-3 (TM-3) (Figure 6, B and C), and its rapid upregulation was subsequently confirmed by immunoblotting with antibody specific for TM-3 (Figure 6, 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).

Pi Induces a Distinct and Sustained Form of Cell Stress through Global Changes in Protein Phosphorylation

No significant Pi-induced oxidative stress or apoptosis was detected in this study, but despite this, a rapid Pi-induced increase in MP output was observed, indicating that apoptosis is not the major source of the MP effect. The MP formation reported here is associated with a distinct and novel form of metabolic stress characterized by global changes in protein phosphorylation. The intracellular Pi signal is sensed in EAhy926 cells through the potent direct inhibition (Figure 4, A and B) of PTPases and phosphoserine/threonine phosphatases by Pi ions that occurs in response to pathologic intracellular Pi concentrations,17–19 culminating in global accumulation of Tyr-phosphorylated and Ser-Thr–phosphorylated proteins that are readily shown using pan-specific P-Tyr and P-Ser/Thr antibodies (Figure 5, A–H). This effect is reversed by siRNA silencing of the Pi transporter PiT1/slc20a1 (Figure 5, E–H), confirming that Pi translocation into cells is needed for the effect. Similar global inhibition of PTPases by the Pi analog orthovanadate and phospho-Serine/Threonine phosphatases by orthovanadate (Figure 4, C and D) closely mimicked the generation of MPs observed with Pi (Figure 1, C and G).
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-1,26 inhibiting the reported ability of death-associated protein kinase-1 to phosphorylate Tropomyosin on Ser-283.) Second, although most phosphoprotein phosphatases with Pi sensitivity that has been reported are inhibited by Pi, at least one such enzyme is activated by Pi.27 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 anti–TM-3 antibody (Figure 6, D–H). Despite the approximately 4-fold increase in total concentration of TM-3, probing of two-dimensional gels with antiphospho-Ser/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,22 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,28 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.23 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),17–19 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.29 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 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 (LMW-PTP) determined by immunoblotting and densitometry. n=3. *P<0.05.
L-glutamine, penicillin (10^2 IU·mL⁻¹), and streptomycin (100 μg·mL⁻¹) at 37°C in a humidified 5% CO₂ atmosphere. Unless otherwise stated, cells were seeded for experiments in 35-mm six-well plates at 3 × 10^5 cells/cm² 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 μg·mL⁻¹) at pH 7.4 with 1.8 mM [Ca²⁺] and 1 mM [Pi]. To model hyperphosphatemia, NaH₂PO₄ 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.

**MP Isolation**

MPS were isolated from the culture medium as described previously with slight modification (Table 1).

Briefly, medium from cultures was centrifuged (step 1) at 1500×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×g at 20°C for 30 minutes to pellet MPs. The top 90% of the superant was then aspirated and the pellet was resuspended in the following 0.2-μm-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 FACS Aria 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.
Table 1. Centrifugation steps applied to conditioned medium from EAhy926 cells

<table>
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<th>Step Number</th>
<th>Starting Material</th>
<th>Centrifugation Applied</th>
<th>Fraction Analyzed</th>
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<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>
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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 an 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 \((2^{-\Delta\Delta C_T})\).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 Iggs/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 µg/mL 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.

Table 2. Primers used for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Primer Sequence</th>
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<tr>
<td>SLC20A1</td>
<td>PIT-1</td>
<td>Forward: CCAACTGTGCAGCAGCATAGAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: TCTTCTCTGTTGTGCTGATT</td>
</tr>
<tr>
<td>SLC20A2</td>
<td>PIT-2</td>
<td>Forward: AGGATTTCTCAGTGGTGCGAA</td>
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<td></td>
<td></td>
<td>Reverse: AGGCCAGACATGAAACCAGA</td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
<td>Forward: CATGTAGTTGAGGTCAAGGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reverse: CGAGCCACATCGCTCAG</td>
</tr>
</tbody>
</table>
ACKNOWLEDGMENTS

Mass spectrometry was performed in the Protein Nucleic Acid Chemistry Laboratory, University of Leicester. We thank Dr. Lucia Pinon (Medical Research Council Toxicology Unit, University of Leicester) for assistance with flow cytometry, Mr. Stefan Hyman and Miss Natalie Alcock (Core Biotechnology Services, Electron Microscopy Laboratory, University of Leicester) for assistance with transmission electron microscopy and scanning electron microscopy, and Mr. Mohammed Ali Alsahl for assistance with calibrated automated thrombocyte assays.

DISCLOSURES

None.

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This article contains supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681/ASN.2014070642/-/DCSupplemental.
Supplemental Text

Supplementary Results

**Microparticle output is not attributable to formation of Calcium Phosphate nanocrystals by the high Pi concentration**

In principle raising the Pi concentration in the medium to 2.5mM may lead to precipitation of Calcium Phosphate nanocrystals which may act on the cells, possibly affecting cell signalling and contributing to MP output. Even though a detectable increase in calcium precipitation in the cell layer was demonstrable in Pi-loaded cultures (by showing deposition of radio-activity from medium labelled with $^{45}\text{Ca}^{2+}$) this effect was enhanced by the Pi analogue PFA (Supplemental Figure 1A). In contrast PFA abolished output of sedimentable protein particles into the medium in the same experiments, indicating that the calcium deposition was not the cause of the particle output.

**Chronic (24h) hyperphosphataemia increases detachment of EAhy926 cells**

Quantification by NTA of MP release during more prolonged (≥ 24h) exposure to 2.5mM Pi initially detected no stimulation of particle output (data not shown). However, centrifugation of the resulting culture medium at 18,000g after 24h exposure of the cells to 2.5mM Pi led to a reproducible increase in sedimentable protein (Supplemental Figure 2A) implying that the sedimented particles were whole cells or large cell fragments exceeding the NTA analyser’s 1.0μm upper detection threshold. This was confirmed by subjecting the particles to fragmentation by freeze-thawing the medium from these experiments and then sedimenting and resuspending the particles. In this way NTA readily detected an increase in particle count following 24h 2.5mM Pi exposure (Supplemental Figure 2C). As with the acute particle release after 90 min (Figure 1C in main manuscript), this cell
detachment effect of 2.5mM Pi was abolished by blocking Pi transport with PFA (Supplemental Figure 1B and 2B).

**Pi has little effect on oxidative stress and cell death**

Elevated Pi concentration has previously been reported to influence endothelial production of labile nitroxide and oxygen species; decreasing nitric oxide synthase activity \(^1\, ^2\) and increasing production of reactive oxygen species (ROS) \(^1\), the latter being a potential cause of apoptosis and consequent MP production. Inducing ROS production in EAhy926 cells with menadione did induce a marked increase in particle output (Supplemental Figure 2A). However, even though decreased nitric oxide synthase activity was readily detected after 90 min of exposure to 2.5mM Pi (Supplemental Figure 3C), no effect of Pi was observed on ROS production. Three independent ROS probes: DM-H2DCFDA (Supplemental Figure 3A and B), Dihydroethidium (DHE) (Supplemental Figure 3D) and Nitro-blue Tetrazolium (NBT) (Supplemental Figure 3E), readily detected a ROS signal within 30 min using a Menadione positive control, but no reproducible response to elevated Pi concentration.

At all time points studied (90 min to 48h), exposure of the cells to 2.5mM Pi induced only a small apparent decline in cell viability assessed by MTT staining (Supplemental Figure 4A); consistent with small increases in pro-apoptotic signals including Caspase-3 cleavage (Supplemental Figure 4B and C), Bax (Supplemental Figure 4B and D), PARP cleavage (Supplemental Figure 4E and F) and DNA laddering (Supplemental Figure 4L). However, more detailed analysis by Propidium iodide/Annexin V flow cytometry, failed to detect significant apoptosis or necrosis (Supplemental Figure 4G, H and I), or cell shrinkage (Supplemental Figure 4J and K) in the cell population as a whole when averaged over 13 independent experiments.
Similarly cell death and apoptosis measured by the TUNEL-coupled Hoechst assay (Supplemental Figure 4M), cellular ATP concentration (Supplemental Figure 5A) and glycolytic rate (lactate output – Supplemental Figure 5B) were also unaffected by elevated Pi, confirming that only minor effects on cell viability were occurring and that the pro-apoptotic signals in Supplemental Figures 4B-F and 4L were confined to a small fraction of the total cells.

This was still the case after 48 hours (data not shown).

**Pi does not enhance CD144 expression in adherent EAhy926 cells**

Even though 24h of exposure to elevated Pi concentration enhanced CD144 expression in MPs (Supplemental Figure 2D) and in detached cells (Supplemental Figure 6) that were released from the cultures, a small decrease in CD144 expression was observed in intact adherent cells (Supplemental Figure 6).

**Effect of Z-VAD-FMK Caspase-3 inhibitor on Pi-induced EMP output**

The effect of a Caspase 3 inhibitor on the acute (90 min) and chronic (24h) effect of elevated extracellular Pi (i.e. 2.5mM [Pi]) on NTA detectable particle release from EAhy926 ECs was studied. Cultures were treated with vehicle alone (DMSO) or Z-VAD-FMK (100μM) (Supplemental Figure 7). Even though Z-VAD-FMK apparently abolished the effect of Pi, a similar blunting was also observed with the DMSO vehicle alone.

**Effect of Rho Kinase inhibitors on Pi-induced EMP output**

As caspase-mediated activation of the Rho/ROCK pathway has been implicated in the activation of microparticle output accompanying apoptosis, the effect of an inhibitor of this pathway (Y-27632) on the acute effect of a high extracellular Pi
concentration (i.e. 2.5mM [Pi]) on NTA detectable particle release from EAhy926 ECs was studied. Some cultures were treated with ROCK inhibitor Y-27632 (10μM) for 1 hour before Pi treatment. Control cultures were treated with 1mM [Pi] in the absence of Y-27632. The Rho-kinase inhibitor did not blunt Pi-induced MP release from EAhy926 endothelial cells but did result in a higher base line of MP output from the medium (Supplemental Figure 8).

The acute effect of Pi on Human Umbilical Vein Endothelial Cells (HUVEC) is similar to that observed in EAhy926 cells

The acute effect of higher extracellular Pi (i.e. 2.5mM [Pi]) on NTA detectable particle release (Supplemental Figure 9), cell layer Pi concentration (Supplemental Figure 10), global protein phosphorylation (Supplemental Figure 11A-D) and Tropomyosin expression (Supplemental Figure 11E and F) in HUVECs was studied and shown to be similar to that observed with EAhy926 cells. Control cultures were treated with 1mM [Pi].

Effect of a wide range of extracellular Pi concentrations on MP output from EAhy926 cells

The effect of a wide range of extracellular Pi (i.e. 0.5, 1, 1.7, and 2.5mM) on the NanoSight detectable particle release from EAhy926 cells was studied after 90 min of exposure of the medium to the cells. Whereas a modest increase in Pi concentration from 1.0mM to 1.7mM had no detectable effect, lowering the concentration to 0.5mM apparently blunted the particle release (Supplemental Figure 12).
Supplementary Discussion

Hyperphosphataemic states, independent of kidney disease, are associated with thrombotic and/or embolic events

Tumor lysis syndrome during cancer chemotherapy provides another possible example of thrombosis associated with hyperphosphatemia. While a number of forms of cancer are associated with increased risk of thrombosis in which MPs expressing tissue factor play a role, this effect is significantly increased during the marked hyperphosphatemia that arises as part of the so-called tumor lysis syndrome during chemotherapy. At least in its early stages this hyperphosphatemia is independent of renal impairment, occurs without accompanying azotemia, and can arise directly from phosphate release from the dying tumor cells.

Endothelial cells but not erythrocytes and skeletal myocytes acutely respond to an increased extracellular Pi concentration.

Here we have shown that endothelial cells exposed to an increased extracellular Pi concentration acutely experience an elevation in intracellular Pi concentration (Figure 2 and Supplemental Figure 10) however this is not observed in other cells like erythrocytes and skeletal myocytes. The molecular basis for this difference is still unclear. In addition to Na+-linked PiT1 (slc20a1) Pi transporters which carry Pi into the cells, mathematical modelling of the regulation of intracellular Pi concentration suggests that the hitherto uncharacterised Pi transporter which carries Pi out of mammalian cells could also have a marked influence on the relationship between intracellular and extracellular Pi concentration. This difference between ECs and other cell types may therefore reside in this Pi efflux transporter.
Supplemental methods

Cell Culture and Incubations

Immortalised human endothelial cell line EAhy926 was used for all experiments between passages 5-20. Cells were maintained in DMEM (Life Technologies ref 11880) with 10% vol/vol heat-inactivated foetal bovine serum (FBS), 2mM L-glutamine, penicillin (10^2 IU.ml^{-1}) and streptomycin (100µg.ml^{-1}) at 37°C in a humidified 5% CO_2 atmosphere. Unless otherwise stated, cells were seeded for experiments in 35mm six-well plates at 3x10^5 cells/cm^2 and used at 70% confluence. Experimental incubations were performed in MEM (Life Technologies Ref 21090) with 2mM L-glutamine, penicillin (10^2 IU.ml^{-1}) and streptomycin (100µg.ml^{-1}), at pH 7.4 with 1.8mM [Ca^{2+}] and 1mM [Pi]. To model hyperphosphataemia NaH_2PO_4 was added to raise the [Pi] to 2.5mM – a concentration which has been used extensively elsewhere^{2-4}. Medium harvested from experimental incubations was subjected to centrifugation as shown in Table 1.

Microparticle Isolation

Microparticles were isolated from the culture medium as described previously^{5} with slight modification (Table 1).

Briefly, medium from cultures was centrifuged (Step 1) at 1,500xg, at 20°C for 20min to remove detached cells and large particles/apoptotic bodies. The top 90% of the supernatant from Step 1 was centrifuged (Step 2) at 18,000xg, at 20°C for 30min to pellet microparticles. The top 90% of the supernatant from this step was aspirated and the pellet resuspended in the following 0.2µm filtered MP-Buffer (145mM NaCl, 2.7mM KCL, 10mM HEPES, pH 7.4) and recentrifuged (Step 3) as before to wash microparticles before resuspending again in MP buffer and storing at -80°C for further analysis.
Isolation and measurement of detached cells following chronic (24h) incubation

Following incubation of cells with medium containing 1mM or 2.5mM [Pi] for 24h, medium was harvested and total particles/detached cells sedimented at 18,000xg, at 20°C, for 30min (Step 4, Fraction 3 in Table 1) followed by precipitation of protein with 0.3M perchloric acid (PCA) and assay of total protein.

Nanoparticle Tracking Analysis (NTA)

The number and size of the particles in fractions isolated as in Table 1 was analysed by Nanoparticle Tracking Analysis (NTA) using a NanoSight LM10 with NTA software v2.2 (NanoSight Ltd, Amesbury, UK) and 90 second video capture as previously described.

Flow Cytometry Analysis of Microparticles

Samples (38μl, Fraction 2; Table 1) were thawed and mixed with 32ul of 1x Annexin V-Binding buffer with calcium (BD Pharmingen Ref 556454). 5ul each of Anti-Human CD144 (VE-Cadherin)-PE (eBioscience), and Annexin V-FITC (BD Pharmingen) was added and samples stored in the dark for 25min followed by dilution to a final volume of 500ul with Annexin V-Binding buffer before analysis on a Flow Cytometer (BD FACSARia™ II; Becton Dickinson, BD Bioscience, San Jose, USA). 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. MPs were initially gated according to their size (i.e. FSC vs. SSC) and subsequently analysed according to their CD144 and Annexin V status as we and others have previously described (please see Figure 3 in and Figure 2-3 in). Gating and compensation adjustments were carried out
using Megamix (BioCytex) beads of 0.5, 0.9, and 3um diameter and single labelling of particles with either CD144-PE or Annexin V-FITC as previously described 16. Events were gated according to size in a Forward/Side scatter dot plot.

**Transmission Electron Microscopy (TEM)**

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

**Scanning Electron Microscopy (SEM)**

Sub-confluent cells on 13mm round coverslips were treated with 1 or 2.5mM [Pi] medium for 90min and then fixed in 2.5% glutaraldehyde in 0.1M Sodium Cacodylate with 2mM Calcium chloride pH7.4, followed by analysis on a Hitachi S3000H scanning electron microscope with an accelerating voltage of 5kV.

**Transfection with siRNA**

Transient silencing of phosphate transporters PiT-1/2 (slc20a1/a2) transporter expression in cells was performed by siRNA targeting the specific genes using Silencer® Select Validated siRNAs (Ambion) with a scrambled non-target Silencer® Select Negative Control siRNA (Ambion) as a negative control. siRNA oligonucleotides were incubated for 4h with the cultures at 10nM 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 quantification by flow cytometry. Gene silencing was confirmed by qRT-PCR using primers specific for PiT-1/2.
RNA extraction and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). Using 1µg of total RNA, cDNA was synthesised 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 an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies) using SYBR green PCR reagent (Applied Biosystems). Relative amounts of mRNA were normalised to the corresponding GAPDH signal for each sample and relative expression is presented as \(2^{\Delta\Delta CT}\).  

Determination of Apoptosis and Cell Death

MTT Assay

Cultures were incubated in Hanks Balanced Salt Solution (HBSS) without phenol red containing 2mg/ml MTT (Sigma M5655) for 4h at 37°C in a culture incubator. Medium was aspirated and the insoluble formazan product in the cell layer was dissolved in DMSO followed by measurement of absorbance at 595nm.

DNA fragmentation

Analysis of DNA fragmentation was performed as described by \(^8\). Briefly, cells were treated with 1mM Pi and 2.5mM Pi for 24h. Cells treated with 100-200µM H₂O₂ were used as positive controls. To suppress DNA fragmentation additional high Pi media supplemented with 100µM Caspase Inhibitor Z-VAD-FMK (R&D Systems) or vehicle control (DMSO) were used. After cell incubation the medium was removed and the adherent monolayer harvested in 250µl of Cell Dissociation Buffer (Sigma) and topped up with their relevant medium followed by centrifugation at 3,000xg, 4°C,
10min. Using a hypotonic lysis buffer (10mM Tris-HCL, pH 8.0, 10mM EDTA, 0.5% Triton X-100) cell pellets were lysed. 0.1 mg/ml RNase A (Sigma-Aldrich) was added to digest the RNA for 30min at 37\(^\circ\)C. Proteinase K (1mg/ml) (Sigma) was added and samples further incubated at 37\(^\circ\)C for 30min. An equal volume of phenol, chloroform, and isoamyl alcohol (25:24:1) (Sigma) was mixed with the nucleic acid solution to extract DNA followed by DNA precipitation in 1:1 v/v isopropyl alcohol at -20\(^\circ\)C for 24h. DNA was collected by centrifugation at 12,000xg, 4\(^\circ\)C, 15min and the DNA pellet was washed in 70% ethanol and re-pelleted as before. DNA pellets were air-dried and resuspended in 25µl 1x TE. DNA concentrations were determined spectrophotometrically. 5µg DNA was mixed with sample buffer (0.25% bromophenol blue, 30% glycerol) and run on 1.5% agarose gel containing 1µg/ml ethidium bromide for 4h and visualised by a UV transilluminator.

**TUNEL-coupled Hoechst Staining**

TUNEL staining was carried out on cultures on glass coverslips using an In Situ Cell Death Detection Kit, Fluorescein (Roche ref 11684795910). Nuclei were stained at the final step with Hoechst 33342 (Sigma) at 1µg/ml for 5min, at room temperature before being analysed by fluorescence microscopy (Nikon Eclipse Ti80 inverted epifluorescence microscope). From each experimental condition four randomly selected fields were examined and fluorescein staining assessed in approximately 50 Hoechst-stained cells per field.

**Flow Cytometry (Annexin V/PI Staining)**

Apoptosis was assessed by Annexin V-FITC (BD Pharmingen) and propidium iodide (BD Pharmingen) staining as described in\(^3\) using a FACSCalibur flow cytometer with Cellquest acquisition software. In the same experiments Forward Angle Light Scattering (FSC) was used as an index of apoptotic cell shrinkage.
**Determination of ROS Generation**

**DCFDA and Flow Cytometry**

Trypsinised cell monolayers were incubated in IMDM-without phenol red (Invitrogen Ref 21056) containing 10µM CM-H₂DCFDA (Invitrogen) at 37°C, under 5% CO₂, in air for 30min and were washed twice in PBS before analysis on a FACSCalibur flow cytometer with Cellquest acquisition software using filter FL1.

**DCFDA-Coupled Hoechst Staining**

Adherent cultures on coverslips were stained with CM-H₂DCFDA as above followed by staining of the nuclei with Hoechst 33342 at 1µg/ml for 5min, at room temperature, before analysis by fluorescence microscopy as in the TUNEL assay above.

**DHE**

Cells were treated with 0.8, 1.8, and 2.5mM [Pi] for 30, 60, and 90min at 37°C, 5% CO₂ in air and humidified atmosphere using HBSS as control medium ([Pi] = 0.8mM). ROS-sensitive dye dihydroethidium (DHE) (10µM) (Sigma) was added to the incubations for the last 30min of the incubations. Cultures were trypsinised, and re-suspended in PBS before being sonicated, followed by determination of fluorescence intensity (Excitation 518nm/Emission 605nm) in a 96-well plate on a Perkin Elmer FluoroCount fluorescent plate scanner.

**NBT**

ROS generation was determined colorimetrically in Pi-treated cells using a nitrobluetetrazolium (NBT) assay (Sigma Ref 74032) as previously described¹. Briefly, HBSS containing 0.2% NBT (Sigma) was set up as control medium (Pi
concentration 0.8mM). After reaching 60-70% confluence, cells seeded on 22mm 12-well culture plates were treated with medium containing 0.8, 1.8, 2.8mM [Pi] for 30min, 60min, and 90min at 37°C, under a humidified 5% CO₂ atmosphere. Cells treated with 0.8mM [Pi] medium supplemented with 30uM Menadione were used as positive control. N-Acetyl-L-cysteine (NAC) (Sigma) at a final concentration of 10mM was used as a negative control suppressing ROS generation. After cell treatment as above, medium was removed and the cell monolayer was gently rinsed twice with PBS to remove extracellular bound formazan. Methanol (200µl per well) was added to fix the cells and then the cells were air-dried before adding 240µl and 280µl of 2M KOH and DMSO respectively per well to solubilise cell membranes and dissolve formazan respectively. To aid solubilisation, plates were placed on a shaker for 15min at room temperature. The absorbance of samples was determined at 650nm.

**Assay of Nitric Oxide Synthase (NOS) activity**

Nitric Oxide Synthase (NOS) activity in intact cultures was assessed from the rate of generation of L-citrulline (as a by-product of conversion of L-arginine to NO)⁹. L-citrulline was determined by high-performance liquid chromatography as described in ¹⁰. Briefly, cells on 35mm six-well plates were treated with 1mM and 2.5mM [Pi] for 90min. Test medium was removed and the monolayer rinsed three times in ice-cold 0.9% NaCl. 150µl of 0.3M PCA was added/well and cells scraped on ice to precipitate proteins. Samples were centrifuged as 3000xg, 4°C for 10min to sediment proteins. The protein pellet used to determine total cell layer protein and an equal volume of Freon-Tri-Octylamine mixture (22% v/v Tri-n-octylamine: 78% v/v 1,1,2-trichlorotrimfluoroethane) was added to the supernatant and agitated vigorously to neutralise the PCA. The neutralised aqueous-top phase was stored at -80°C for determination of L-citrulline by HPLC.
Immunoblotting and Gel Staining

Cell lysates were subjected to SDS-PAGE (20µg protein per lane) followed by either immunoblotting or in-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), Caspase 3, Bax, global P-Tyr (all from Santa Cruz), PARP (Sigma-Aldrich), global P-Ser/Thr (Antibodies-online, BD), LMW-PTP (Thermo Scientific), and β-actin (Abcam). Polyclonal Rabbit Anti-Mouse and Goat Anti-Rabbit Immunoglobulins/HRP (DakoCytomation) were used as secondary antibodies as appropriate and HRP-labelled proteins were detected by chemiluminescence (ECL-Amersham). Band intensities were quantified by Image Studio Software v 4.0.21 (LI-COR Biosciences, Lincoln, Nebraska, USA) and data are presented as the ratio of intensity for the protein of interest/housekeeping protein expressed as a % of the corresponding ratio under control conditions.

2-D Electrophoresis (2-DE)

Cell lysates were desalted using Protein Desalting Spin Columns (Thermo 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, BD).
**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 analysed by Scaffold 3 software and MALDI-TOF MS data by the Mascot search engine.

**Measurement of Cell Layer Pi**

Pi was determined in neutralised de-proteinised cell extracts as previously described \(^{11}\). Briefly, medium was aspirated and adherent cells rinsed three times in ice-cold 0.9% NaCl on ice. 150µl of 0.3M perchloric acid (PCA) was added per well and cells were scraped. Extracts were deproteinised by standing samples on ice for a 30min followed by centrifugation at 3000xg, 4°C for 10min. The protein pellet was dissolved in 200µl of 0.5M NaOH and stored at -80°C for total cell protein determination. 3µl of Universal Indicator was added to the deproteinised PCA supernatant and the pH was adjusted to approximately 7 by adding appropriate volumes of 4.3M KOH/0.6M Imidazole. Samples were snap-spun to precipitate potassium perchloride and 145µl of neutralised deproteinised supernatant transferred to a clean tube and mixed with 655µl of H₂O, 200 µl of Acidified Molybdate\(^{11}\), and 200 µl of mixture of 2-methyl propan-1-ol and light petroleum (IBPE; 4:1 v/v) \(^{11}\). Samples were vortexed and briefly centrifuged to separate the phases. 75 µl of the top pink organic phase was transferred to a clean tube and mixed with 150 µl of absolute ethanol and 10 µl of 4% w/v SnCl₂. The absorbance of the resulting blue species was determined at 750nm.
32Pi transport assays

Pi transport into intact cell monolayers was assayed from the rate of uptake of 32Pi as described in 12 but the cells were incubated in Hapes Buffered Saline (HBS) (140mM NaCl, 20mM Hapes, 2.5mM MgSO4·7H2O, 5mM KCl, 1mM CaCl2·2H2O, 10mg/L Phenol Red) with 32Pi at room temperature for only 5min, and the total Pi concentration in the assay was 0.1mM. To demonstrate the Na-dependence of Pi transport, NaCl in the HBS was replaced with 140mM Choline Chloride.

Determination of Inhibitory effect of Pi on Tyrosine Phosphatase (PTPase) Activity

PTPase activity in EAhy926 cell lysates was determined using a Tyrosine Phosphatase Assay System (Promega Ref V2471) as described in the manufacturer’s instructions.

Thrombin Generation Assay (TGA) using Calibrated Automated Thrombography (CAT)

The ability of Pi-derived MPs to enhance thrombin generation was determined using CAT using PRP Reagent (Diagnostica Stago, UK) containing 1pM tissue factor. Briefly, 10^6 cells were seeded into a 75cm^2 culture plate and, at 60-70% confluence, were treated with 1mM and 2.5mM [Pi] for 24h. The medium was collected and microparticles isolated as described (Table 1) by serial centrifugation steps. One volume of the MP suspension (Fraction 2; Table 1) was mixed with 4 volumes of filtered pooled plasma. 20µl of PRP Reagent (Diagnostica Stago, UK) containing 1pM tissue factor was added to 80µl of MPs in PRP in Immulon 2HB round-bottomed microtitre plates and incubated for an hour at 37°C in a fluorescent plate reader equipped with Thrombinoscope software (Thrombinoscope, Synapse BV, Netherlands) with continuous monitoring.
ATP measurement

Intracellular ATP was determined by high-performance liquid chromatography as described in\textsuperscript{10}. Briefly, cells on 35mm six-well plates were treated with 1mM and 2.5mM [Pi] for 90min, 8h, 24h and 48h. Test medium was removed and the monolayer rinsed three times in ice-cold 0.9% NaCl. 150µl of 0.3M perchloric acid (PCA) was added/well and cells scraped on ice to precipitate proteins. Samples were centrifuged at 3000xg, 4°C for 10min to sediment proteins. The protein pellet was used to determine total cell layer protein and an equal volume of Freon-Tri-Octylamine mixture (22% v/v Tri-n-octylamine: 78% v/v 1,1,2-trichlorotrifluoroethane) was added to the supernatant and agitated vigorously to neutralise the PCA. The neutralised aqueous-top phase was stored at -80°C for determination of ATP by HPLC.

Glycolytic rate (Lactate production measurement)

The rate of glycolysis was determined in cells treated with 1mM Pi and 2.5mM Pi over time by measuring the rate of lactate production in culture medium from cells. Lactate was measured spectrophotometrically with lactate dehydrogenase. Briefly; 55µl of standards (0, 400, 800, 1200, 1600 nmoles Lactate/ml) and/or samples (medium from cells) was pipetted into 12mm diameter disposable tubes and to these 350µl of a glycine/hydrazine/NAD\textsuperscript{+} mixture (comprising 10mg NAD\textsuperscript{+} (Sigma N7004) mixed with 2ml of glycine/hydrazine (Sigma G5418) and 4ml of water) was added. Tubes were agitated and 190µl from each tube transferred to a cuvette and the OD read at 340nm. Lactate Dehydrogenase (LDH) suspension (4µl of Sigma L3916) was added to 215µl of the glycine/hydrazine/NAD\textsuperscript{+} mixture above and incubated at room temperature until a new stable OD was obtained (about 30min). 190µl from each tube was transferred to a cuvette and the OD read again at 340nm. The increase in
OD before and after adding LDH used to estimate the concentration of lactate in the samples by comparison with a linear calibration curve obtained using lactate standards.

**Statistical Analysis**

Data are presented as the Mean±SEM and were analysed using GraphPad Prism 6.0. Two group data comparisons were analysed by *t* test (for normally distributed data) or by 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.
References cited in Supplemental Text and in Supplemental Methods


Supplemental Figures

Supplemental Figure 1. Particle release from Pi loaded EAhy926 endothelial cells in vitro is independent of Calcium Phosphate (CaPi) deposition on the cell monolayer. (A) Calcium ($^{45}$Ca) deposition on EAhy926 cells after Pi loading; t=24h; *P<0.05 (n=6) (B) Effect of Pi and PFA on protein particle release from the medium of Pi and PFA loaded EAhy926 cells (For the duration of the 24h incubation, the culture medium was supplemented with $^{45}$Ca at 27nCi/ml); t=24h; **P<0.001 (n=3).
Supplemental Figure 2. Chronic protein particle release/cell detachment from EAhy926 endothelial cells after exposure for 24h to medium (2ml per 35mm culture well) with control (1mM) Pi or high (2.5mM) Pi. (A) Total protein determined in particles sedimented from the medium at 18,000g (Fraction 3 in Table 1). 30µM Menadione was used as a positive control. (n=36) ****P<0.0001. (B) Blunting of the Pi-induced sedimentable protein particle release by loading the medium with Pi analogue PFA (an inhibitor of sodium-dependent-Pi transporters PiT1/2) (Fraction 3 in Table 1). (n=4) *P<0.01, ****P<0.0001. (C) Detection of MP-size particles by Nanoparticle Tracking Analysis (NTA) by harvesting of the medium after incubation with the cells for 24h at the stated Pi concentration, followed by freeze-thaw and 1,500xg centrifugation cycle (see Table 1) followed by resuspension of the sedimented material in the same volume as the original medium. (Fraction 1 in Table 1). (n=3) *P<0.05. (D) Flow cytometry data showing the number of particles (obtained after incubation of cells with medium for t = 24h at the specified Pi concentration) which dual-labelled with anti-CD144-PE antibody and Annexin V-FITC (Left) and Annexin V-FITC only (Right). Using medium from a 75cm² culture flask, particles were prepared (Fraction 2 in Table 1), suspended in 500µl MP-Buffer (145mM NaCl, 2.7mM KCL, 10mM Heps, pH 7.4) and 38µl of this suspension was subjected to FACS analysis as described in Methods (n=3), *P<0.05, **P<0.01 versus 1mM Pi control.
Supplemental Figure 3. Effect of hyperphosphataemia on ROS generation in EAhy926 endothelial cells. (A) Representative fluorescent microscopy images (Left) and analysis (Right) of ROS DCFDA positive cells (Green fluorescence Arrows). Nuclei were counterstained with Hoechst dye (Blue). Cells were treated (t=90min) with 1mM Pi, or 2.5mM Pi, or 1mM Pi + 30µM Menadione as a positive control in the presence and absence of DCFDA probe; Scale Bar: 50µm; (n=3) ***P<0.001, ****P<0.0001.

(B) Representative histogram (Left) and corresponding analysis of the effect of high Pi on intracellular ROS generation using DCFDA probe analysed by Flow Cytometry; t=90min, (n=3). NS: Not Statistically Significant.

(C) HPLC analysis of citrulline generation by cells treated with control and high phosphate for 90min. (Citrulline measured as a by-product of the conversion of L-arginine to nitric oxide (NO)): data are from four replicate cultures from a representative experiment; **P<0.01.

(D) Detection of ROS using DHE fluorescence as a probe. Cells were treated in medium at the stated Pi concentration for 90min. Menadione (30µM) and ROS scavenger N-Acetyl Cysteine (10mM) were used as positive and negative controls respectively. Data are pooled from two independent experiments.

(E) Colorimetric detection of ROS using Nitro Blue Tetrazolium (NBT) in cells treated in medium at the stated Pi concentration for 90min. (n=3) ****P<0.0001 (Menadione and NAC were added as in (D)).
Supplemental Figure 4. Effect of hyperphosphataemia on cell viability, apoptosis and pro-apoptotic signals in EAhy926 cells. (A) Time course of MTT cell viability staining of control (1mM Pi) and 2.5mM Pi-treated cells. (n=4) *P<0.05, **P<0.01. (B) Representative immunobLOTS showing cleavage of caspase-3 and increase in pro-apoptotic Bax expression in cells treated with 2.5mM Pi; t=24h. (C and D) Densitometry analysis of cleaved 17/19kDa caspase-3 fragment (C) and Bax expression (D); t=24h (n=3) *P<0.05. (E and F) Representative immunoblot and corresponding densitometry analysis showing increased cleavage of PARP at 85kDa; t=24h, (n=3) *P<0.05. (G-I) Representative Flow Cytometry cytograms (G) and analysis of Annexin V binding and PI staining of cells (H-I) exposed to 1mM and 2.5mM Pi for 90min and 24h showing failure to detect an effect of Pi-loading on necrosis, early apoptosis or late apoptosis in spite of clear effects with a 30μM Menadione positive control. (H) Different apoptosis stages at 90min and I: at 24h (n=13) *P<0.05, **P<0.001, ***P<0.0001. (J, K) Forward angle light scatter (an indication of cell size) detects apoptotic cell shrinkage in response to 30μM Menadione positive control (n=5) but no response to Pi loading (neither at 90min: J nor at 24h: K): *P<0.05. (Similar data were obtained at 8h and 48h: - not shown). (L) Representative agarose gel electrophoresis from 3 experiments showing the presence of DNA fragmentation in 2.5mM Pi-treated and Positive control (H2O2-treated) cells. Caspase inhibitor Z-VAD-FMK prevented the effect of high Pi; t=24h. (M and N) Representative (M) Fluorescent Microscopy images and (N) analysis of apoptosis index in Pi treated cells as determined by TUNEL assay (Green- Arrows). Nuclei were counterstained (Blue) and cells treated with H2O2 as a positive control; Scale Bar 50μm, t=24h (n=3) ****P<0.0001.
Supplemental Figure 5. *In vitro* effect of Pi on intracellular adenosine triphosphate (ATP) and glycolytic rate (Lactate production).

(A) Time course of the effect of extracellular Pi load on intracellular ATP level in EAhy926 cells determined by HPLC (Three replicates from a representative experiment)

(B) Glycolytic rate (Lactate production) in Pi loaded EAhy926 endothelial cells over time (Three replicates from a representative experiment).
Supplemental Figure 6. Reciprocal modulatory effect of Pi on CD144 level in adherent EAhy926 endothelial cells vs. detached cells in vitro sedimented after centrifugation at 1,500g for 30min. Flow Cytometry performed on intact adherent EAhy926 cells in high Pi and control Pi medium showed a phosphate-induced decrease in CD144-positive cells (n=3 at t=24h) *P<0.05, contrasting with the increase observed in detached cells ***P<0.001.
Supplemental Figure 7. Effect of Pi and caspase-3 inhibitor Z-VAD-FMK on EAhy926 ECs function and membrane integrity. (A) Nanoparticle Tracking Analysis (NTA) performed on uncentrifuged medium showing particle concentration expressed as millions (E6) per ml indicating that both Z-VAD-FMK and vehicle (0.5% v/v DMSO) blunt MP output. (n=3) *P<0.05. (B) Chronic protein particle release from Pi treated cells in the presence or absence of inhibitor or vehicle showing abolition of sedimentable protein particles from the medium with both Z-VAD-FMK and vehicle alone **P<0.01 (n=3).
Supplemental Figure 8. Acute release of microparticles from EAhy926 endothelial cells incubated for 90 min with control (1 mM) and high (2.5 mM) [Pi] medium in the presence or absence of Rho kinase inhibitor (ROCK; Y-27632-10 μM). Nanoparticle Tracking Analysis (NTA) was performed on uncentrifuged medium showing no inhibitory effect of the inhibitor on MP output at 90 min (particle concentration expressed as millions (E6) per ml) (n=3) **P<0.01.
Supplemental Figure 9. Acute release of microparticles from HUVECs incubated for 90 min with control (1mM) and high (2.5mM) [Pi] medium. Nanoparticle Tracking Analysis (NTA) was performed on uncentrifuged medium (particle concentration expressed as millions (E6 per ml) (n=3) ***P<0.001, ****P<0.0001.
Supplemental Figure 10. Relationship between extracellular Pi concentration and Pi detected in the cell layer in HUVECs incubated for 90 min with control (1mM) and high (2.5mM) [Pi] medium. This indicates an acute increase in intracellular Pi. (n=3) **P<0.01.
Supplemental Figure 11. Net effects of hyperphosphataemia on global protein phosphorylation, and on the expression of Tropomyosin-3 (TM-3) in HUVECs. (A, B) Representative immunoblots and quantitative analysis by densitometry of protein tyrosine phosphorylation probed with pan-specific anti-phosphotyrosine antibody. (C, D) Corresponding immunoblots and densitometry of protein serine/threonine phosphorylation probed with pan-specific anti-phosphoserine/threonine antibody. Densitometry is shown for cells incubated for 1.5h in medium with 1 or 2.5mM Pi. For tyrosine phosphorylation (n=3), *P<0.05, **P<0.01. For serine/threonine phosphorylation (n=3), *P<0.05, **P<0.01. (E) Tropomyosin immunoblots (representative of 3 independent experiments) obtained from cells incubated in medium with 1 or 2.5mM Pi for 1.5h, probed using anti-TM-3 antibody. (F) Corresponding densitometry analysis on TM-3 immunoblots at 1.5h. (n=3) *P<0.05.
Supplemental Figure 12. Effect of wide range of extracellular Pi on MP output from EA.hy926 cells at 90min. EA.hy926 cells were incubated in the medium containing 0.5, 1, 1.7, and 2.5mM Pi for 90min. Medium from cells was collected after 90min incubation and particles from the medium counted by NTA. Medium containing 1mM [Pi] was set up as the control. (n=3) *P<0.05, **P<0.01.