Increased inorganic phosphate induces human endothelial cell apoptosis in vitro

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Di Marco GS, Hausberg M, Hillebrand U, Rustemeyer P, Wittkowski W, Lang D, Pavenstädt H. Increased inorganic phosphate induces human endothelial cell apoptosis in vitro. Am J Physiol Renal Physiol 294: F1381–F1387, 2008. First published April 2, 2008; doi:10.1152/ajprenal.00003.2008.—Chronic kidney disease with hyperphosphatemia is associated with accelerated atherosclerosis and endothelial dysfunction. However, the contribution of high serum phosphate levels to endothelial injury is incompletely understood. The aim of this work was to evaluate the responses of endothelial cells to elevated levels of extracellular phosphate in vitro. High phosphate in concentrations similar to those observed in uremia-associated hyperphosphatemia (>2.5 mM) induced apoptosis in two endothelial cell lines (EAHy926 cells and GM-7373 cells). This effect was enhanced when cells were incubated for 24 h in the presence of 2.8 mM calcium instead of 1.8 mM. By treating cells with 0.5 or 1.0 mM phosphonoformic acid, an inhibitor of the phosphate transporter, death was completely prevented. The process of phosphate-induced apoptosis was further characterized by increased oxidative stress, as detected by increased ROS generation and disruption of the mitochondrial membrane potential at ~2 h after treatment, followed by caspase activation. These findings show that hyperphosphatemia causes endothelial cell apoptosis, a process that impairs endothelial integrity. Endothelial cell injury induced by high phosphate concentrations may be an initial event leading to vascular complications in patients with chronic kidney disease.

VASCULAR CHANGES IN CHRONIC kidney disease and end-stage renal failure are associated with increased atherosclerosis, ischemic heart disease, and vascular stiffening. Prospective studies and registry data suggest a strong role of hyperphosphatemia and an increased calcium-phosphate product as significant predictors of cardiovascular mortality, potentially acting as progression factors of unwanted atherosclerosis and calcifications in uremia (26). In addition, it has been very recently shown that an increased phosphate concentration is an independent risk factor for higher mortality during the predialysis phase, suggesting that phosphate levels within the normal range are likely of vital importance in predialysis patients (32).

Moreover, it is now widely accepted that oxidative stress and the ensuing endothelial dysfunction play a key role in the pathogenesis of atherosclerosis and cardiovascular disease. Specifically, endothelial dysfunction caused by an excess of reactive oxygen species (ROS) precedes and promotes atherosclerosis (18, 25). The high turnover of endothelial cells in atherosclerosis also suggests that apoptosis may contribute to the pathology (4). In this context, common risk factors for atherosclerosis are associated with increased generation of ROS and mitochondrial dysfunction, which can lead to activation of the mitochondrial apoptotic pathway (4, 18, 25). Thus mitochondria have been proposed as an important link among risk factors, oxidative damage, endothelial dysfunction, and apoptosis, and the initiation and development of atherosclerotic lesions.

If a number of observations confirm the importance of endothelial cell apoptosis in the pathogenesis of atherosclerosis (8, 14, 25, 29), there are only sparse data on the effects of phosphate, in concentrations as observed in uremia-related hyperphosphatemia, on endothelial cell function and death. Thus, in the present study, we aimed to ascertain whether increased phosphate concentrations alone or in combination with increased calcium concentrations are able to modulate endothelial cell apoptosis and to explore the mechanisms involved in this process.

MATERIALS AND METHODS

All experiments were performed under institutional ethical approval of the protocol.

Endothelial cell culture. Human endothelial cells [EAHy926; a permanent human cell line derived from human umbilical vein endothelial cells that express highly differentiated functions characteristic of human vascular endothelium] (1, 5, 6, 24) and bovine aortic endothelial cell GM-7373 cells (DSMZ, Braunschweig, Germany) (24) were used.

EAHy926 cells were grown in DMEM (Biochrom, Berlin, Germany) containing 5% FCS (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine, and 50 U/ml each of penicillin/streptomycin at 37°C in an atmosphere of 5% CO2 in air. GM-7373 cells were cultured in MEM supplemented with 20% FCS, nonessential amino acids, MEM vitamins, and penicillin/streptomycin as recommended by the manufacturer. Cell cultures between passages 10 and 20 and 8 and 13 were used, respectively. For most of the experiments, cells were seeded in 24-well plates. Twenty-four hours before apoptosis induction, the growth medium was replaced; and just before each specific treatment, cells were rinsed with fresh medium and treated for the indicated times.

Treatment protocols. Medium supplemented with 5% FCS was set as the control (phosphate and calcium concentrations in the culture medium are 1.0 and 1.8 mM, respectively). In treated groups, control medium was supplemented with 133 μl of a stock solution of sodium phosphate (1.0 M Na2HPO4; 0.6 M PO43−) and 217 μl of a stock solution of calcium gluconate/calcium saccharate (0.23 M Ca2+) to raise the phosphate concentration to 2.5 mM and calcium concentration to 2.8
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Fig. 1. High phosphate concentration induces endothelial cell apoptosis. A: morphological features of cells exposed to 1.0 mM phosphate and 1.8 mM calcium (Control), 2.5 mM phosphate and 1.8 mM calcium (Pi), and 2.5 mM phosphate and 2.8 mM calcium (CaPi). The photographs were taken 24 h after exposure and evidenced changes such as cell shrinkage, rounding, and loss of cell contact with and attachment to the substratum. Images are the result of overlaying F-actin (red fluorescence) and integrin-β1 (green fluorescence) stainings. Magnification: ×63. B: effect of phosphate and calcium on phosphatidylserine externalization. Cells were treated for 24 h with increasing phosphate concentration (1.0–7.0 mM) and calcium (1.8 or 2.8 mM). Apoptosis was assessed by annexin V staining followed by flow cytometric analyses. Values are means ± SE (n = 4–6) expressed as control-fold increase. Control values (1 mM phosphate, 1.8 mM calcium) were used to normalize all experimental data. *P < 0.05 compared with control. §P < 0.05 compared with the correspondent 1.8 mM calcium-treated cells. C: 4′,6-diamidino-2′-phenylindol-dihydrochloride (DAPI) staining and transmission electron microscopy. Death through apoptosis was further confirmed by the presence of changes in nuclear morphology, such as the presence of pyknotic nuclei, condensed chromatin at the periphery of the nucleus and in the nucleoplasm, apoptotic bodies, and intense vacuolization after 24-h incubation with 2.5 mM phosphate independently of calcium concentration. Magnification: ×63 for DAPI staining and ×4,600 for electron microscopy.

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Phosphate induces apoptosis. We first exposed endothelial cells to phosphate at 2.5 mM, a concentration that correlates with serum levels found in uremia-related hyperphosphatemia. Morphologically, by confocal microscopy, cells exposed for 24 h to high phosphate in the presence of normal (1.8 mM) or increased (2.8 mM) calcium concentration exhibited a shrinkage in size, membrane blebbing, and loss of their attachment to the substratum compared with untreated control cells (1.0 mM phosphate and 1.8 mM calcium) (Fig. 1A). In control cells, integrin-β1 (green fluorescence) is mainly expressed in the filopodia, playing an important role in the attachment of cells to other cells and substratum. In treated cells, we can observe retrieval of the filopodia and loss of cell contact; however, integrins are still expressed in the cell membrane, only presenting a distinct distribution compared with control situation.

For quantitative assessment of phosphate-induced apoptosis, cells were incubated for 24 h with increasing phosphate concentrations (1–7 mM) and analyzed by means of phosphatidylserine externalization. We observed that physiological levels of phosphate (<1.4 mM) present no effects on endothelial cell death. The phosphate threshold level for inducing apoptosis was 2.5 mM (Fig. 1B); with an increase of almost 40% in the number of apoptotic cells compared with control levels. An increase of >100% could be observed when cells were incubated at concentrations of phosphate >5 mM.

Concerning the influence of extracellular calcium, we noted that at a phosphate concentration of 1 or 1.5 mM in the culture medium, an increase in the calcium level from 1.8 to 2.8 mM did not affect the extent of endothelial cell death.
However, the elevation of phosphate concentration to levels >2.5 mM and a simultaneous elevation of the calcium concentration from 1.8 to 2.8 mM resulted in a significant increase in apoptosis compared with elevated phosphate concentration only (Fig. 1B).

At an ultramicroscopic level, transmission electron microscopic images of the treated cells provide further evidence that high phosphate concentration (2.5 mM) alone and in combination with elevated calcium (2.8 mM) mediated endothelial cell apoptosis. Following treatment, endothelial cells showed condensed chromatin, intense vacuolization, and numerous secretory vacuoles and ribosomes, as well as the presence of apoptotic bodies (Fig. 1C, bottom). Changes in nuclear morphology can also be observed by DAPI staining (Fig. 1C, top).

Expression of sodium-phosphate transporter on endothelial cells and effect on phosphate transport blockage on phosphate- and calcium-phosphate−induced apoptosis. Figure 2A shows that human endothelial cells (EAhy926) express at the mRNA level at least one kind of phosphate transporter, Pit-1 (sodium-phosphate transporter type III).

To determine whether apoptosis is phosphate dependent, we incubated cells with PFA, an inhibitor of phosphate transport. Specifically for inhibition studies, cells were treated with high phosphate concentration (5 mM) or with calcium-phosphate (5 mM phosphate, 2.8 mM calcium), and apoptosis was analyzed in the presence of increasing concentrations (0.1–1 mM) of PFA. Figure 2, B and C, shows that PFA caused a dose-dependent decrease in the percentage of apoptotic cells in phosphate- and calcium-phosphate-treated cells. At 0.5 or 1.0 mM, PFA was able to abolish apoptosis in both treated cell groups (n = 6) (Fig. 2B). Incubation with PFA alone had no effect on cell apoptosis (data not shown).

To ascertain that apoptosis inhibition by PFA was specifically related to phosphate transport, we analyzed the effect of PFA on staurosporine-induced apoptosis. Figure 2D shows that treatment with 1 mM PFA was not able to prevent endothelial cell apoptosis induced by 1 μM staurosporine (n = 6).

Disruption of mitochondrial membrane potential and ROS generation. To explore the mechanism of apoptosis, control and treated cells were probed with TMRE, a mitochondrial membrane voltage dye, and DHE, a ROS reporter. Figure 3A shows that cells treated with phosphate (2.5 mM) or calcium-phosphate (2.5 mM phosphate, 2.8 mM calcium) presented a significant reduction in TMRE uptake (n = 6/group) compared with control (n = 8), evidencing changes in mitochondrial membrane potential 2 h after incubation. As expected, the disruption of mitochondrial membrane potential was preceded by induction of ROS generation. Figure 3B shows that, in the presence of both treatments, the relative levels of ROS started...
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Elevating at 60 min after treatment, presenting a great increase at 75 min. The levels of ROS in untreated control cells were unchanged over all of the incubation time periods (n = 7–14). Figure 3C contains typical fluorescence histograms showing that the exposure to 2.5 mM phosphate (in the presence or not of increased calcium concentration) induces a rightward shift of the DHE fluorescence curve, which is avoided in the presence of 1 mM PFA. Figure 3D shows in situ staining of DHE in control and cells treated with 2.5 mM phosphate in the presence or not of DMTU, a superoxide scavenger. Weak superoxide signals were detected in control and DMTU-treated cells, while intense production was observed when cells were treated with a high phosphate concentration for 75 min.

Effect of caspase inhibition on phosphate- and calcium-phosphate-induced apoptosis and mitochondrial membrane potential. As a subsequent step in the sequence of events related to the mitochondrial apoptotic pathway is the activation of caspases, we preincubated endothelial cells with the pan-caspase inhibitor Z-VAD-FMK for 45 min before adding phosphate and calcium-phosphate to the culture medium. We thus observed that the inhibitor was able to avoid endothelial cell apoptosis as measured by means of phosphatidylserine externalization (n = 6 for all groups) (Fig. 4A).

In contrast, under the same experimental condition, we found that Z-VAD-FMK did not avoid the disruption of mitochondrial membrane potential induced by high phosphate concentrations (Fig. 4B, n = 5 for Z-VAD-FMK-treated group and n = 8 for control and phosphate-treated groups). The mitochondrial membrane depolarization was only avoided when cells were incubated in the presence of 1 mM PFA (Fig. 4B, n = 5). These two lines of data suggest that caspase activation, which is responsible for phosphatidylserine externalization, is a downstream event resulting from ROS and disruption of mitochondrial function.

Discussion

Endothelial cell dysfunction and apoptosis are crucial events in the pathogenesis of atherosclerosis. Apoptosis of endothelial cells in atherosclerosis may be caused by the generation of oxidative stress and/or inflammatory mediators, for example (18, 25, 27). In uremia, hyperphosphatemia is highly correlated with the extent of vascular calcification and vascular disease (15–17). Moreover, the effect of hyperphosphatemia may not be limited to renal disease patients because, also in patients with normal renal function and coronary heart disease, the serum phosphate concentration has been suggested to be a strong predictor of the severity of vascular constriction, suggesting a more general role of serum phosphate in the development of coronary plaques (22).

Because endothelial cells first sense phosphate variations occurring in the plasma and may respond initially by modulation of their functions, we examined the role of high phosphate concentrations in endothelial cell injury. We demonstrated here that phosphate induces endothelial cell apoptosis, an effect that is substantially enhanced by increased calcium concentrations, as verified from morphological observations and the assessment of phosphatidylserine externalization by FACS analysis. The induction of apoptosis by high phosphate is not only the effect of binding and precipitation of calcium, since acute effects of hyperphosphatemia on cell shrinkage and stiffening can be discriminated from apoptosis (Hillebrand U, Lang D, unpublished observations). In addition, our experiments showed disruption of mitochondrial function, via the observed elevation of ROS production, and subsequent caspase activation.

The time course studies showed that ROS generation and disruption of the mitochondrial membrane potential occurred as early as 75–120 min post-exposure to high phosphate or calcium-phosphate concentrations, indicating that these events were earlier than that of the apoptotic execution phase, such as phosphatidylserine externalization. In addition, we found that all of these events were inhibited in the presence of PFA, a well-known phosphate transport blocker, suggesting that an increase in the intracellular phosphate concentration is a primary and essential death signal.

In this study, we chose to use a permanent human cell line (EAhy926) derived from human umbilical vein endothelial cell dysfunction and apoptosis.
cells because 1) it is extensively described in the literature that these cells express highly differentiated functional characteristic of human vascular endothelium (1, 5, 6); and 2) they can be used as a homogeneous experimental cell line, which permits more consistent responses to specific variables and greater reproducibility of data. Moreover, using bovine aortic endothelial cells (GM-7373), we were able to confirm that the responses to high phosphate (or calcium-phosphate) observed here probably reflect endothelial behavior, at least at in vitro level.

Since the intracellular concentrations of phosphate and calcium are regulated by the mitochondria, and mitochondrial dysfunction is known to trigger apoptosis (7, 33), we examined mitochondrial function in phosphate- and calcium-phosphate-treated endothelial cells. Indeed, ROS, which are the mitochondrial by-products of normal cellular oxidative processes, have been suggested as regulating the process involved in the initiation of mitochondrial apoptotic signaling (30).

In this study, we demonstrate that phosphate and calcium-phosphate exposure results in an elevation of ROS generation in a time dependent manner. However, it is not exactly known how these treatments could directly induce ROS generation. As it occurs before there is any evidence of apoptosis, including any noticeable disruption in the mitochondrial membrane potential, it is unlikely to be produced by mitochondrial dysfunction. However, as ROS generation was blocked in the presence of PFA, it is evident here that an increase in ROS is related to the accumulation of phosphate in the cells and that ROS accumulation will lead to oxidative stress.

However, these phenomena, increased ROS generation and mitochondrial dysfunction, seem to be a normal response of mitochondria to increased intracellular phosphate. A number of studies show that isolated mitochondria treated with elevated phosphate concentrations present irreversible changes in mitochondrial oxidative-phosphorylating activity, inducing common steps in the apoptosis pathway (3, 9, 11).

Thus, to prove that caspase activation was also involved in phosphate- and calcium-phosphate-mediated apoptosis, we used the cell-permeable caspase inhibitor Z-VD-FMK. This inhibitor avoided apoptosis in endothelial cells, indicating that phosphate and calcium-phosphate trigger caspase-dependent pathways in these cells. However, caspase inhibition was not able to prevent the loss of mitochondrial membrane potential, confirming that caspase activation is downstream of ROS generation and mitochondrial dysfunction in phosphate-induced apoptosis in endothelial cells (13). Our data support the concept that mitochondrial damage represents a key step in the course of apoptosis induced by phosphate and calcium-phosphate.

Finally, the use of PFA provided interesting insights into the mechanism of phosphate action. At 0.5 or 1.0 mM PFA, endothelial cells were protected against phosphate-induced apoptosis, suggesting that phosphate, as well as phosphate in the presence of increased calcium concentrations, mediates apoptosis through a plasma membrane transport mechanism. These results are in line with previous studies showing that phosphate and the ion pair calcium-phosphate are a potent apopptogen for osteoblasts, chondrocytes, and vascular smooth muscle cells and that PFA was able to block phosphate-induced apoptosis in all these different cell types (19, 20, 28). Herein, as we have not aimed to determine the specific requirement for Pit-1, but rather the effect of decreased phosphate uptake on endothelial cell apoptosis, we opted to use PFA instead of the RNA interference approach. As recently published by Villa-Bellosta et al. (31), RNA interference for Pit-1 in vascular smooth muscle cells correlates with only a 23% inhibition of total phosphate uptake, suggesting that this small amount of transport inhibition observed is enough to bring the phosphate transport beneath a “threshold” such that the osteogenic signaling pathways are not activated. This would explain then the prevention of smooth muscle cell calcification with PFA, despite the fact that PFA is described as a weak inhibitor of type III phosphate transporters. The same line of thought could be applied to our findings, which support the idea that exposure to high extracellular phosphate concentration causes a rise in the intracellular phosphate level, leading to endothelial cell apoptosis.

In summary, human endothelial cells challenged with high phosphate and calcium-phosphate concentrations present increased ROS generation, mitochondrial membrane depolarization, and, consequently, caspase activation. Since endothelial cell apoptosis could be blocked by PFA, it is likely that phosphate mediates cell death by raising the intracellular phosphate concentration and activating downstream effectors of the apoptotic process.

Thus it is conceivable that hyperphosphatemia, by inducing endothelial cell apoptosis, may provide an early contribution to atherogenesis. Indeed, additional insights into the molecular basis of endothelial cell apoptosis could facilitate specific therapeutic interventions slowing the progression of uremia-associated vascular disease.

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