Hydrogen Phosphate Selectively Induces MDA-MB-231 Breast Cancer Cell Death *in vitro*

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Abstract

Phosphate ions are the most abundant anions inside the cells, and they are increasingly gaining attention as key modulators of cellular function and gene expression. However, little is known about the effect of inorganic phosphate ions on cancer cells, particularly breast cancer cells. Here, we investigated the toxicity of different phosphate compounds to triple-negative human breast cancer cells (MDA-MB-231) and compared it to that of human monocytes (THP-1). We found that, unlike dihydrogen phosphate (H_2PO_4^-), hydrogen phosphate (HPO_4^{2-}) at 20 mM or lower concentrations induced breast cancer (MDA-MB-231) cell death more than immune (THP-1) cell death. We correlate this effect to the fact that phosphate in the form of HPO_4^{2-} raises pH levels to alkaline levels which are not optimum for transport of phosphate into cancer cells. The results in this study highlight the importance of further exploring hydrogen phosphate (HPO_4^{2-}) as a potential therapeutic for the treatment of breast cancer.

Introduction

Inorganic phosphate (P_i) is a vital nutrient for all living things. It plays a crucial role in various physiological processes such as energy metabolism, lipid biosynthesis, nucleic acid biosynthesis, cellular differentiation, cellular repair and cellular signaling^{1,2}. The main source of P_i intake for humans is the diet, where P_i exists in one of three anionic forms; HPO_4^{2-} , H_2PO_4^- or H_3PO_4 ². In the body, serum P_i levels are precisely maintained within a specific range via the interplay between several organs including intestine, kidney, parathyroid gland and bones. Similarly, extracellular P_i levels are maintained within a narrow range; between 0.7 and 1.55 mM²⁻⁴.

Many studies have proposed the existence of a phosphate sensing mechanism in the body capable of detecting both serum and extracellular phosphate fluctuations and subsequently relaying this information to the cells, the local environment and/or the whole body⁵⁻⁷. Interestingly, several findings have revealed that extracellular phosphate in itself carries out this function by acting as a signaling molecule^{6,8-12}. The interior environment of the cell is electronegative compared to the exterior one, thus, the transport of P_i into the cell does not happen by simple diffusion, but instead is mediated by Na^+ -coupled P_i cotransporters, which are highly regulated proteins^{4,5,13}. Furthermore, P_i can initiate signal transduction pathways, alter gene expression and regulate diverse cellular functions^{7,9,14,15}. All these evidence serve to pinpoint the importance of P_i in the body and to highlight the significance of regulating P_i levels.

Recently, high P_i intake and high P_i serum levels were associated with higher morbidity and mortality rates^{16,17}. In fact, high serum concentrations are associated with kidney disease, perturbed brain growth, vascular calcification and cardiovascular events¹⁶⁻¹⁹. However, the mechanisms by which high P_i concentrations are linked to tissue damage are not completely understood. Shuto et.al showed that P_i acutely impairs endothelial function by increasing production of reactive oxygen species and decreasing

production of nitric oxide ²⁰. On the other hand, how phosphate affects diseased cells, particularly cancer cells, is only minimally explored ^{21,22}. For instance, Spina et.al demonstrated that P_i inhibits proliferation of human osteosarcoma U2OS cells as well as MDA-MB-231 cells but did not explore its effect on normal, healthy cells ^{14,23,24}.

In this study, we investigated the toxicity of different phosphate compounds to triple-negative human breast cancer cells (MDA-MB-231) and to human monocytes (THP-1). We demonstrated that, unlike phosphate in the form of dihydrogen phosphate ($H_2PO_4^-$), hydrogen phosphate (HPO_4^{2-}) induces breast cancer cell death but not immune cell death. We attribute this effect to the fact that phosphate in the form of HPO_4^{2-} raises pH levels to alkaline levels which are not optimum for phosphate transport into cancer cells. Taken together, these results indicate the significance of further exploring HPO_4^{2-} as potential therapeutic for the treatment of breast cancer.

Results

Cytotoxicity of Different Phosphate Compounds

In order to assess the effect of different phosphate compounds on the cell viability of MDA-MB-231 and THP-1 cells, three inorganic phosphates (NaH_2PO_4 , Na_2HPO_4 , and KH_2PO_4) and three organic phosphates (adenosine triphosphate (ATP), adenosine diphosphate (ADP) and adenosine monophosphate (AMP)) were tested. Both MDA-MB-231 and THP-1 cells were incubated with 20 mM of NaH_2PO_4 , Na_2HPO_4 , KH_2PO_4 , ATP, ADP and AMP for 48 h, then assessed for their viability via MTT assay. As shown in Figure 1, almost all phosphate-containing compounds were toxic (viability < 80 %) to both cell types and their toxicity was more pronounced on THP-1 cells than it was on MDA-MB-231 cells. Interestingly, only Na_2HPO_4 was more toxic to MDA-MB-231 cells than it was to THP-1 cells. In particular, the viability of MDA-MB-231 cells incubated with 20 mM Na_2HPO_4 was 37.5 % lower than that of THP-1 (Relative viability of MDA-MB-231 was 54.1 % while that of THP-1 was 86.6 %). This indicates that the toxicity of Na_2HPO_4 is selective to the cell type.

In order to understand the difference in the effect of the various phosphate compounds on the two cell types, the pH of the different phosphate solutions was measured. In Figure 2, all phosphate compounds produced acidic solutions except Na_2HPO_4 which produced a basic one.

This, as well as the fact that Na_2HPO_4 is the only compound containing phosphate in the form of HPO_4^{2-} , were a drive to further explore Na_2HPO_4 compound.

In the medium, inorganic phosphate compounds are dissociated into ions.

Na_2HPO_4 produces phosphate ion in the form of HPO_4^{2-} , i.e. hydrogen phosphate.

Effect of hydrogen phosphate (HPO_4^{2-}) on MDA-MB-231 and THP-1 cell viability

In order to further examine the effect of hydrogen phosphate (HPO_4^{2-}) on viability of MDA-MB-231 and THP-1 cells, cells were incubated with different amounts of NaHPO_4 for 48 h then assessed for their viability via MTT assay. Figure 3 demonstrated that, at low concentrations, HPO_4^{2-} was more toxic to MDA-MB-231 cells than it was to THP-1 cells. Interestingly, for MDA-MB-231, even low concentrations (< 20 mM) caused a significant decrease in cell viability. For example, 5 mM Na_2HPO_4 reduced the viability of MDA-MB-231 by almost 20 %. However, for THP-1 cells, there was no significant decrease in the viability of cells incubated with 5, 10 and 20 mM NaHPO_4 compared to control cells. Only concentrations above 20 mM produced a significant decrease in viability.

In order to confirm that the observed toxicity of Na_2HPO_4 on MDA-MB-231 was induced by phosphate and not by sodium in NaHPO_4 or by simple osmotic pressure, the toxicity of NaCl, sucrose and sodium bicarbonate on MDA-MB-231 was assessed. In Figure 4, none of the compounds caused a significant decrease in the viability of cells which indicates that the phosphate is the probable cause for the induced toxicity observed.

In order to explore the possible mechanisms of action in which HPO_4^{2-} induces its toxicity, the effect of HPO_4^{2-} on pH of the media was assessed. For that, phosphate solutions at different concentrations (5, 10, 20, 40, 80, 100 and 120 mM) were prepared in media then their pH measured. As seen in Figure 5, results indicate that as the concentration of HPO_4^{2-} increases, the pH of the solutions increases.

Tolerance of MDA-MB-231 and THP-1 cells to different pH levels

As HPO_4^{2-} was found to alter the pH of the media, the difference in the tolerance of MDA-MB-231 and THP-1 cells to different pH levels was examined. For this, cells were incubated in media solutions at pH values equal to 5, 6, 7, 8 and 9 for 48 h then their viability determined using MTT assay. As seen in Figure 6, results demonstrated that both cell types were more tolerant to basic conditions than they were to acidic conditions, and that MDA-MB-231 cells were slightly more tolerant to pH fluctuations than THP-1 cells were.

Effect of Sodium Phosphate Dibasic at Physiological pH

If MDA-MB-231 cells are more tolerant to pH fluctuations than THP-1 cells are, there should be another reason why Na_2HPO_4 is more toxic to MDA-MB-231 than it is to THP-1 cells. Our hypothesis is that this toxicity is related to the way phosphate is transported into cancer cells, particularly breast cancer cells. Several studies showed that breast cancer cells have high levels of P_i transporters compared to normal tissue^{25–27}. These transporters, according to Forster et al., Russo-Abrahão et.al and Takeda, are sodium dependent and bind with high affinity to inorganic phosphate^{1,28,29}. Interestingly, they have high affinity for P_i in its diprotic form (H_2PO_4), which exists at physiological pH, and low affinity for P_i in its monoprotic form (HPO_4^{2-}), which exists at alkaline pH¹. Since triple negative breast cancer cells are known to require elevated amounts of phosphate to meet their metabolic demands, failure to efficiently transport phosphate into cancer cells alters their functioning and probably leads to cellular death^{30,31}. This study demonstrates that phosphate in its monoprotic form (HPO_4^{2-}) causes significant death in MDA-MB-231 cells but not in THP-1 cells, unlike phosphate in its diprotic form.

Since phosphate in general is a tryptotic acid and, thus, has different physiological forms according to the pH range in which it is found, the effect of adding Na_2HPO_4 to MDA-MB-231 and THP-1 cells and then adjusting the pH to a physiological one (pH = 7.4) was also explored³². As seen in Figure 7, results demonstrated that adjusting the pH to physiological range after adding HPO_4^{2-} slightly enhanced viability of MDA-MB-231 cells but had no effect on viability of THP-1 cells. The level of enhancement in viability was also pH dependent. These results support the hypothesis that cancer cells seem to favor diprotic form of phosphate than monoprotic one.

Discussion

In this study, we demonstrated the potential of HPO_4^{2-} to contribute to the treatment of breast cancer cells. This is quite significant as there is no effective therapy for triple-negative breast cancer to date and it continues to acclaim the lives of its patients^{33,34}. P_i is emerging as a crucial signaling molecule capable of altering signal transduction pathways, gene expression, and protein abundance in many cell types^{9,35–37}. Interestingly, its effect on cells is not universal but varies according to the cellular type and cellular background. For instance, P_i was shown to increase cell proliferation in some cell types, such as preosteoblastic cells, lung cells, and epidermal JB6 cells while decreasing proliferation in other cell types such as human osteosarcoma U2OS cells and MDA-MB-231 cells^{3,19,23,23,35, 38–41}. In addition, P_i was shown to induce apoptosis in MO6-G3 odontoblast-like cells⁴². Interestingly, Spina et.al showed that P_i inhibits proliferation in MDA-MB-231 cells but not in MCF-7 breast cancer cells, which are not “triple negative” and express estrogen and progesterone receptors¹⁴. These results strongly suggest that P_i has discrete effects on cells depending on their type and background. Here, we also show that the specific type of phosphate available to the cells has a great influence on its response to it. In particular, hydrogen phosphate causes significant death in MDA-MB-231 cells but not in THP-1 cells, unlike dihydrogen phosphate. Schematic diagram of Figure 8 showed the effect of hydrogen phosphate on the cell viability of MDA-MB-231 and THP-1 cells. We correlate this effect to the fact that MDA-MB-231 cells require high

amounts of phosphate to meet their metabolic needs but are unable to attain it due to the inability of phosphate transporters to carry phosphate into the cells when it is in the form of HPO_4^{2-} ⁴³. Studies show that cancer cells depend on several transporters to transport phosphate into the cells, but the key ones are sodium dependent transporters and hydrogen dependent transporters, both of which are highly overexpressed in MDA-MB-231 cells^{1,2,28,29}. Interestingly, sodium dependent phosphate transporters have higher affinity for dihydrogen phosphate (H_2PO_4) than they do to hydrogen phosphate (HPO_4^{2-})¹. In addition, hydrogen dependent transporters tend to transport phosphate more efficiently in acidic conditions than they do in basic conditions due to the greater availability of protons (H^+) in acidic environments compared to basic environments². These findings are, in fact, in consistence with the results obtained from this study.

Finally, we suggest that hydrogen phosphate is a suitable candidate for further investigation as a therapeutic for the treatment of breast cancer, due to its selective ability to induce cell death in cancer cells but not in immune cells. This is quite crucial as effective cancer therapeutics require high antitumor activity and minimal toxicity to normal tissues.

Materials And Methods

Cell culture

MDA-MB-231 (ATCC, Manassas, VA, USA) cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L D-glucose, 0.584 g/L L-Glutamine and 0.11 g/L Sodium Pyruvate (Gibco, Fisher Scientific, Waltham, MA, USA). The media was supplemented with 10 % Fetal Bovine Serum (FBS) (Gibco) and 1% penicillin–streptomycin (Gibco). The cells were incubated at 37°C and 5 % CO_2 .

THP-1 (ATCC) cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium containing 4.5 g/L D-glucose, 0.3 g/L L-glutamine, and 0.11 g/L sodium pyruvate (Gibco). The media was supplemented with 10 % heat inactivated fetal bovine serum (FBS) and 1 % penicillin–streptomycin (Gibco).

All cells were incubated at 37°C and 5 % CO_2 .

Cytotoxicity of Different Phosphate Compounds

To assess the toxicity of different phosphate compounds on MDA-MB-231 cells and on THP-1 cells, an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, St. Louis, MO, USA) reduction assay was performed. In brief, cells were seeded in 96 well plates at a concentration of 6×10^5 cells/mL and incubated for 24 h at 37°C and 5 % CO_2 . After 24 h of incubation, 20 mM of NaH_2PO_4 , KH_2PO_4 , Na_2HPO_4 , ATP, ADP and AMP were added to cells. Cells with media only and no phosphate were kept as a control. Cells were then incubated for an additional 48 h. After 48 h, MTT was added to the cells and the cells were subsequently incubated for extra 4 h. During this incubation time, yellow MTT solution was reduced to purple formazan in living cells. After 4 h, media containing MTT was removed from all

wells and replaced by DMSO. The absorbance of each sample was then measured at 570 nm using the microplate reader (Tecan Trading AG, Zurich, Switzerland). Percentage of viable cells was calculated by comparing the absorbance of the control cells (cells with medium only and no phosphate) to that of cells with the phosphate. Percentage cell viability was calculated as: Percentage cell viability = (cells with phosphate and MTT) / (control cells with MTT but no phosphate) × 100.

Cytotoxicity of Sodium Phosphate Dibasic

To further examine the effect of phosphate in the form of HPO_4^{2-} on MDA-MB-231 cells and on THP-1 cells, cells were incubated with different concentrations of Na_2HPO_4 for 48 h then their viability assessed. In brief, cells were seeded in 96 well plates at a concentration of 6×10^5 cells/mL and incubated for 24 h at 37°C and 5 % CO_2 . After 24 h of incubation, Na_2HPO_4 was added to cells at variable concentrations (5, 10, 20, 40, 80, 100 and 120 mM). Cells with media only without Na_2HPO_4 were kept as a control. Cells were then incubated for an additional 48 h. After 48 h, MTT was added to cells and cells were subsequently incubated for extra 4 h. After 4 h, the absorbance of each sample was measured and subsequent cellular viability determined.

To confirm that any observed effects are due to phosphate and not to osmotic pressure or to sodium in Na_2HPO_4 , MTT assay was performed to quantify the toxicity of sodium and the effect of osmotic pressure. In particular, cells were incubated with 20 mM, 40 mM, 60 mM sucrose to examine the effect of osmotic pressure and with 20 mM, 40 mM NaCl and 20 mM sodium bicarbonate to examine effect of sodium.

Measurement of pH

In order to investigate the possible ways in which phosphate influences MDA-MB-231 and THP-1 cell survival, the pH of the resultant solutions (after adding different phosphate compounds and after adding different concentrations of Na_2HPO_4) were measured using a pH meter (ORION STAR A211, Thermo Scientific, Waltham, MA, USA).

Tolerance of MDA-MB-231 and THP-1 cells to different pH levels

To study the tolerance of MDA-MB-231 and THP-1 cells to different pH levels, cells were incubated for 48 h in media having pH levels ranging from 5-9 then their viability was assessed using MTT assay. In brief, MDA-MB-231 and THP-1 cells were seeded in 96 well plates at a concentration of 6×10^5 cells/mL and incubated for 24 h at 37°C and 5 % CO_2 . After 24 h of incubation, the old media was replaced by new media having its pH adjusted by HCl (Sigma-Aldrich) or NaOH (Sigma-Aldrich) to obtain a specific pH; either 5, 6, 8 or 9. Media without any addition of acid or base was kept as a control (pH 7.2). The cells were incubated for 48 hours. After 48 hours, MTT was added to cells and their viability was quantified.

Toxicity of Sodium Phosphate Dibasic at Physiological pH

To study the toxicity of Na₂HPO₄ on MDA-MB-231 and THP-1 cells at physiological pH, cells were incubated for 48 h with 20 mM sodium phosphate dibasic (Na₂HPO₄) solutions having their pH adjusted to 7.4 then their viability was assessed via MTT assay. In brief, cells were seeded in 96 well plates at a concentration of 6×10^5 cells/mL and incubated for 24 h at 37°C and 5 % CO₂. After 24 h of incubation, 20 mM Na₂HPO₄ at pH 8.1 and at pH 7.4 were added to the cells. Two controls were maintained for this experiment: cells kept in media only (negative control) and cells kept in media having its pH adjusted to 8 or 8.5 by NaOH (Sigma-Aldrich) (positive control). The cells were incubated for 48 hours. After 48 h, MTT was added to cells and their viability was quantified.

Data Analysis

All experiments were done at least in triplicates. The data is presented as mean $\pm$ standard error. Student t-test was performed to determine significance. $P < 0.05$ was regarded as significant.

Declarations

Author Contributions

Conceptualization, A.S. and S.L.; methodology, A.S. and S.L.; validation, A.S., K.A. and S.L.; investigation, A.S., K.A., S.L.; resources, S.L.; data curation, S.L.; writing—original draft preparation, A.S.; writing—review and editing, K.A. and S.L.; visualization, A.S. and S.L.; supervision, S.L., C.S.; project administration, S.L. and C.S.; funding acquisition, S.L. and C.S. All authors have read and agreed to the published version of the manuscript.

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Figures

Figure 1

Toxicity of different phosphate compounds to (a) THP-1, human monocyte cell line, and (b) MDA-MB-231, human breast cancer cell line. All phosphate-containing compounds reduced viability of both cell types significantly ($p < 0.05$) except Na₂HPO₄, which only reduced the viability of MDA-MB-231 cells but not that of THP-1 cells.

Figure 2

pH values for different phosphate compounds. All compounds produced acidic solutions except Na_2HPO_4 which produced a basic solution.

Figure 3

Toxicity of Sodium Phosphate Dibasic to (a) THP-1 and (b) MDA-MB-231 cells. At low concentrations (< or = 20 mM), Na₂HPO₄ is more toxic to MDA-MB-231 cells than it is to THP-1 cells. At high concentrations (>20 mM), Na₂HPO₄ is toxic to both cell types.

Figure 4

Effect of Sodium and osmotic pressure on MDA-MB-231 viability. None of the compounds caused a significant decrease in the viability of cells.

Figure 5

pH values for different concentrations of Sodium phosphate dibasic. As the concentration of HPO₄²⁻ increases, the pH of the solutions increases

Figure 6

Viability of (a) THP-1 and (b) MDA-MB-231 cells at different pH levels. Acidic conditions caused a significant decrease in the viability of both cell types. For basic conditions, at pH 8, the viability of THP-1 cells decreased significantly but that of MDA-MB-231 did not. At pH 9, viability of both cell types decreased significantly.

Figure 7

Viability of (a) THP-1 and (b) MDA-MB-231 cells after adding Sodium Phosphate Dibasic and adjusting pH to Physiological one. Adjusting the pH to physiological range (7.4-7.5) after adding Na_2HPO_4 increased the viability of cells by 18 %. Media only and media with NaOH are controls

Figure 8

Schematic diagram – Effect of hydrogen phosphate on the cell viability of MDA-MB-231 and THP-1 cells

Supplementary Files

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