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Hydrolysis of phosphatidylcholine by cerium(IV) releases significant amounts of choline and inorganic phosphate at lysosomal pH

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Niemann–Pick disease and drug-induced phospholipidosis are examples of lysosomal storage disorders in which serious respiratory infections are brought on by high levels of the phospholipid phosphatidylcholine in the acidic lamellar bodies and lysosomes of pulmonary cells. One approach to developing an effective therapeutic agent could involve the use of a metal to preferentially hydrolyze phospholipid phosphate ester bonds at mildly acidic, lysosomal pH values (~pH 4.8). Towards this end, here we have investigated phosphatidylcholine hydrolysis by twelve metal ion salts at 60 °C. Using a malachite green/molybdate-based colorimetric assay to detect inorganic phosphate released upon metal-assisted phosphate ester bond hydrolysis, Ce(IV) was shown to possess outstanding reactivity in comparison to the eleven other metals. We then utilized cerium(IV) to hydrolyze phosphatidylcholine at normal, core body temperature (37 °C). The malachite green/molybdate assay was used to quantitate free phosphate and an Amplex® Red-based colorimetric assay and matrix-assisted laser desorption ionization time-of-flight mass spectrometry were employed to detect choline. Ce(IV) hydrolyzed phosphatidylcholine more efficiently at lysosomal pH: *i.e.*, at a Triton X-100:phosphatidylcholine molar mixing ratio of 1.57, yields of choline and phosphate were 51 ± 4% and 40 ± 4% at ~pH 4.8, compared to 28 ± 4% and 27 ± 5% at ~pH 7.2.

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1. Introduction

A lysosome is a mildly acidic organelle found in animal cells. It is responsible for the degradation of cellular macromolecules (*e.g.*, glycoproteins, glycolipids, muccopolysaccharides, and phospholipids) into original building blocks that can be recycled by the cell. This task is accomplished by a lysosomal protein called an acid hydrolase, a hydrolytic enzyme that functions optimally at the pH values found in the interior of the lysosome (~pH 4.8). When a particular acid hydrolase is defective, pathogenic levels of the corresponding macromolecular substrate (*e.g.*, a phospholipid) build-up within lysosomes, causing lysosomal storage disease [1,2]. The resulting clinical manifestations are often serious.

A major complication in common to the inherited, metabolic lysosomal storage disorders Niemann–Pick disease types A and B, Sandhoff disease, and Gaucher disease type I is compromised pulmonary function [3–12]. Bronchoalveolar lavage fluid collected from Sandhoff disease, and Gaucher disease type I patients [11] and from Niemann–Pick and Sandhoff mouse models [8,9] contains elevated amounts of the phospholipid phosphatidylcholine (**1**, PC), suggesting that high PC levels in pulmonary surfactant are the underlying cause of the pulmonary complications associated with these illnesses. Similarly, in drug-induced phospholipidosis, an acquired lysosomal storage disorder that is triggered by the administration of cationic, amphiphilic medications [13], pulmonary complications are thought to be related to increased quantities of phosphatidylcholine [14]. In these lysosomal storage diseases, the high pulmonary PC levels are associated with two phenomena. The catabolism of PC within the acidic lysosomes of alveolar macrophages is significantly decreased [8]. The resulting accumulation of PC within the alveolar macrophage lysosomes converts the macrophages into "foam cells" that congest free air spaces in the lungs [8,13–16]. Secondly, the phosphatidylcholine in the pulmonary surfactant impairs the normal secretion of PC from the acidic lamellar bodies [17] of type II granular pneumocytes [15,18]. This causes the PC rich lamellar bodies within the pneumocytes to increase is number [13,14].

In humans, the acquired disorder drug-induced phospholipidosis is caused by administration of the antiarrhythmic drug amiodarone, the antidepressant fluoxetine, the aminoglycoside antibiotic gentamicin, and the antianginal agent perhexiline [13–15,19,20]. The inhibition of lysosomal acid hydrolase activity by these drugs results in the accumulation of the phospholipids phosphatidylcholine, and to a lesser extent, phosphatidylinositol, phosphatidylethanolamine, sphingomyelin, and phosphatidylserine, in the cellular lysosomes of a number of organ systems including the lung [13,15]. As mentioned above, pulmonary complications result from large quantities of phosphatidylcholine in the acidic lysosomal compartments of alveolar

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macrophages and acidic lamellar bodies of type II granular pneumocytes [14]. Alternatively, in the case of the inherited, metabolic lysosomal storage disorders Niemann–Pick disease types A and B, Sandhoff disease, and Gaucher disease type I, there is no direct explanation linking the genetically compromised acid hydrolase to the observed build-up of phosphatidylcholine in pulmonary lysosomes. Niemann–Pick disease types A and B are caused by mutations in the gene encoding the lysosomal acid hydrolase sphingomyelinase, a phosphodiesterase that acts on the phospholipid sphingomyelin [1]. In Sandhoff disease, defective activity of the acid hydrolase β-hexosaminidase results in the lysosomal accumulation of the gangliosides GM_2 and GA_2, while in Gaucher disease type I, lysosomal accumulation of glucosylceramide is due to reduced activity of the acid hydrolase glucocerebrosidase. Notwithstanding, the lung pathology associated with lysosomal build-up of PC in Niemann–Pick disease types A and B, Sandhoff disease, and Gaucher disease can result in serious clinical manifestations which include reduced pulmonary function, respiratory failure, and frequent respiratory infections that can lead to death [3,5–12].

The pulmonary symptoms associated with drug-induced phospholipidosis can be reversed by terminating drug administration. In the case of the inherited forms of lysosomal storage disease, the symptoms can be alleviated with whole-lung or partial branchoalveolar lavage, procedures in which a bronchoscope is used to remove excess fluid from the lungs [11,21,22]. A less invasive approach would be to employ a therapeutic agent to assist with the hydrolysis of the excess PC in alveolar macrophage lysosomes and in type II granular pneumocyte lamellar bodies. The pH of the interior of lysosomes is approximately 4.8, and is thus substantially more acidic than the surrounding cytoplasm (~pH 7.2). Therefore, an ideal therapeutic agent would be relatively unreactive at neutral pH, but would efficiently hydrolyze the phosphate ester bonds of phosphatidylcholine at mildly acidic pH values. If this were the case, any therapeutic agent molecules "leaking" out of the lysosomes would be less likely to damage the rest of the cell.

One approach to cleaving phosphodiester bonds is to employ metal ions. For example, a wide range of metal complexes has been used to hydrolyze the phosphate ester bonds of nucleic acids [23–28]. Impressive rate enhancement factors for the cleavage of double-standed DNA have been obtained (*e.g.*, 10^7 fold for Co(III) [25]; 10^8 fold for Cu(II) [25]; and 10^{10} fold for Ce(IV) [27]). Although metal-assisted hydrolysis of phospholipids has not been extensively studied, there are some promising examples [29–34]. In these experiments, the rare earth metal ions Ce(III), Er(III), Eu(III), La(III), Lu(III), Tb(III), Tm(III), Y(III), and/or Yb(III) (at 25 °C to 45 °C and pH 7.0 to 8.5) were utilized to cleave unactivated, phosphate ester bonds of naturally occurring phospholipids [29,30] and the "activated" *p*-nitrophenyl phosphate ester bonds of synthetic phospholipid analogs [31–34]. (The *p*-nitrophenol chromophore allows for the hydrolytic reaction to be monitored spectrophotometrically, and makes the phosphodiester bond more reactive towards hydrolysis.) Until now, an application that has not been addressed by researchers is the development of metals as therapeutic agents to target disease-related phospholipids.

In this paper, we have tested unactivated phosphatidylcholine (**1**) against Ce(IV), Zr(IV), Hf(IV), Co(II), Cu(II), Eu(III), La(III), Ni(II), Pd (II), Y(III), Yb(III), and Zn(II), metal ions which have been used to hydrolyze activated and unactivated amide and/or phosphate ester bonds in peptides, nucleic acids, and other in compounds [23–41]. To the best of our knowledge, the present work represents the first example of a research study that has focused on metal-assisted cleavage of unactivated phospholipids at lysosomal pH (~4.8). Hydrolysis reactions were conducted at 60 °C and 37 °C. Using MALDI-TOF mass spectrometry in combination with colorimetric assays to detect inorganic phosphate (**2**) and choline (**3**), we have shown that cerium(IV) produces superior levels of phosphate ester bond hydrolysis at ~pH 4.8 compared to near neutral, cytoplamic pH (~7.2). The high cleavage levels provide a strong rationale to develop cerium(IV) complexes as potential therapeutic agents to reduce phospholipid accumulation in lysosomal storage disease.

2. Experimental

2.1. Materials

De-ionized, distilled water was used in the preparation of all buffers and all aqueous reactions. Chemicals were of the highest available purity and were used without further purification. L-α-phosphatidylcholine (Egg, Chicken; catalog number 840051P, MW = 760.19 $g mol^{-1}$) was purchased from Avanti Polar Lipids, USA. The metal ion salts $Ce(NH_4)_2(NO_3)_6$, $ZrCl_4$, $HfCl_4$, $CoCl_2 \cdot H_2O$, $CuCl_2 \cdot 2H_2O$, $EuCl_3 \cdot 6H_2O$, $LaCl_3 \cdot H_2O$, $NiCl_2 \cdot 6H_2O$, K_2PdCl_4, $YCl_3 \cdot 6H_2O$, $YbCl_3 \cdot 6H_2O$, and $ZnCl_2$ were obtained from The Aldrich Chemical Company (purity > 99%). Triton X-100 and piperazine were from Fluka (Sigma-Aldrich, USA). A malachite green/molybdate-based colorimetric QuantiChrom™ Assay Kit (catalog number POMG-25 H) was purchased from BioAssay Systems, USA. An Amplex® Red Sphingomyelinase Assay Kit (catalog number A12220) was from Invitrogen, USA. Calcium phosphocholine chloride tetrahydrate, choline chloride, tris(hydroxymethyl)aminomethane (Tris) and 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich.

2.2. Preparation of lipid vesicles and micelles

A total of 25 mg of chicken egg L-α-phosphatidylcholine (32.89 μmol) was added to a 10 mL round bottomed flask and was dissolved in 1 mL of chloroform. The chloroform was evaporated to dryness *in vacuo* (12 h). In order to form lipid vesicles, a total of 274 μL of pre-heated water (65 °C) was added to the dried phospholipid (120 mM final concentration) and the solution was sonicated for 20 min at 65 °C, a temperature above the highest phase transition temperatures of chicken egg L-α-phosphatidylcholine (41 °C for PC 16:0 and 55 °C for PC 18:0 [42,43]). A total of 0 μL, 1.3 μL or 20 μL of Triton X-100 (10% in water (w/v), 0.16 M) was added to 17 μL of the 120 mM phosphatidylcholine solution and was allowed to react for 30 min at room temperature to convert the phospholipid vesicles (0 mM Triton X-100, final concentration) to mixed lipid vesicles (11.4 mM Triton X-100, final concentration), and to mixed micelles (86.5 mM Triton X-100, final concentration) [44]. The Triton X-100:phosphatidylcholine molar mixing ratios of these solutions equaled 0 (0 mM:120 mM); 0.10 (11.4 mM:111.5 mM); and 1.57 (86.5 mM:55.1 mM).

2.3. Phosphatidylcholine hydrolysis

A total of 700 μL of a 100 mM aqueous solution of a metal ion salt, ($Ce(NH_4)_2(NO_3)_6$, $ZrCl_4$, $HfCl_4$, $CoCl_2 \cdot H_2O$, $CuCl_2 \cdot 2H_2O$, $EuCl_3 \cdot 6H_2O$, $LaCl_3 \cdot H_2O$, $NiCl_2 \cdot 6H_2O$, K_2PdCl_4, $YCl_3 \cdot 6H_2O$, $YbCl_3 \cdot 6H_2O$, or $ZnCl_2$, was combined with 700 μL of a 200 mM aqueous buffer solution (piperazine or HEPES) to prepare a series of metal/buffer cocktails. The piperazine and HEPES cocktails were respectively adjusted to final pH values of ~5.2 and 7.2 by adding concentrated HCl and/or 50% NaOH (w/v) (total volume added, 2 μL to 5 μL). Two hundred microliters of each pH adjusted cocktail were then transferred to either 17 μL of the 0 mM Triton X-100 solution, 18.3 μL of the 11.4 mM Triton X-100 solution, or 37 μL of the 86.5 mM Triton X-100 solution. Deionized, distilled H_2O was used to bring the volume of each reaction to 1000 μL. The resulting solutions were allowed to react at 60 °C for 0 h and 20 h (2 mM phosphatidylcholine, 0 mM, 0.2 mM or 3.2 mM Triton X-100, 10 mM metal ion salt, 20 mM piperazine ~ pH 4.8 or 20 mM HEPES ~ pH 7.2). In control reactions, dissolved metal ion salts were replaced by equivalent volumes of ddH_2O. For K_2PdCl_4, an

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