A novel method for the purification of inositol phosphates from biological samples reveals that no phytate is present in human plasma or urine

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1. Summary

Inositol phosphates are a large and diverse family of signalling molecules. While genetic studies have discovered important functions for them, the biochemistry behind these roles is often not fully characterized. A key obstacle in inositol phosphate research in mammalian cells has been the lack of straightforward techniques for their purification and analysis. Here we describe the ability of titanium dioxide (TiO₂) beads to bind inositol phosphates. This discovery allowed the development of a new purification protocol that, coupled with gel analysis, permitted easy identification and quantification of InsP₆ (phytate), its pyrophosphate derivatives InsP₇ and InsP₈, and the nucleotides ATP and GTP from cell or tissue extracts. Using this approach, InsP₆, InsP₇ and InsP₈ were visualized in Dictyostelium extracts and a variety of mammalian cell lines and tissues, and the effects of metabolic perturbation on these were explored. TiO₂ bead purification also enabled us to quantify InsP₆ in human plasma and urine, which led to two distinct but related observations. Firstly, there is an active InsP₆ phosphatase in human plasma, and secondly, InsP₆ is undetectable in either fluid. These observations seriously question reports that InsP₆ is present in human biofluids and the advisability of using InsP₆ as a dietary supplement.

2. Background

Inositol is present in all eukaryotes, most archaea and some bacteria [1], but only nucleated cells have taken advantage of the metabolic stability of this sugar to evolve the complex array of phosphorylated signalling molecules known as inositol phosphates (if water soluble) or inositides (if lipid-bound) [2]. Attention has been drawn to the soluble inositol phosphates by the elucidation of the signalling pathway that connects receptor activation, via phospholipase C, to the release of the second messenger Ins(1,4,5)P₃ [3]. A variety of inositol phosphates are also generated using Ins(1,4,5)P₃ as precursor through a series of kinases and phosphatases [2]. Notably, the sequential action of the kinases IPMK (inositol polyphosphate multikinase, also known as Ipk2) [4] and IP₇-2kinase (also known as IPPK or Ipk1) [5] convert Ins(1,4,5)P₃ to InsP₆ (inositol hexakisphosphate; phytic acid) [6,7]. InsP₆ was originally discovered as a phosphate storage molecule in plant seeds but is now known to be present in all eukaryotic cells. It is the most abundant intracellular inositol phosphate species, with concentrations ranging between 10 and 50 μM in mammalian cells [8,9]. The social amoeba Dictyostelium discoideum has the highest known (non-plant seed) concentration, where InsP₆ levels can reach 0.5 mM [8,10,11]. Biophysical studies have indicated that at cytoplasmic pH and salt concentrations, InsP₆ exists in a neutral...
pentamagnesium form with a solubility limit of 49 μM [12], so it is likely that in D. discoideum some of the InsP₆ is compartmentalized in vesicles, or that this organism has unusual cytoplasmic conditions permitting higher InsP₆ solubility.

In mammals, were extracellular InsP₆ to exist, it should only do so complexed to proteins, since it would be expected to precipitate in the prevailing salt and pH conditions. The suggested presence of InsP₆ in human biofluids is currently relevant as ‘natural products’ companies are selling InsP₆ as a supposedly beneficial dietary supplement. The claim is that dietary InsP₆ is absorbed by the intestinal mucosa and transported in plasma, both of which assumptions have proved highly divisive. Specific InsP₆ transporters are yet to be identified in mammals, and a molecule as polar as InsP₆ cannot diffuse through the plasma membrane; thus its intestinal absorption is unlikely. By a variety of assays, the InsP₆ concentration in human plasma has been calculated as 0.2–0.5 μM [13,14], but a more direct and highly specific mass assay was unable to confirm these values [8], showing that InsP₆ could be present in human plasma at only sub-nanomolar concentrations, if at all.

Many signalling roles have been attributed to InsP₆, notably a role in the control of nuclear–cytoplasm mRNA export [15]. The InsP₆-derived inositol pyrophosphates have their own signalling roles and have been implicated in the pathophysiology of important human diseases such as diabetes, obesity, cancer, blood coagulation and viral infection (for reviews, see [16–18]). In these studies, the cellular biochemistry of InsP₆, InsP₇ and InsP₈ is often incompletely characterized, however. This is due to the technical difficulty of accurate measurement, which requires radioactive metabolic labelling and HPLC analysis [19]. These cumbersome techniques have held back InsP₆, InsP₇ and InsP₈ extraction, however, the lower concentrations of inositol phosphates extracted from mammalian cells and tissues. Since this newly developed technology allows the extraction of inositol phosphates from large sample volumes, we also used it to test for the presence of InsP₆ in human biofluids in an attempt to resolve the controversy [8,22,23] surrounding this issue.

3. Material and methods

3.1. Cell culture and treatment

Mammalian, plant and fly cells used were gifts from several different laboratories and were grown in standard conditions for each cell type. HCT116 cells were cultured in DMEM (Invitrogen) supplemented with 10% FBS, in 5% CO₂. Vegetative state Dictyostelium discoideum cells were grown at 22°C in a shaking HLF medium supplemented with 100 U ml⁻¹ penicillin and 100 μg ml⁻¹ streptomycin (Invitrogen). For sodium fluoride treatment, 90% confluent HeLa, MCF7 and HCT116 cells (2 x 14 cm dishes) were treated with 10 mM sodium fluoride (Sigma) for 1 h before harvesting by trypsinization. For oligomycin treatment, the cells were pre-treated with glucose-free DMEM for 30 min, before addition of 5 μM oligomycin (Sigma) for 3 h. Cells were harvested by trypsinization.

3.2. Titanium dioxide bead extraction

All steps in the extraction until elution were performed at 4°C to avoid acid degradation of inositol pyrophosphates. First, the titanium dioxide (TiO₂) beads (Titansphere TiO 5 M; GL Sciences) were weighed and prepared by washing once in water then once in 1 M perchloric acid (PA). Generally 4–5 mg of beads was used for each sample. After centrifuging at 3500g for 1 min, the beads were resuspended in PA. Cells were harvested as appropriate and washed in PBS. A small aliquot was removed for later protein quantification, enabling normalization. The cells were pelleted and extracted using 800 μl PA (pH 1). After resuspension in the acid, samples were kept on ice with vortexing for 10 min, then centrifuged at 18 000g for 5 min, at 4°C. The supernatants were removed into new eppendorfs and TiO₂ beads added (4 mg in 50 μl PA). Samples were vortexed briefly then rotated at 4°C for 15 min; the inositol phosphates and other molecules were adsorbed onto the beads at this point. Beads were pelleted by centrifuging at 3500g for 1 min, and then washed twice in PA with supernatants discarded. To elute, 200 μl 10% ammonium hydroxide (pH 10) was added to the beads. Samples were vortexed briefly before rotation for 5 min. After centrifuging, the supernatants (containing the inositol phosphates) were transferred into new eppendorfs. The elution procedure was repeated on the beads to ensure full recovery, and the second supernatants added to the first. The samples were then vacuum evaporated to 50 μl for PAGE or other further analysis. Alternatively, samples were evaporated until at pH 7 then stored at 4°C or –20°C.

The protocol used for TiO₂ extraction from Dictyostelium PA extracts, diluted InsP₆ standards and radioactive [³²P]InsP₆ (PerkinElmer) or [³H]InsP₆ (Amersham) was the same as above, except that the standards were directly added to 1 ml PA. For the radioactive experiments, 5 ml of Ultima Gold (PerkinElmer) scintillation cocktail was added to the TiO₂ eluate and the samples were counted in a β-counter.

Mouse liver and brain were collected from newborn (P1) pups and rapidly frozen. PA (2 ml) was added to approximately 0.5 g of tissues, equivalent to one liver or two brains. The organs were rapidly homogenized in an electric blender and incubated in ice for 10 min. The samples were centrifuged at more than 15 000g for 15 min and the supernatant used for TiO₂ bead extraction.

3.3. Plasma, serum and urine extraction

Bovine and horse serum and plasma were bought from Life Technologies and Sigma Aldrich. Human serum and plasma were bought from TCS Biosciences and Sigma Aldrich. Alternatively, human plasma was prepared from anonymous...
tides and polyP standards were bought from Sigma Aldrich, [19]. Initially, a specific amount of InsP6 diluted in PA was incubated with TiO2 beads for 30 min. After two washes with PA, InsP6 was eluted from the beads by a pH change induced by hydroxide and reducing the volume using a centrifugal evaporator. The ability of titanium dioxide (TiO2) to bind with very high affinity to phosphate groups is used in phosphopeptide enrichment protocols, an essential step in modern phosphoproteomic studies. We used this TiO2 property [25] to develop a simple enrichment method (schematized in figure 1) to extract inositol phosphates from acidic solutions, normally 1 M PA with toluidine blue staining, demonstrating an almost complete recovery of the input InsP6 (figure 1b). We next tested this procedure on a D. discoideum extract, and recovered quantifiable levels of InsP6 and its pyrophosphate derivatives InsP7 and InsP8 (figure 1c). To precisely quantify the inositol phosphate recovery, radioactive 3H-InsP1,4,5P3 and 3H-InsP2 tracers were each mixed with 2 nmol of InsP1,4,5P3 and InsP2 and subjected to TiO2 enrichment. These experiments demonstrated that 87 ± 4.6 and 84 ± 3.5% (average ± s.d., n = 4) for InsP1,4,5P3 and InsP2, respectively, of input radioactivity was recovered after TiO2 elution, while about 2–4% remained attached to the TiO2 beads (figure 1d). The TiO2 beads are in fact completely efficient at binding and releasing inositol phosphates; the small loss is intrinsic to the manual handling involved.

4.2. Titanium dioxide purifies inositol phosphates and nucleotides from mammalian cell extracts

The lower concentration of inositol phosphates in mammalian cells has previously rendered extracts from these cells impracticable for PAGE analysis. Either the volume is too large to load onto the gel, or volume reduction by evaporation results in salt concentrations that cause aberrant gel migration. The ability to concentrate inositol phosphates using TiO2 beads overcomes these limitations.

We tested TiO2 enrichment on extracts from HCT116 cells (human colon cancer cell line). PAGE analysis (figure 2a) of the phosphate-rich molecules extracted revealed the presence of three inositol phosphate bands that co-migrate with D. discoideum-extracted InsP6, InsP7 and InsP8 [10]. Interestingly, unlike D. discoideum extracts, mammalian cell extracts revealed extra bands that co-migrate with the nucleotide standards ATP and GTP. We confirmed the identity of the bands presumed to be ATP and GTP by treating the TiO2-purified samples with apyrase, an enzyme that specifically hydrolyses nucleotides (figure 2b). We also detected a faint, slower migrating band of unknown nature (labelled Unk), which is particularly abundant in liver extract (figure 3c). This band does not represent an InsP4 species since it is not fully degraded after phytase treatment unlike the InsP6, InsP7 and InsP8 bands (data not shown). The partial action of phytase on this unknown band suggests a complex molecule containing an inositol phosphate group. Inositol pyrophosphates in mammalian cells are known to be dramatically regulated by sodium fluoride (NaF) [26]. To confirm the identity of the observed InsP7 and InsP8 bands, we incubated three human cell lines with NaF: firstly, MCF7 cells, which usually have undetectable levels of inositol pyrophosphates; secondly, HeLa cells, which have detectable levels of InsP7 and thirdly, HCT116 cells, which have detectable levels of InsP7 and InsP8. PAGE analysis after TiO2 extraction revealed that NaF treatment increases inositol pyrophosphate levels, and decreased the level of their precursor InsP6, in all three cell lines (figure 2c).

4.3. Screening of mammalian cell lines and tissues for the presence of inositol pyrophosphates

Next, we decided to screen 27 mammalian, one plant and one Drosophila cell line for the presence of inositol pyrophosphates, to identify the best cell line(s) for studying the different aspects of inositol pyrophosphate metabolism. TiO2 purification was done. Two 20 ml samples of blood from each volunteer were collected into tubes pre-filled with 1.6 mg of EDTA per ml of blood (5.5 mM) and immediately cooled on ice for 10 min. One of the samples was then spiked with 1 nmol of InsP6 prior to removal of cells and platelets by centrifugation at 1500g for 10 min at 4°C. This yielded plasma for analysis. To extract InsP6, half volume of 2 M PA was added to 10–20 ml of serum or plasma and the sample rotated at 4°C for 30 min. The denatured proteins were removed by centrifugation at 15 000g at 4°C for 30 min. The supernatant was subjected to the extraction procedure as described above, using 5 mg of TiO2 beads.

Human urine was obtained from anonymous donors. After centrifugation at 2000g at 4°C for 5 min to remove any epithelial cells, which were discarded, the samples were split in half; one half was spiked with 2 nmol InsP6. Concentrated PA was added to a final concentration of 1 M and rotated at 4°C for 30 min. The denatured proteins were removed by centrifugation at 15 000g at 4°C for 30 min. The supernatant was subjected to the extraction procedure as described above.

3.4. Enzymatic treatment

Cell extracts were treated with apyrase (New England Biolabs) following the manufacturer’s instructions.

3.5. Polyacrylamide gel electrophoresis of inositol phosphates

PAGE was performed as previously described [20]. Briefly, 35% polyacrylamide/TBE gels were used to resolve the TiO2-extracted samples. Samples were mixed with either orange G or bromophenol blue loading buffers. The gels were pre-run for 30 min at 300 V and run overnight at 4°C at 600 V and 6 mA, until the orange G had run through two-thirds of the gel. Gels were stained as previously described [20] with either DAPI or toluidine blue. Gels stained with toluidine blue were scanned using a desktop computer scanner for image analysis. IMAGEJ was used for densitometry (n = 3 per experiment), and amounts of inositol pyrophosphates are expressed as a ratio of their band density over InsP6. Nucleotides and polyP standards were bought from Sigma Aldrich, while InsP6, InsP7, InsP8 and InsP3 were bought from Sichem.

4. Results

4.1. Titanium dioxide binds to inositol phosphates

The ability of titanium dioxide (TiO2) to bind with very high affinity to phosphate groups is used in phosphopeptide enrichment protocols, an essential step in modern phosphoproteomic studies [24]. We used this TiO2 property [25] to develop a simple enrichment method (schematized in figure 1a) to extract inositol phosphates from acidic solutions, normally 1 M PA [19]. Initially, a specific amount of InsP6, diluted in PA was incubated with TiO2 beads for 30 min. After two washes with PA, InsP6 was eluted from the beads by a pH change induced by 10% ammonium hydroxide. After removing the ammonium hydroxide and reducing the volume using a centrifugal evaporator, the samples were resolved by PAGE and visualized with toluidine blue staining, demonstrating an almost
performed from 90% confluent cells grown in two 14 cm adherent culture dishes or shaking culture, as appropriate. The use of DAPI to better visualize inositol pyrophosphates [20] revealed the presence of InsP7 in almost all cells analysed (electronic supplementary material, figure S1), while InsP8 is easily detectable in mouse ES cells, Drosophila S2 and the human HCT116 cell line, where inositol pyrophosphates seem to be particularly abundant. An exact comparative analysis cannot be achieved since cell density, size and shape differ greatly between cell lines. Furthermore, DAPI staining is not linear, unlike toluidine blue, the staining intensity of which depends only on the number of phosphates [10]. Therefore, we chose several human cell lines to investigate their relative inositol pyrophosphate levels more thoroughly, normalizing the different extracts by protein mass. The normalized analysis of six mammalian cell lines by DAPI confirmed the screening result (figure 3a). Parallel toluidine blue staining confirmed a variable amount of InsP7 and especially InsP8 between the cell lines (figure 3b).

4.4. Effect of altered energetic metabolism on inositol pyrophosphates levels

The ability to enrich and analyse InsP2 and InsP5 from mammalian extracts has the potential to revolutionize this field of research. As inositol pyrophosphates have been linked to cellular and organismal metabolism [27–29], we took advantage of the TiO2 method to observe their changes...
after metabolic perturbation. Inositol pyrophosphates were TiO$_2$-extracted from glucose-starved HCT116 cells treated with the oxidative phosphorylation inhibitor oligomycin for 3 h. PAGE analysis showed the disappearance of InsP$_8$ and a substantial reduction in InsP$_7$, with a concomitant increase in InsP$_6$ (figure 3d).
4.5. Absence of InsP₆ in human blood revealed by titanium dioxide extraction

Since large volumes of acidified fluid can be subject to TiO₂ bead extraction, this gave us the opportunity to assay InsP₆ in biofluids. Initially, we used commercially available serum from bovine, equine and human sources. We extracted 20 ml of serum with TiO₂ beads and analysed the extracts by PAGE. While we were able to detect an almost complete InsP₆ recovery in the spiked samples, we did not recover any InsP₆ in non-spiked serum (electronic supplementary material, figure S2A,B). We next analysed human plasma from a commercial source. Similar to serum, TiO₂ extraction and PAGE analysis showed that InsP₆ could not be recovered from non-spiked samples of human plasma (figure 4a). The lower limit of InsP₆ standard detection on PAGE is about 0.25 nmol (figures 4a,b and 5), therefore TiO₂-extracting 20 ml of plasma with a recovery of approximately 85% (figure 1c) indicates that the lower limit of plasma InsP₆ we are able to extract and detect is approximately 15 nM. Consequently, we conclude that substantially less than 15 nM InsP₆ is present in human plasma, in agreement with the enzymatic radio-assay previously reported [8].

Conversely, using less direct assays, others have suggested that the InsP₆ concentration in human plasma is in the 0.2–0.5 μM range [13,14]. They suggested that the previous failure [8] to detect InsP₆ in plasma was due to losses during plasma preparation [23]. To investigate this possibility, we here prepared plasma from anonymous donors following the extraction protocol described in [13,14] (which uses EDTA as an anti-coagulant [23]). While we were able to detect a good recovery of InsP₆ from plasma when whole blood was spiked with 1 nmol (0.05 μM) of InsP₆, we were again unable to detect any InsP₆ in non-spiked human plasma (figure 4b). Interestingly, in the spiked samples, other faster migrating bands of weak intensity, probably lower forms of InsPₓ, were detected besides InsP₆, and we only recovered spiked InsP₆ with a high efficiency if the blood was cooled on ice before spiking. These observations suggested the presence of a phosphatase activity in plasma. To test this directly, we incubated InsP₆-spiked human plasma at 37°C. Just 5 min of incubation resulted in substantial conversion of InsP₆ to InsP₅ (figure 4c); 20 min led to the complete removal of InsP₆, while after 40 min all the exogenous InsP₆ was converted to InsP₃ and even lower forms of inositol phosphates. It is likely that a recently reported secreted mammalian phosphatase, MINPP1

Figure 4. Absence of InsP₆ and presence of inositol phosphate phosphatase in human plasma. All the extracts were resolved by PAGE and visualized with toluidine blue staining. (a) EDTA was added to 20 ml of commercial human plasma from male (MP) and female (FP); InsP₆ was added to the spiked aliquot (InsP₆; 2 nmol). The samples were then acidified and subjected to TiO₂ enrichment. (b) Plasma from healthy anonymous donors (D1 to D4) was prepared as described in Material and methods, with spiking (InsP₆; 1 nmol), and subjected to TiO₂ extraction. (c) 4 nmol of InsP₆ was added to 1 ml of human plasma and incubated at 37°C for the indicated time before acidification and extraction of inositol phosphates with the TiO₂ procedure. Standards: InsP₆ (4 nmol); InsP₅ (6 nmol of Ins(1,3,4,5,6)P₅); InsP₄ (5 nmol of Ins(1,4,5,6)P₄); InsP₃ (20 nmol of Ins(1,4,5,6)P₃). (d) 4 nmol of InsP₆ was added to 1 ml of a different source of human plasma (HP) and bovine plasma (BP) before incubation at 37°C for the indicated time, followed by acidification of the samples and TiO₂ extraction. The gels presented are representative of experiments performed two to four times.
Figure 5. Absence of InsP₆ in human urine. Freshly collected urine from healthy anonymous donors (D1 – D3) was centrifuged to remove any epithelial cells. The samples were then divided into two aliquots (25 ml each for D1 and D2, 10 ml for D3), EDTA was added and InsP₆ (2 nmol) was supplemented into the spiked (InsP₆) aliquots. The samples were then PA extracted and subjected to TiO₂ enrichment. The extracted inositol phosphates were resolved by PAGE and visualized with toluidine blue staining. The gel presented is representative of three experimental repeats.

4.6. Absence of InsP₆ in human urine revealed by titanium dioxide extraction

Another human biofluid in which InsP₆ has been contensively reported is urine, with some literature indicating that it reaches 1 – 3 μM concentration [31]. Therefore, we processed 10 – 25 ml of urine from anonymous donors using TiO₂ and visualized the extraction on PAGE. Similar to the serum and plasma experiments, we were unable to detect any InsP₆ in non-spiked urine (figure 5). Thus, less than 12 nM InsP₆ is present in human urine, a maximal value in accordance with the earlier report using a specific enzymatic assay [8].

5. Discussion

TiO₂ beads and pre-packed columns are useful tools for the enrichment of phosphopeptides and have contributed hugely to the development and application of phosphoproteomic studies [24]. We have taken advantage of this TiO₂ phosphate binding property [25] to develop a new inositol phosphate purification protocol. Crucially, it allows for the purification of inositol phosphates from large volumes of acidified extracts and so makes feasible the extraction/enrichment of the low concentration inositol phosphates in mammalian extracts.

The direct analysis of mammalian InsP₆ and its pyrophosphate derivatives InsP₇ and InsP₈ by coupling TiO₂ enrichment with PAGE analysis negates the requirement for HPLC and ³H-inositol labelling. This new procedure therefore simplifies inositol pyrophosphate analysis in particular and also solves the often forgotten but ever-present problem of determining the labelling time necessary for metabolic equilibrium. Among the new research possibilities opened up by TiO₂ purification is the analysis of inositol phosphates from animal tissues. Animal welfare and monetary considerations made this previously troublesome, as it would require treating the live animal with ³H-inositol tracer; analysis of human tissues and fluids was completely unattainable. The method described permits the extraction and analysis of inositol phosphates from animal organs including but not limited to mouse brain or liver, where InsP₆ and InsP₇ can be detected, or chicken egg white, where InsP₅ and InsP₆ are particularly abundant (Saiardi laboratory, unpublished data).

The ability of InsP₆ to bind to TiO₂ indicates that lower phosphorylated inositol species can also be purified from biological samples. As these stain poorly by toluidine blue they cannot be quantified by gel electrophoresis, but mass assays for other inositol phosphates exist that, coupled with TiO₂ purification, can be used in quantifying lower phosphorylated inositol species from biological specimens. Beyond the soluble inositol phosphates, it will be interesting to take advantage of this newly discovered ability of TiO₂ to bind and purify phosphate groups attached to an inositol ring to develop new inositol lipid purification methods, since the current approaches are essentially adaptations of Folch’s extraction method, developed more than 60 years ago [32].

The parallel analysis of D. discoideum and mammalian cell extracts revealed quite different patterns of extracted molecules. While InsP₆, InsP₇ and InsP₈ are extracted from both amoeba and mammalian cells, the nucleotides ATP and GTP are only visible in the mammalian extract. A quantitative comparison between the two experimental models is inappropriate since the number of cells extracted and analysed is different (in figure 2, 4 × 10⁶ cells for D. discoideum were compared to 80 × 10⁶ human HCT116 cells). However, the relative proportion between inositol pyrophosphate and nucleotides is unquestionably different in the two experimental models analysed. Since inositol pyrophosphates are able to regulate energetic metabolism and specifically are inversely connected with ATP level [27], we might speculate that the high levels of inositol pyrophosphates present in D. discoideum are lowering nucleotide levels. This hypothesis is currently under investigation.

We have also taken advantage of the high efficiency of TiO₂ extraction to independently re-address the question of whether human body fluids such as plasma and urine contain any InsP₆ (the relevance of this question is discussed below). In contrast with some other groups (e.g. [13,14]), but in agreement with an earlier study that used a specific
and sensitive enzyme-based \textit{InsP}_6 assay [8], we find that there is no \textit{InsP}_6 present, with a detection limit more than an order of magnitude below the levels claimed to be there by others. We have carefully controlled for \textit{InsP}_6 recovery using \textit{InsP}_6-spiked controls (including adding exogenous \textit{InsP}_6 to whole fresh blood), and it is important to stress that our discovery of a highly active phosphatase in human (and bovine) plasma, probably secreted MINPP1 [30], which hydrolyses any spiked \textit{InsP}_6, within minutes at 37°C, in itself rules out the possibility of any \textit{InsP}_6 being present in plasma \textit{in vivo}. Eiseman \textit{et al.} [33] reported that in rats the half-life of intravenously injected radiolabelled \textit{InsP}_6 is 8 min, suggesting that the presence of an active \textit{InsP}_6 phosphatase in plasma is not confined to humans and cattle. Given the potent ion-chelating power of \textit{InsP}_6, it actually makes evolutionary sense to immediately remove such a compound (which might be released by cell lysis or damage) from extracellular fluids. We should add that the available evidence suggests that ingested \textit{InsP}_6, if absorbed through the gut, enters the blood plasma exclusively as inositol [33], with possibly also some small amounts of inositol monophosphate [34]; the dephosphorylation of \textit{InsP}_6 before absorption is apparently caused by gut flora [35].

These observations have relevance for the reported effects of dietary \textit{InsP}_6 on, for example, kidney stone formation [36,37] and other calcifications [38], and on cancer growth [39]. Understanding these effects does not need to invoke \textit{InsP}_6 in extracellular fluids, as they can readily be explained either by \textit{InsP}_6 acting as a chelator of cations (e.g. Ca^{2+}, Fe^{3+}) in the gut and thus altering uptake [8], or because \textit{InsP}_6 is a major source of our dietary inositol [8]. In the latter context, in studies on cancer where inositol has been compared directly with \textit{InsP}_6, the two have similar efficacies (e.g. [40] and see [39] for other references).

This discussion then points to a key question: if all the effects of dietary \textit{InsP}_6 (other than as a source of inositol) are mediated by modulating cation absorption from the gut, could taking \textit{InsP}_6 supplements ever be harmful? In humans on a poor diet the answer is clearly ‘yes’ [41], and there is a significant effort in the plant breeding world to produce low \textit{InsP}_6 varieties of maize and rice to reduce these deleterious effects [42]. Shamshuddin [43] has argued that the ion-chelating effect of \textit{InsP}_6 in the gut is not harmful in well-fed individuals, but this has only been examined for a few divalent cations (e.g. Zn^{2+} and Cu^{2+} [44,45]), while trivalent metals, whose affinity for \textit{InsP}_6 is very much higher than divalents [46] and which are essential dietary components (e.g. Cr^{3+} [47]), have not been studied in this context. Overall, this leads us to the conclusion that chronically altering cation absorption from the gut by artificially loading the diet with a non-specific chelator [39] in the hope that it might impact indirectly on cancer or other pathologies seems highly inadvisable.

**Ethics statement.** All volunteers gave informed consent prior to donating blood. These experiments were carried out in accordance with the University of Cambridge Research Ethics Committee guidance ‘University of Cambridge Policy on the Ethics of Research Involving Human Participants and Personal Data’.

**Acknowledgements.** We thank the members of the Saiardi laboratory for discussion, suggestions and helpful comments.

**Author contributions.** M.S.C.W., S.J.B., R.F.I. and A.S. designed the research. M.S.C.W., S.J.B., F.P. and A.S. performed experiments. M.S.C.W., S.J.B., R.F.I. and A.S. wrote the manuscript.

**Funding statement.** The work is supported by the Medical Research Council (MRC) core support to the MRC/UCL Laboratory for Molecular Cell Biology University Unit (MC_U122680443). S.J.B. was supported by the British Pharmacological Society and the Wellcome Trust.

**Conflict of interests.** The authors declare no competing financial interests.

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