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Evaluation of inositol phosphates in urine after topical administration of myo-inositol hexaphosphate to female Wistar rats

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ABSTRACT

Aims: Previous studies demonstrated a remarkable increase of urinary $InsP_6$ by topical administration. However, the methodology used for $InsP_6$ analysis was not specific. The aim of this paper is to measure urinary inositol phosphates InsPs using more advanced methodologies and to compare the results with those obtained by the non-specific method.

Materials and methods: We fed 12 female rats with a diet without $InsP_6$ for 16 days. Then, we administered a topical $InsP_6$ gel at high doses for 7 days (50 mg $InsP_6$/day) or at low doses for 28 days (20 mg $InsP_6$/day). We measured urine levels InsPs using a nonspecific method (based on the ability of InsPs to complex Al^{3+}) and levels of $InsP_6$ by a specific method (using polyacrylamide gel electrophoresis). Identification of different InsPs was performed by MS.

Key findings: At baseline, after dietary deprivation of $InsP_6$, rats only excreted $InsP_2$ in their urine, and there was no detectable $InsP_6$ or other InsPs. Rats given the high dose treatment for 7 days had abundant urinary $InsP_6$, but also had other InsPs in their urine; cessation of $InsP_6$ administration led to decreased levels of urinary InsPs. Rats given the low dose treatment for 28 days had increasing levels of urinary InsPs over time. The maximum urinary $InsP_6$ was at 21 days, after which InsPs excretion decreased.

Significance: We conclude that the skin can absorb $InsP_6$ from a topical gel, and that $InsP_6$ is excreted in the urine, along with other InsPs ($InsP_5$, $InsP_4$, $InsP_3$, and $InsP_2$).

1. Introduction

Myo-inositol hexaphosphate ($InsP_6$, phytate) is abundant in plant seeds [1]. Consumption of $InsP_6$ as a supplement or in the diet may provide important health benefits, such as prevention of renal calculi [2–4], prevention of cardiovascular calcifications and other pathological calcifications [5,6], antioxidant effects [7,8], and prevention of some cancers [9–11] and osteoporosis [12]. The chemistry of inositol phosphates (InsPs) *in vivo* is complicated because there are multiple forms ($InsP_1$ to $InsP_6$) and multiple isomers of the different forms, due to the dephosphorylation of $InsP_6$ by phosphatases and the *de novo* synthesis within cells [13–14].

Interestingly, several InsPs also have physiological effects, such as the ability to inhibit the crystallization of calcium salts [15–17]. Some of these other physiological effects are more specific. For example, inositol 1,4,5-triphosphate functions as a secondary messenger in multiple signaling pathways [18].

The most common analytical methods for determination of $InsP_6$ are based on nonspecific measurements of total inorganic phosphate or formation of phosphate complexes, and the measured amount is usually attributed to $InsP_6$ [19]. In some cases, the method includes initial separation procedures. These methods may overestimate the level of $InsP_6$ when other InsPs are present in the sample, because these other InsPs have similar activities as $InsP_6$ in some analyses [20]. Direct and specific methodologies for measurement of $InsP_6$ are complex, especially when measuring levels in biological fluids, such as blood and urine, and use of initial separation processes is likely to lead to a loss of some $InsP_6$ [21]. At present, it seems that coupling of separation

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techniques (high performance liquid chromatography [HPLC] with mass spectrometry [MS]) can provide reliable results [22]. For example, an analysis of InsPs consisting of anion-exchange HPLC coupled with electrospray ionization MS demonstrated that $InsP_6$ was the dominant InsP in almond meal and almond brown skins, but there were also significant amounts of other InsPs ($InsP_1$-$InsP_5$) [23,24].

Our previous studies indicated that a moisturizing $InsP_6$-based cream (containing phytate as a sodium salt) was highly absorbed, and led to significant $InsP_6$ urinary excretion [25,26]. However, the $InsP_6$ analysis was performed using a non-specific method; there was an initial separation using an anion exchange resin, and $InsP_6$ evaluation was based on measurement of total phosphorus by inductively coupled plasma (ICP) atomic emission spectrometry [27]. In the present paper, we re-evaluated the urinary excretion of $InsP_6$ and other InsPs in rats after topical administration of an $InsP_6$ moisturizing gel, using more specific analytical strategies to evaluate the presence of $InsP_6$ and other InsPs, and compared the results with a nonspecific method based on the formation of InsPs complexes.

2. Materials and methods

2.1. Animals, diets and experimental design

Twelve female Wistar rats, weighing 35–55 g when weaning, were used to evaluate InsPs urinary excretion after topical administration of $InsP_6$. The animals were housed in collective cages (3 animals per cage) in a temperature-controlled room (23 ± 1 °C) with a 12 h light-dark cycle, and received a standard food (UAR-A03, Panlab, Barcelona, Spain) and tap water *ad libitum*. At age 120 days, when the rats weighed about 240 g, they were transferred to individual cages and were switched to an AIN-76A diet (Harlan, Indianapolis, IN, USA), a synthetic purified diet in which $InsP_6$ or other InsPs are undetectable (Table 1).

After 16 days of consuming the AIN-76A diet, urine was collected over 24 h using an individual metabolic cage. The levels of total InsPs were determined by a non-specific methodology (see Section 2.5 below). After this period, animals were randomly assigned to a high-dose $InsP_6$ group or a low-dose $InsP_6$ group, with 6 rats per group.

Rats in the high-dose group were given the AIN-76A diet and daily topical treatment with 1.25 g of a gel (LoLi Pharma, Rome, Italy), which contains 4% $InsP_6$ as a sodium salt (corresponding to 50 mg of $InsP_6$) for 7 days. The gel was applied over a surface of about 4 cm^2 on the back of the animal that was shaved with an electric shaver every 4 days. Samples of 24 h urine were collected on day 7 of treatment for evaluation of InsPs. Rats in the low-dose group were given daily topical treatment with 0.5 g of the gel (corresponding to 20 mg of $InsP_6$) for 28 days. Samples of 24 h urine were collected on days 7, 14, 21 and 28.

All procedures were carried out according to the Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes. Official permission to perform the animal experiments was provided by the ethical committee of our university (Exp. 2016/19/AEXP).

Table 1
Composition of AIN-76A diet according to the manufacturer (Harlan).

	Amount (g/kg dry weight)
Phosphorous	6.7
Calcium	6.2
Magnesium	0.69
Manganese	0.056
Iron	0.056
Zinc	0.045
Phytate	< 0.01

2.2. Extraction of InsPs with TiO_2

The extraction of InsPs was performed as previously described [28]. First, 50 mg of TiO_2 beads (Titansphere TiO 5 μm; GL Sciences) were added to an Eppendorf tube, prepared by washing with 1 mL of milli-Q water, then with 1 mL of 1 M perchloric acid (PA), and finally re-suspended in 1 mL of 1 M PA. A 100 μL sample of this suspension (corresponding to 5 mg TiO_2) was used for each urine sample.

Rat urine was centrifuged at 2600*g* for 5 min. The urine sample was 10 mL, when available. Concentrated PA was added, so the final concentration was 1 M, the sample was mixed for 15 min, and was then centrifuged at 2600*g* for 5 min. The supernatant was transferred into a 50 mL Falcon tube, and 100 μL of the TiO_2 suspension was then added. The mixture was mixed again for 15 min, and then centrifuged at 2600*g* for 5 min. The supernatant was discarded, and the TiO_2 with adsorbed InsPs was transferred into an Eppendorf tube, which was centrifuged to remove the supernatant. Then, the TiO_2 was washed twice with 1 mL of 1 M PA, and once with 1 mL of water, and the supernatants were discarded after 2 min of centrifugation at 10,000*g*. Finally, the InsPs were eluted with two fractions of 200 μL of 6 M NH_3, with mixing of each elution for 5 min. Then, 400 μL of eluate was evaporated on a SpeedVac Concentrator (Thermo Fisher Scientific, Waltham, USA) until the final volume was 10 μL. This sample was then reconstituted with 1 mL of water.

2.3. Identification of InsPs by MS

About 200 μL of the reconstituted eluate was injected into a Q Exactive Orbitrap high-resolution mass spectrometer equipped with a heated electrospray ionization (HESI) probe (Thermo Fisher Scientific, Waltham, USA), which was operated in a negative ionization mode.

2.4. Identification and semi-quantification of $InsP_6$ by polyacrylamide gel electrophoresis

An appropriate volume of the reconstituted eluate was evaporated until a volume of 30 μL was obtained for use in polyacrylamide gel electrophoresis (PAGE), following a previously described procedure [29]. For these experiments, 35% polyacrylamide/TBE gels were used to resolve the TiO_2-extracted samples. Samples were mixed with a bromophenol blue loading buffer and the gels were pre-run for 30 min at 4 °C at 300 V, and then for 6.5 h at 4 °C at 500 V and 6 mA. Gels were stained with toluidine blue and scanned with a GS-800 Calibrated Imaging Densitometer (Bio-Rad). The Quantity One image analysis program (Bio-Rad) was used for quantitation relative to an $InsP_6$ standard. $InsP_6$ (phytic acid dipotassium salt, MW = 736.22 g/mol) was from Sigma Aldrich.

2.5. Nonspecific quantification of $InsP_6$

The nonspecific quantitation of urinary $InsP_6$ measured the ability of InsPs to form complexes with Al^{3+}, and displace it from the aluminum-xylenol orange dye, as previously described [21]. For these measurements, 5 mL of fresh urine was mixed with 5 mL of EDTA and 10 mL of milli-Q water. This solution was adjusted to pH 6, transferred to a 50 mL Corning tube, and centrifuged at 2600*g* for 10 min. The supernatant was acidified with HCl to pH 3, and then quantitatively transferred to 150 mL beakers, each containing 0.25 g of AG1-X8 resin without previous conditioning. Each mixture was then stirred at 160 rpm for 15 min using an orbital stirrer. Resin and urine were transferred to an empty 20 mL SPE tube, through which urine was allowed to pass. The resin was washed with 300 mL of 100 mM HCl and 2 × 5 mL of milli-Q water. Finally, InsPs were eluted from the resin by 5 fractions of 0.5 mL of 2 M NaCl, and stirred at 180 rpm for 5 min, followed by collection of the eluate. InsPs were determined by indirect phytate equivalent analysis of this eluate using the aluminum-xylenol