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ORIGINAL PAPER

High calcium concentration and calcium oxalate crystals cause significant inaccuracies in the measurement of urinary osteopontin by enzyme linked immunosorbent assay

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Abstract Strong evidence that osteopontin (OPN) is a determinant of urolithiasis has prompted studies comparing the protein's urinary excretion in healthy subjects and stone formers. However, reported mean urinary values have varied widely, from <1 μg/mL to more than 20 times that value. Since OPN binds to CaOx crystals, the presence of crystals in urine may cause underestimation of its urinary levels. Using a commercial ELISA, we measured urinary OPN levels in the presence of endogenous or exogenous CaOx monohydrate (COM) and dihydrate (COD) crystals. OPN concentrations decreased in the presence of endogenous and exogenous CaOx crystals, but never below 2 μg/mL. Increasing the urinary calcium concentration decreased detectable OPN levels, possibly as a result of changes in the three-dimensional conformation of the protein. Because calcium concentration and the formation of CaOx crystals cannot be controlled in urine, the use of urinary OPN levels as a biomarker for any human pathology must be seriously questioned, but particularly for the investigation of stone formers in whom hypercalciuria and crystalluria are more common than in healthy subjects.

Keywords Biomarker · Calcium oxalate · Enzyme linked immunosorbent assay (ELISA) · Osteopontin · Urine · Urolithiasis

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Introduction

A secreted phosphoprotein with a high content of serine, aspartate and glutamate residues [1], osteopontin (OPN) contains a specific segment consisting almost entirely of aspartic acid residues, which is assumed to comprise its mineral binding domain [2]. OPN belongs to a large group of bone proteins referred to as small integrin binding ligand N-linked glycoproteins [3], which are highly flexible along their entire length in solution—a distinctive property thought to play a crucial role in their function [4]. Although usually associated with bone metabolism, for some years OPN has been implicated in the formation of human kidney stones, most of which are composed of crystals of calcium oxalate (CaOx).

Osteopontin is present in kidney stones [5] and is secreted in the luminal epithelia of the distal nephron [6] and the thick ascending loop of Henle [7], where urine is most concentrated and the likelihood of nucleation is highest [5]. It inhibits CaOx crystal nucleation [8, 9], growth [6] and aggregation [8] in inorganic solutions, in which it directs the preferential formation of CaOx dihydrate (COD) crystals, which adhere less avidly to renal epithelial cells than do CaOx monohydrate (COM) crystals [10]. The protein has been reported to block [11], but also to stimulate [12] the binding of CaOx crystals to cultured renal epithelial cells. This process, which is mitigated by agents that interfere with the cellular expression of OPN, has led to the conclusion that extracellular OPN is the principal cause of CaOx crystal deposition on the cell surface [13]. However, this seems at odds with the observation that OPN-deficient mice have significant deposits of CaOx in their kidneys following ethylene glycol ingestion [14] and that OPN mRNA levels increase in experimental nephrolithiasis [15].

Irrespective of whether OPN promotes or inhibits crystal attachment to cells, empirical and experimental evidence

clearly points to its having some involvement in stone formation, which has prompted studies comparing the protein's urinary excretion in healthy subjects and stone formers, using ELISAs. However, reported urinary concentrations, even in healthy controls, have varied widely [8, 9, 16–20], ranging from as low as 0.76 mg/L [16] to as high as 10 mg/L [18]. Such large discrepancies could result from the protein's susceptibility to proteolytic cleavage [16], conformational changes induced by calcium [4], differences in antibody specificity and reactivity [19], or additional factors, including interactions with other urinary components, storage conditions, crystalluria, and stones in situ [20]. The measured urinary concentration of prothrombin fragment 1 (PTF1) decreases in the presence of COM crystals [21] because it binds irreversibly to the COM crystal surface [22]. OPN also binds to COM [23] and COD [22] crystals. The presence of CaOx crystals could therefore reduce measurable amounts of urinary OPN and cause underestimation of its urinary concentration and excretion. The aim of this study was to examine the effect of exogenous and endogenous COM and COD crystals on the measurement of urinary OPN concentration.

Methods

All chemicals, which were of the highest grade obtainable, were purchased from Sigma Chemical Company (St Louis, MO, USA) unless otherwise indicated. All solutions were prepared with high quality water from a "hi-pure" water purification system (Permutit Australia, Brookvale, NSW, Australia).

Urine collection and processing

Urine was collected in the presence of thimerosal (0.1%) from healthy laboratory colleagues (four women, two men). Urine was refrigerated during the collection period, and the absence of nitrites and haematuria was confirmed by dipstick (Combur® test strips, Roche Diagnostics, Germany). It was then pooled and centrifuged at 10,000×*g* for 20 min at 10°C (Beckman J2-21 M/E centrifuge, Beckman instruments, Palo Alto, USA), followed by pre-filtration (RW0314250; Millipore Corporation, Bedford, MA, USA) and filtration (0.22 µm; GVWP14250; Millipore). Calcium concentration was measured using the *o*-cresophthalein complexone technique and then adjusted to the desired concentration with calcium chloride solution.

Preparation of inorganic CaOx crystals

COM crystals Using a peristaltic pump (P-3, Pharmacia Fine Chemicals), 100 mL of 0.15 M $CaCl_2$ and 100 mL of 0.15 M sodium oxalate (NaOx) were simultaneously added to 600 mL of distilled water at 20 mL per hour and incubated for 2 h in a water bath shaking at 100 opm at 37°C. Crystals were harvested by filtration through 0.22 µm Durapore® membrane filters (GVWP04700, Millipore), washed lightly with distilled water and lyophilized.

COD crystals Inorganic COD crystals were prepared using the method of Brown et al. [24]: 500 mL of 25.1 mM $CaCl_2$ and 500 mL 6.4 mM NaOx were added drop-wise to a solution containing 38.5 mM tri-sodium citrate, 46.2 mM $MgSO_4$ and 254.8 mM KCl. Crystals were collected as above. Crystals were identified by field emission electron microscopy (FESEM).

Measurement of urinary OPN by ELISA

Osteopontin ELISA kits from IBL-Hamburg (Gunma, Japan; cat. No.17158) were used according to the manufacturer's recommendations. Urine was diluted 200-fold with the enzyme immunoassay buffer supplied with the kits. The coefficients of variation for ten replicate samples of two different urine specimens were 3.9 and 5.0%.

Effect of exogenous inorganic COM and COD crystals on urinary OPN concentration

CaOx monohydrate typically precipitates from urine at a calcium concentration of 2 mM, while principally COD is deposited at 8 mM [22]. All experiments involving COM were therefore performed at 2 mM Ca, while those using COD were carried out at 8 mM. Crystals were added to the urine samples at final suspension densities ranging from 0 to 250 mg/mL and incubated for 2 h in a shaking water bath at 37°C. Before addition to the urine, the slurries were incubated at 37°C on a rotary mixer to ensure thorough mixing. After 2 h, crystals were recovered as above. The supernatant was diluted 200 times with buffer as before and analysed for OPN. Each measurement was performed in triplicate and the experiment was repeated using two additional urine samples.

Effect of endogenously formed COM and COD crystals on urinary OPN concentration

Urine samples were divided into two portions and the calcium concentration adjusted to either 2 or 8 mM. The metastable limit (MSL), defined as the minimum amount of oxalate required to induce CaOx crystallization [25], was detected using light microscopy. A measure of 30 µL of NaOx solution was added to 3 mL aliquots of urine to give final concentrations of 45, 60, 75, 90 and 120 mM at 8 mM Ca, and 135, 150, 165, 180, 195 and 210 mM at 2 mM, and

the samples were incubated for 2 h in a shaking water bath at 37°C. Crystals were harvested and examined by FESEM, and the supernatant was diluted 200-fold and assayed for OPN, as described above Each measurement was performed in triplicate and the experiment was repeated using two additional urine samples.

Field emission scanning electron microscopy (FESEM)

Small samples of crystal suspensions were filtered (0.22 μm) after incubation and washed using copious amounts of distilled water. The filtration membranes were dried overnight at 37°C, mounted on aluminium stubs and coated with carbon to 3 nm thickness using a high vacuum evaporator (DV-502, Denton Vacuum Inc., Moorestown, NJ, USA). The stubs were examined using a Philips XL30 FEGSEM field-emission scanning electron microscope at 10 kV accelerating voltage and 10 mm working distance.

Results

The effect of inorganic COM and COD crystals on urinary OPN concentration

Field emission electron microscopy showed that the inorganic crystals were monoclinic COM (Fig. 1a) and tetrahedral bipyramidal COD (Fig. 1b), while those precipitated from urine at 2 and 8 mM Ca showed the typical morphology of urinary COM (Fig. 1c) and COD (Fig. 1d) crystals, respectively.

Figure 2 shows the effects on OPN concentration of incubating inorganic COD crystals in urine at 8 mM Ca and inorganic COM crystals in the same urine at 2 mM Ca. In the absence of added crystals, at 8 mM Ca the measured OPN concentration was 2.6 μg/mL, which fell to 2.2 μg/mL at a suspension density of 0.025 mg/mL before increasing to 3.3 μg/mL (a rise of 50% from the initial value) and falling gradually thereafter to 1.9 μg/mL at a suspension density of 10 mg/mL. At 2 mM Ca in the absence of crystals, the OPN concentration was 3.3 μg/mL, but this fluctuated between 4.1, 2.8, 4.5 and 2.7 μg/mL at suspension densities ranging from 0.025 to 0.2 mg/mL, before gradually declining to a final concentration of 1.9 μg/mL at a crystal concentration of 10 mg/mL. The highest measured concentration (4.5 μg/mL) represents an increase of 36.4% above the initial value measured in the absence of crystals. Fluctuations in OPN concentration were observed with every urine specimen studied. It is noteworthy that the final OPN concentration was identical at both 2 and 8 mM calcium, but more particularly, that it did not fall below ~2 μg/mL. To determine whether this resulted from insufficient crystalline material to bind all the OPN in solution, a further experiment was performed with a different pooled urine sample using higher suspension concentrations.

With no added COM crystals the measured OPN concentration at 2 mM calcium was 5.22 μg/mL (Fig. 3), which steadily decreased to a final value of 3.1 μg/mL at a crystal

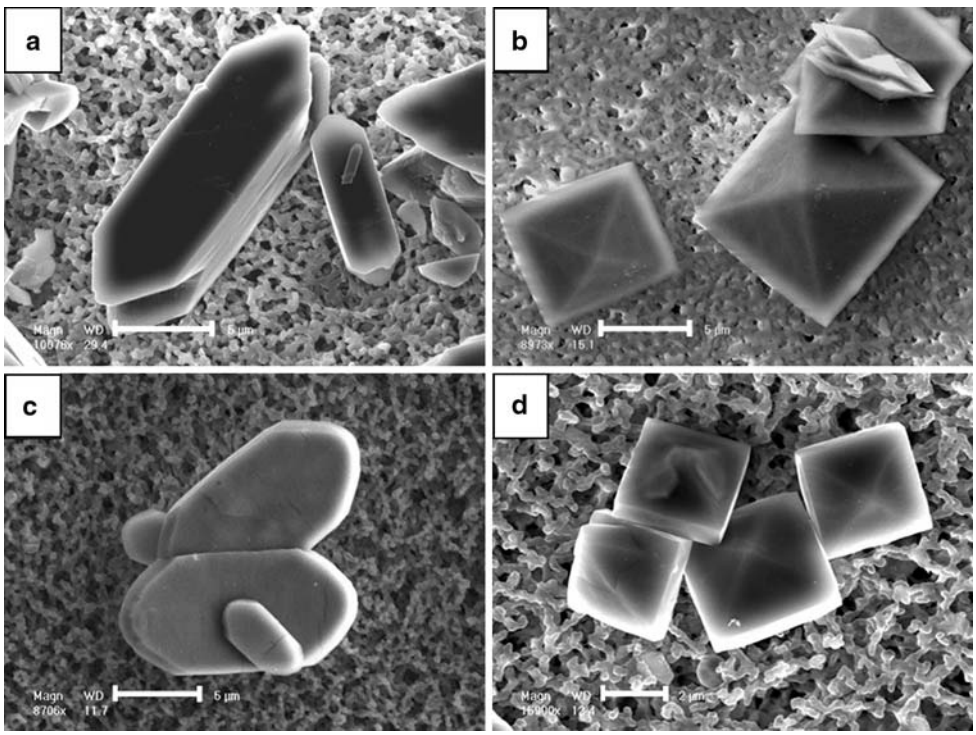

Fig. 1 Calcium oxalate crystals used in the experiments. **a** Inorganic COM, **b** inorganic COD, **c** urinary COM, **d** urinary COD

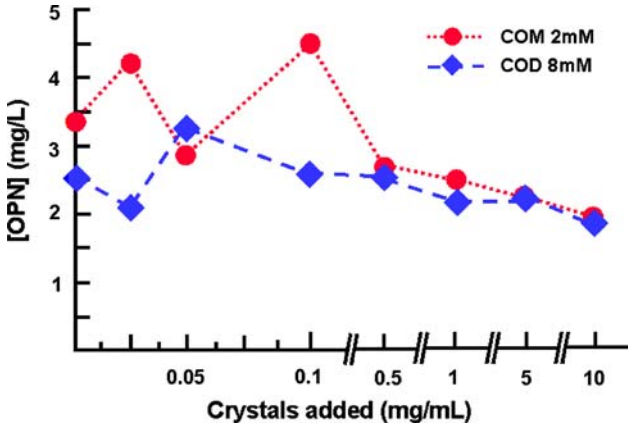

Fig. 2 The effect of adding increasing quantities of exogenous inorganic COM and COD crystals on the measured urinary OPN concentration. Data are summarised in the *row* marked *Exogenous A* in Table 1

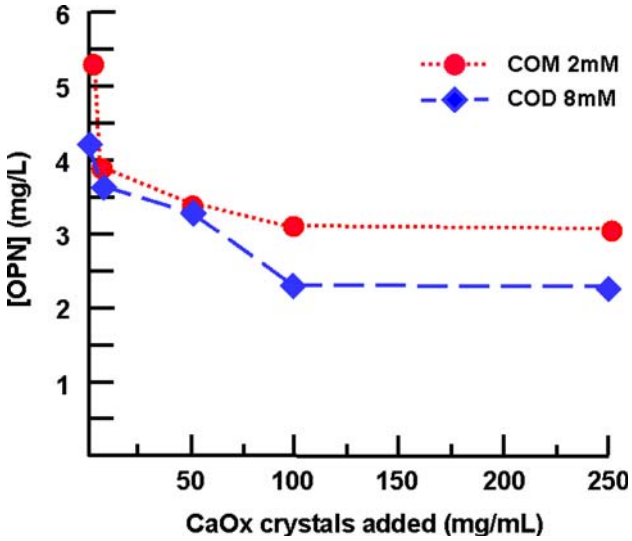

Fig. 3 The effect of adding large increasing quantities of exogenous inorganic COM and COD crystals on the measured urinary OPN concentration. Data are summarised in the *row* marked *Exogenous B* in Table 1

suspension density of 250 mg/mL. A similar reduction occurred with increasing COD crystals. Addition of COD crystals at 8 mM calcium elicited a decrease from 4.1 μg/mL in the absence of crystals, to 2.3 μg/mL at a suspension density of 250 mg/mL. With COM and COD, OPN concentration reached plateaux of 3.1 and 2.3 μg/mL, respectively, between crystal densities of 100 and 250 mg/mL.

Effect of ambient calcium concentration on OPN measurement

Because the data presented in Figs. 2 and 3 suggested a dependence of measured OPN level on the ambient calcium concentration, the effect of increasing calcium concentration on OPN determination was tested in an additional pooled urine sample without added crystals. The OPN concentrations at 1 and 2 mM calcium (Fig. 4) were 6.0 and 6.2 μg/mL, respectively, but fell to 4.8 μg/mL at 4 mM. Between 5 and 10 mM calcium, OPN concentration varied only slightly, with differences being within experimental error for the method. The drop in OPN concentration between 1 and 10 mM calcium represents a decrease of almost 25%.

The effect of endogenous COM and COD precipitation on urinary OPN levels

Figure 5 shows the effect of adding increasing amounts of oxalate to the urine. At 2 mM Ca crystals were first visible by light microscopy after addition of 10 mmol of oxalate, and at 8 mM Ca, crystallization was first detectable after addition of 2 mmol. Without added oxalate, at 2 mM Ca, the OPN level was 5.8 μg/mL, compared to 3.4 μg/mL at 8 mM. At both calcium concentrations, there was an initial increase in the measured OPN concentration, followed by a gradual decrease that began at around the point at which crystallization was first detected. At 2 mM Ca the OPN concentration reached a plateau of approximately 2.7 μg/mL, while at 8 mM Ca the OPN concentration stabilised at approximately 2.2 μg/mL.

To facilitate comparison of the effects of exogenous and endogenous CaOx crystals on OPN concentration, the data from Figs. 2 (exogenous A), 3 (exogenous B) and 5 (endogenous) are summarised in Table 1. Three points are noteworthy. First, initial OPN concentration was consistently greater at 2 mM Ca than at 8 mM, which is in keeping with the data presented in Fig. 4. Second, irrespective of whether crystals were added to, or were precipitated in the urine, the final OPN concentration never fell to zero—even in the

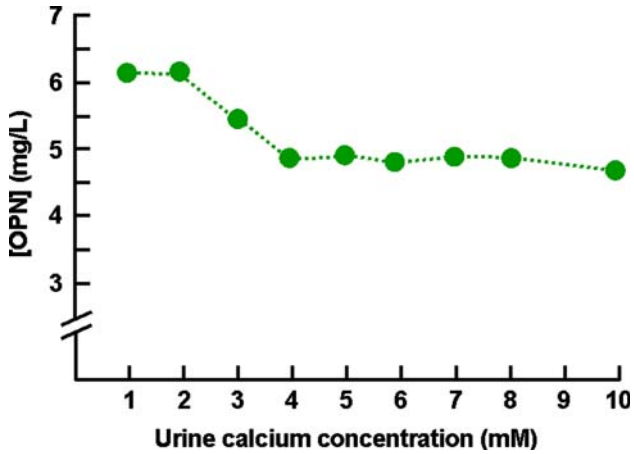

Fig. 4 The effect of urinary calcium concentration on the measured OPN concentration

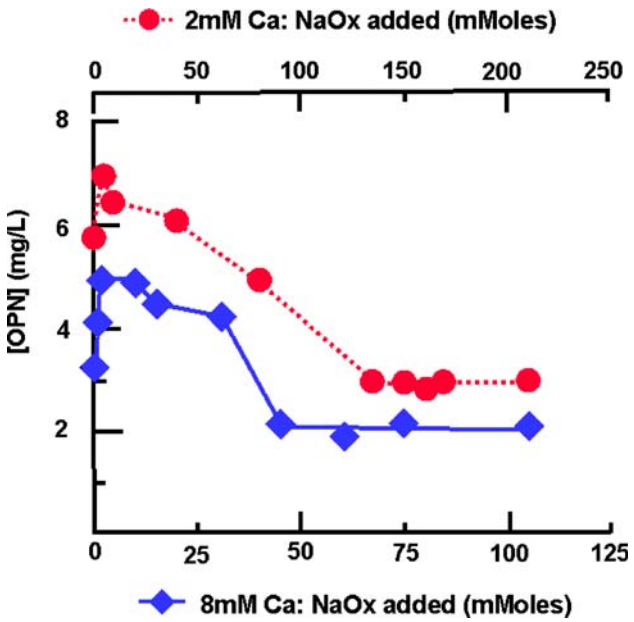

Fig. 5 The effect on urinary OPN concentration of increasing quantities of endogenously generated COM and COD crystals, precipitated by the addition of increasing amounts of oxalate. Data are summarised in the *row* marked *Endogenous* in Table 1

Table 1 Summary of the effects on the measured OPN concentration of inorganic exogenous COM and COD at low (*Exogenous A*) and high (*Exogenous B*) crystal suspension densities, and endogenously precipitated COM and COD crystals (*Endogenous*) at the indicated calcium concentrations

Crystal source	[Calcium] (mM)	Initial [OPN] (μg/mL)	Final [OPN] (μg/mL)	% decrease
COM				
Endogenous	2	5.8	2.7	53.4
Exogenous A	2	3.3	1.9	42.4
Exogenous B	2	5.4	3.1	42.5
COD				
Endogenous	8	2.4	2.2	8.3
Exogenous A	8	2.6	2.0	21.6
Exogenous B	8	4.2	2.3	45.2

presence of excessive amounts of CaOx that would never be formed in vivo. Third, the magnitudes of the deviations in concentrations measured in the presence of exogenous and endogenous crystals were considerable—the lowest value being 8.3%, which is larger than the coefficient of variation of the method.

Discussion

Osteopontin has been implicated in urolithiasis since Hoyer and his colleagues [6] reported that it potently inhibited CaOx crystal growth under inorganic conditions. The protein has since been shown to be present in kidney stones [5], to reduce CaOx crystal growth, nucleation and aggregation [8] in aqueous media at physiological concentrations, and to influence the binding of CaOx crystals to cultured renal cells [11, 12]. OPN message also rises during experimental urolithiasis [15] and its production is stimulated in cultured renal cells exposed to CaOx crystals [26]. Because these observations suggested that stone formation might result from abnormally low urinary concentrations of OPN, the protein has been regarded as a potential biomarker for assessing stone risk.

Three studies demonstrated that stone formers excrete significantly lower amounts of the protein than control subjects [18, 20, 27], while a fourth [16] showed no difference. Four other papers reported measurements of urinary OPN concentrations in healthy controls [8, 9, 17, 19], but values differed widely, with levels in one study [16] being more than tenfold lower than those in another [18]. These differences may reflect the use of different ELISA assays or antibodies (see below), or the fact that in none of these eight studies was the possibility considered that CaOx or calcium phosphate (CaP) crystals may have formed in the urine samples before or after voiding. Hoyer et al. [5] suggested that reduced urinary OPN might result from adsorption of the protein on to larger quantities of CaOx crystals present in the urine of stone formers, while Yasui et al. [20] attributed the reduction to binding of the protein to stones in situ. Our aim was to determine the effect of crystalline CaOx on measured OPN urinary levels.

At 2 mM Ca, addition of inorganic COM crystals at crystal suspension densities up to 10 mg/mL caused the OPN concentration to drop more than 40%, which was almost identical to that occurring in the presence of much larger amounts of COM crystals. Similar effects also occurred when exogenous inorganic COD crystals were added to the same urine specimen after adjustment of the calcium concentration to 8 mM. At suspension densities up to 10 and 250 mg/mL, the reductions were ~23 and 45%, respectively. Falls in OPN concentration of a similar magnitude were also observed when COM and COD crystals were generated endogenously. OPN binds to CaP crystals precipitated from urine [28], to pre-formed inorganic COM crystals in aqueous media [23] and to inorganic COM crystals incubated in human urine, from which it can be removed by copious washing [29]. However, while the protein attaches to and is interred within COD crystals formed in urine [22, 29], it is not an intracrystalline component of urinary COM crystals [22, 29]. Our finding here that endogenous and exogenous COM crystals can significantly reduce the measured OPN concentration was therefore not unexpected, since the crystals will remove the protein from the urine and prevent its quantification by any technique

that requires it to be in solution. Three other important observations also emerged from the results presented in Figs. 2 and 3.

1. OPN concentration fluctuated in the presence of small amounts of CaOx crystals

Addition of small quantities of COM and COD crystals (up to 0.1 mg/mL) caused fluctuations in the OPN concentration. These fluctuations were not caused by experimental variability, since they occurred in all of three different urine specimens tested and because their magnitudes greatly exceeded the coefficient of variation of the ELISA. It is probable that urine contains one or more urinary components that interfere with the attachment of OPN to the ELISA antibody and which also bind to the CaOx crystal surface. Interference could occur directly, or more likely, by chelating calcium ions required to maintain the OPN molecule in a three-dimensional configuration needed for docking to the antibody. Either mechanism would allow more OPN to bind to the antibody and increase its measured concentration, at least until the imbalance was reversed by the addition of sufficient crystals to reduce the measured OPN concentration. Interfering agents are likely to be calcium-binding macromolecules or low molecular mass species such as citrate, pyrophosphate or magnesium, all of which bind calcium ions and adsorb to CaOx crystal surfaces [30].

2. OPN concentration never reached zero even with large amounts of CaOx crystals

Although CaOx crystals caused reductions in the OPN level of up to 45%, the concentration never fell below ~2 mg/L, indicating that a significant proportion of OPN detected by the ELISA antibody does not bind to CaOx crystals. This is perhaps not unexpected, given that alterations in the OPN molecule, particularly the degree of phosphorylation, can affect the molecule's functional properties [31]. The predicted native size of OPN, based on its amino acid sequence, is ~32 kDa [1], but this can alter considerably depending upon RNA splicing and post-translational modifications, including glycosylation, phosphorylation, sulphation and *trans*-glutamination. In human urine OPN migrates on SDS-PAGE as three or four bands with molecular weights ranging from ~40 to 70 kDa [16, 17, 32]. The number of bands is affected by storage conditions [17] and the presence of endogenous serine proteases [16], which can cleave OPN to produce a fragment of Mr < 40 kDa.

The existence of multiple OPN fragments in urine is significant, since individual OPN fragments can exhibit different cell and receptor-binding properties [19], possibly because proteolysis alters the molecule's steric configuration [19]. Consequently, fragments of OPN with dissimilar post-translational modifications, or resulting from cleavage, can exhibit variable specificity and degrees of sensitivity to monoclonal antibodies. Observed differences in ELISA measurements may therefore result from heterogeneity in epitopes recognised by monoclonal antibodies, as well as structural differences in the target molecule [33]. It is not surprising then that different anti-OPN monoclonal antibodies show differential binding to OPN secreted by various cell lines and also to the many urinary forms of the protein [31] or that urinary fragments resulting from cleavage by thrombin are undetectable by some antibodies [16]. Indeed, different commercial ELISA systems produce such widely divergent results for plasma OPN levels that caution must be exercised when comparing results, even using the same ELISA [34]. Thus the antibody provided with the commercial ELISA kit used in our study almost certainly detected OPN isoforms and fragments that were not able to bind to COM crystals.

3. Measurements of OPN changed with alterations in urinary calcium concentration

When testing the effects of COM and COD crystals we noted that concentrations of the protein were consistently higher at 2 mM than at 8 mM. The effect of calcium concentration on the measured OPN value was therefore tested in the absence of crystals. An overall drop of ~25% in OPN concentration occurred over the range 1–10 mM calcium, but particularly between calcium concentrations of 2 and 4 mM, which fall in the range commonly encountered in clinical practice. This indicates that binding of OPN to the ELISA antibody in the ELISA depended on the ambient calcium concentration. This was probably determined principally by Ca-induced changes in the steric configuration of the OPN molecule, whose folding is profoundly affected by the ambient calcium level, particularly in the region between the *N*-glycosylation site and the thrombin cleavage site [1]. It is likely therefore that the three-dimensional conformation adopted by the molecule at higher calcium concentrations conceals an epitope essential for the protein's attachment to the antibody, which is exposed at low calcium concentrations. The epitope through which OPN binds to the antibody probably differs from the site through which it attaches to the CaOx crystal surface, since binding of OPN to both COM and COD is stronger at higher than at lower calcium concentrations [22]. This is in keeping with a report that calcium ions increase the fraction of the OPN molecule adopting a β-sheet configuration [35], which facilitates binding of proteins to crystal surfaces [36]. This would also explain why measured OPN levels in the presence of COD crystals were lower than with COM crystals, whose effects were tested at calcium concentrations of 8 and 2 mM, respectively.

Summary and conclusion

It is clear that OPN is exceedingly difficult to quantify reproducibly and accurately, particularly in urine, in which its measured values are significantly affected by both the ambient calcium concentration and CaOx crystals. Further, OPN concentration will also fall in the presence of CaP crystals, to which it binds [28], and which, like CaOx, commonly occur in human urine. Although this is particularly important for investigating patients with urolithiasis, it also raises the possibility that forms of the protein relevant to other pathologies may be detected and measured, even though they may not be functionally active. Quantification of urinary OPN has already been considered for the investigation of type 2 diabetes [37], IgA nephropathy [38] and ovarian cancer [39]. However, our results demonstrate clearly that urinary OPN is an unsuitable biomarker for any pathology. It is obviously impossible to control the concentration of calcium in urine or to guarantee that crystals of CaOx or CaP do not form in vivo or in frozen or refrigerated urine samples during storage before batch analysis [19, 38, 39]. These insurmountable problems clearly negate the usefulness of measuring urinary OPN for investigating any human pathology, but pose particular difficulties for assessing patients with urolithiasis, in whom hypercalciuria and crystalluria are especially prevalent.

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