Pyridoxamine lowers kidney crystals in experimental hyperoxaluria: A potential therapy for primary hyperoxaluria

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Pyridoxamine lowers kidney crystals in experimental hyperoxaluria: A potential therapy for primary hyperoxaluria.

Background. Primary hyperoxaluria is a rare genetic disorder of glyoxylate metabolism that results in overproduction of oxalate. The disease is characterized by severe calcium oxalate nephrolithiasis and nephrocalcinosis, resulting in end-stage renal disease (ESRD) early in life. Most patients eventually require dialysis and kidney transplantation, usually in combination with the replacement of the liver. Reduction of urinary oxalate levels can efficiently decrease calcium oxalate depositions; yet, no treatment is available that targets oxalate biosynthesis. In previous in vitro studies, we demonstrated that pyridoxamine can trap reactive carbonyl compounds, including intermediates of oxalate biosynthesis.

Methods. The effect of PM on urinary oxalate excretion and kidney crystal formation was determined using the ethylene glycol rat model of hyperoxaluria. Animals were given 0.75% to 0.8% ethylene glycol in drinking water to establish and maintain hyperoxaluria. After 2 weeks, pyridoxamine treatment (180 mg/day/kg body weight) started and continued for an additional 2 weeks. Urinary creatinine, glycolate, oxalate, and calcium were measured along with the microscopic analysis of kidney tissues for the presence of calcium oxalate crystals.

Results. Pyridoxamine treatment resulted in significantly lower (by ∼50%) levels of urinary glycolate and oxalate excretion compared to untreated hyperoxaluric animals. This was accompanied by a significant reduction in calcium oxalate crystal formation in papillary and medullary areas of the kidney.

Conclusion. These results, coupled with favorable toxicity profiles of pyridoxamine in humans, show promise for therapeutic use of pyridoxamine in primary hyperoxaluria and other kidney stone diseases.

Primary hyperoxaluria is a rare autosomal-recessive disorder of glyoxylate metabolism that results in overproduction of oxalate. The most severe form of the disease is primary hyperoxaluria type 1 characterized by the absence or deficiency of the liver peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT), which catalyses the transamination of glyoxylate to glycine using pyridoxal phosphate as coenzyme (Fig. 1). A number of mutations have been identified in AGT, which are associated with inhibition of coenzyme binding, accelerated degradation, aggregation, or peroxisome-mitochondrion mistargeting [1]. Most commonly, the disease is characterized by severe calcium oxalate nephrolithiasis and nephrocalcinosis, resulting in end-stage renal disease (ESRD) early in life [2]. A decline in glomerular filtration leads to the deposition of calcium oxalate in almost every tissue throughout the body. A minority of the patients with a milder course of the disease, which, presumably, carries a mutation associated with residual AGT activity, can be at least partially treated with pharmacologic amounts of pyridoxine [3]. However, most patients eventually require dialysis and kidney transplantation, usually in combination with the replacement of the liver [2]. A second form of the disease, primary hyperoxaluria type 2 is caused by a deficiency of enzyme glyoxylate reductase/hydroxypyruvate reductase [4]. It is almost always milder than primary hyperoxaluria type 1 but still manifests nephrolithiasis and sometimes renal failure [5].

Deposition of calcium oxalate is also observed in idiopathic kidney stone disease where it accounts for ∼70% of stones formed [6]. This multifactorial disease affects about 2% to 3% of general population in the industrialized countries and is often accompanied by hyperoxaluria [7]. Although, the extracorporeal shock wave lithotripsy has significantly simplified kidney stone removal, the recurrence rates remain high, reaching 50% to 70% in 10 years [8, 9].

The control of concentrations of oxalate and/or calcium in urine is an important part of medical treatment.

Key words: primary hyperoxaluria, kidney stone disease, pyridoxamine.

Received for publication March 8, 2004 and in revised form May 28, 2004, and July 14, 2004
Accepted for publication July 27, 2004

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programs designed to inhibit or prevent calcium oxalate stone formation. In this regard, the lowering of urinary oxalate level has a number of advantages. In hyperoxaluria, the contribution of oxalate to calcium oxalate supersaturation is considerably greater than that of calcium. As a result, a relatively small decrease in oxalate concentration could lower the calcium oxalate level below saturation, and thus prevent crystal formation. Dietary control of oxalate can produce only a partial effect since a majority of it is synthesized endogenously, mainly in liver [10, 11] (Fig. 1). In primary hyperoxaluria, contribution of dietary oxalate to urinary oxalate is very small. However, even in absorptive hyperoxaluria, a reduction in endogenous oxalate synthesis would decrease total oxalate excretion. Thus, oxalate biosynthesis is a potential target for the design of drug therapy that decreases urinary oxalate excretion in model animals. To minimize possible chemical degradation of pyridoxamine, a light-sensitive compound, fresh solution was prepared daily and administered in water bottles wrapped in aluminum foil. The length of treatment was determined based on data by Khan [17], suggesting that after about 35 days of experimental hyperoxaluria rats may have some evidence of microscopic nephrolithiasis, but their renal function remains normal. Animals were randomized on day 1 to receive either ethylene glycol (0.75% vol/vol in drinking water) (ethylene glycol group) or water (control group). After day 14 animals within each group

**METHODS**

Reagents

Ethylene glycol, glycolic acid, glycolate oxidase, glyoxylic acid, glycolaldehyde, trinitrobenzenesulfonic acid were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Pyridoxamine was generously provided by Biosstratum, Inc. (Durham, NC, USA)

**Ethylene glycol model of hyperoxaluria and pyridoxamine treatment**

We employed an established rat model of experimental hyperoxaluria, the ethylene glycol model [17, 18]. Although rats do not spontaneously develop stones, hyperoxaluria can be induced in rats, and, as in humans, their oxalate synthesis occurs primarily via glyoxylate pathway [17]. Because ethylene glycol is converted to glycolaldehyde, an intermediate in glyoxylate pathway, its administration results in increased urinary oxalate levels [17, 18] (Fig. 1).

Animal experiments were performed at the AAALAC-accredited animal facilities at Vanderbilt University Medical Center and University of Kansas Medical Center according to institutional guidelines and IACUC-approved experimental protocol. Sprague-Dawley male rats (49 to 52 days old) (Harlan Bioproducts, Inc., Indianapolis, IN, USA) were housed individually and fed standard powdered stock ration (Purina Mill Inc., St. Louis, MO, USA). The number of animals in each experiment is indicated in the figure legends. The temperature was kept at 22 ± 2°C with the lights set at a 12-hour light/darkness cycle. For uniform administration of ethylene glycol and pyridoxamine, water supply to all animals was limited to 45 mL/day. Pyridoxamine was given to animals in drinking water after a 2-week adaptation period to establish elevated constant levels of urinary oxalate excretion in model animals. To minimize possible chemical degradation of pyridoxamine, a light-sensitive compound, fresh solution was prepared daily and administered in water bottles wrapped in aluminum foil. The length of treatment was determined based on data by Khan [17], suggesting that after about 35 days of experimental hyperoxaluria rats may have some evidence of microscopic nephrolithiasis, but their renal function remains normal. Animals were randomized on day 1 to receive either ethylene glycol (0.75% vol/vol in drinking water) (ethylene glycol group) or water (control group). After day 14 animals within each group

**Fig. 1. Glyoxylate pathway of oxalate biosynthesis in liver** [42]. 1 is aldehyde dehydrogenase; 2 is glycolate oxidase; 3 is lactate dehydrogenase; and 4 is glyoxylate reductase/hydroxy pyruvate reductase.
or stored at −70°C until further analysis.

Analysis of urine samples

Urinary oxalate was measured by the oxalate oxidase method. Briefly, the method is based on the conversion of oxalate to hydrogen peroxide and carbon dioxide by oxalate oxidase. The former is then determined enzymatically with horseradish peroxidase by oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone with N,N-dimethylaniline. The resulting colored product is determined spectrophotometrically at 595 nm [19]. Urinary calcium was measured using the Calcium Assay Kit (Diagnostic Chemical Ltd., Charlottetown, Canada). Urinary creatinine was determined using the Creatinine Kit (Sigma-Aldrich Co.) based on the Jaffé colorimetric assay, with modifications to improve specificity. Urinary concentration of glycolic acid was determined by the method described by Petrarulo et al [20]. The method is based on enzymatic conversion of glycolic acid to glyoxylic acid followed by derivatization with phenylhydrazine, separation of reaction products by reverse-phase high performance liquid chromatography (HPLC) (NovaPack-C18 column) (Waters Co., Milford, MA, USA), and spectrophotometric detection at 324 nm.

Statistical analysis

Effects were tested by post-hoc Student-Newman-Keuls comparisons. Relationships between the urine chemistries and crystal scores were assessed with Pearson correlations. All statistical analyses were performed using SPSS version 9.0 (SPSS, Chicago, IL, USA). P ≤ 0.05 was taken to signify statistical significance.

RESULTS

Effect of pyridoxamine treatment on urinary oxalate excretion

In our experiments, the dose of pyridoxamine for animal treatment (180 mg/day/kg body weight) was chosen based on the results of long-term animal studies in a diabetic rat model, where similar or higher pyridoxamine doses were safe and had therapeutic affects [22, 23]. Animals in all experimental groups showed no adverse effects. No significant differences in weight gain were detected for the duration of the experiment (data not shown). Animals with experimental hyperoxaluria (ethylene glycol group) exhibited about a fourfold increase in urinary oxalate excretion, consistent with previously published data [17]. Pyridoxamine treatment of these animals (ethylene glycol + pyridoxamine group) caused dramatic and sustained decrease in urinary oxalate excretion (Fig. 2A). In a separate experiment, pyridoxamine was discontinued after a significant reduction in oxalate excretion was achieved (Fig. 2B). This caused an increase in urinary oxalate excretion to the levels found in untreated hyperoxaluric animals, confirming that the observed
Fig. 2. Effect of pyridoxamine (PM) treatment on urinary oxalate excretion in animals with experimental hyperoxaluria. Sprague-Dawley male rats (49 to 52 days old) were randomized on day 1 to receive either ethylene glycol (0.75% vol/vol in drinking water (450 mg/day/kg body weight) (ethylene glycol group) or no treatment (control group). After day 14, animals within each group [control (○) or ethylene glycol (■)] were pair-matched according to their oxalate level. One member of each pair was then randomly assigned to receive pyridoxamine (3 mg/mL or 180 mg/day/kg body weight) either in drinking water [pyridoxamine (△)] or in 0.75% ethylene glycol [ethylene glycol + pyridoxamine (♦)]. Urine samples were collected under toluene (to inhibit bacteria growth) in 50 mL tubes with hydrochloric acid to minimize spontaneous breakdown of urinary ascorbic acid to oxalate. Urinary oxalate was measured as described in the Methods section. Each symbol represents mean value ± SE (N = 5); at the time points indicated by asterisks, differences between ethylene glycol group and ethylene glycol + pyridoxamine group were statistically significant (P < 0.05). (A) Pyridoxamine was given continuously starting from day 14. (B) In a separate experiment, pyridoxamine treatment started on day 14 and was discontinued on day 28. In this experiment, the elevated levels of oxalate excretion in control group on day 3 are most likely related to animal adaptation. Note that the oxalate levels went down and leveled off before the beginning of pyridoxamine treatment.

Fig. 3. Effect of pyridoxamine (PM) treatment on urinary calcium, creatinine, glycolate, and oxalate. The timing and dosing of treatment were as in Figure 2B, except ethylene glycol (EG) was 0.8% vol/vol in drinking water and pyridoxamine treatment continued until the end of the experiment. Urinary calcium, creatinine, glycolate and oxalate were determined in 24-hour urine samples collected on day 28 of pyridoxamine treatment as described in the Methods section. Each bar represents mean value ± SE; asterisks indicate a significant difference between ethylene glycol group and ethylene glycol + pyridoxamine group (P < 0.05). Control group, N = 3; ethylene glycol group, N = 9; ethylene glycol + pyridoxamine group, N = 8.

effect is dependent on the pyridoxamine treatment (Fig. 2B). In the ethylene glycol group, increase in oxalate excretion was accompanied by the elevated excretion of oxalate precursor glycolate (Fig. 3C and D). Elevated levels of urinary glycolate are often present in primary hyperoxaluria type 1 patients [24]. The pyridoxamine treatment lowered excretion of glycolate along with excretion of oxalate suggesting that pyridoxamine interferes with the flow of intermediates through glyoxylate pathway (Fig. 3C and D). The pyridoxamine treatment did not significantly affect either urinary creatinine or urinary calcium concentrations (Fig. 3A and B).
With the proposed experimental design, we were concerned about possible interference between pyridoxamine and ethylene glycol because they were both administered in drinking water. If significant spontaneous oxidation of ethylene glycol occurs in solution, the resulting carbonyl moieties may react with the pyridoxamine amino group. To address this question, we measured the amount of reactive pyridoxamine amino groups in incubations with ethylene glycol using trinitrobenzenesulfonic acid (TNBS) assay [25]. No change in amino groups was detected after 24 hours at room temperature (data not shown).

**Effect of pyridoxamine treatment on kidney crystal formation**

The microscopic analysis of kidney tissue sections under the polarized light showed a dramatic increase in crystal formation in the ethylene glycol group compared to control group (Fig. 4). There was also an apparent decrease in crystal formation in hyperoxaluric animals upon pyridoxamine treatment (Fig. 4) (ethylene glycol + pyridoxamine group). In the ethylene glycol group, the most crystals were formed in papilla followed by medulla (Table 1). The least crystals were formed in cortex, where the difference was not statistically significant compared to the control group.

There was a direct relationship between urinary oxalate and kidney crystal deposition, most prominently in papilla (Fig. 5), with the correlation coefficient ($r=0.77$) approaching the overall reliability of scoring ($r=0.84$). The data from the three treatment groups formed three clusters with the exception of one outlier data point in the ethylene glycol + pyridoxamine group (Fig. 5, closed squares), indicating that the pyridoxamine treatment was effective in eight out of nine animals (Fig. 5,
Table 1. Scores of kidney crystal deposition in different treatment groups

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Kidney anatomic area</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Papilla</td>
</tr>
<tr>
<td>Control (N = 3)</td>
<td>0.00</td>
</tr>
<tr>
<td>Ethylene glycol (N = 9)</td>
<td>1.292 ± 0.855a</td>
</tr>
<tr>
<td>Ethylene glycol + pyridoxamine (N = 8)</td>
<td>0.391 ± 0.461b</td>
</tr>
</tbody>
</table>

Animals from the experiment described in Figure 3 were euthanized on day 28 of pyridoxamine treatment and the kidneys removed. The crystals were analyzed in hematoxylin–eosin–stained paraffin kidney sections under polarized light as described in the Methods section. Data represent the mean ± SD.

aDifference between control group and ethylene glycol group is significant (P < 0.02).
bDifference between ethylene glycol group and ethylene glycol + pyridoxamine group is significant (P < 0.02).

Fig. 5. Correlation between urinary oxalate excretion and papillary crystal deposition. Symbols represent values from individual animals in the same experiment as in Figures 3 and 4. EG is ethylene glycol; EG + PM is ethylene glycol + pyridoxamine. The outlier was apparently due to lack of responsiveness to pyridoxamine in this single animal, since its treatment or behavior (including water consumption or urine output) were similar to the rest of the group. Because the urinary oxalate reading of the outlier data point was outside the margin of 2 SD of the mean, this data point was removed from statistical analysis. The statistical analysis of the data demonstrated that the pyridoxamine treatment of hyperoxaluric animals (ethylene glycol + pyridoxamine group) resulted in a significant decrease in crystal formation compared to the ethylene glycol group in both papilla and medulla of the kidney (Table 1).

Trapping of glyoxylate by pyridoxamine

We have previously demonstrated that pyridoxamine can react with low-molecular-weight carbonyl compounds, including oxalate precursor glycolaldehyde [14]. These results suggested that pyridoxamine may lower urinary oxalate excretion by trapping carbonyl intermediates of oxalate biosynthesis. In the present study, we demonstrated that another oxalate precursor, glyoxylate, can potentially be a target for pyridoxamine. In the in vitro incubations, pyridoxamine trapped glyoxylate via reaction involving the carbonyl group (Fig. 6).

Fig. 6. Trapping of glyoxylate by pyridoxamine. Glyoxylate (10 mmol/L) was incubated either alone (■) or with 15 mmol/L pyridoxamine (●). The carbonyl groups of glyoxylate were determined using 2,4-dinitrophenylhydrazine (DNPH) as described in the Methods section.

DISCUSSION

A number of pharmacological approaches have been tested in an attempt to develop a therapy for hyperoxaluria that targets oxalate biosynthesis. One approach is to inhibit the enzymes involved in glyoxylate pathway (Fig. 1). Several inhibitors of either aldehyde dehydrogenase or glycolate oxidase have been tested in animals and in humans with mixed results [26, 27]. Newer inhibitors of aldehyde dehydrogenase such as fomepizole were recently proposed for treatment of ethylene glycol poisoning [28] and may potentially be used for kidney stone therapy. However, this intravenous drug is not appropriate for a long-term use, and may produce alcohol intolerance. Another drawback of this approach may be the accumulation of glycolaldehyde, a potential cytotoxic agent.

An alternative approach to reduction of urinary oxalate concentration is based on the use of pyridoxine, a precursor of pyridoxal-5'-phosphate (PLP), to enhance the activity of AGT (Fig. 1). The mechanism of this effect is not entirely clear, but may relate to the ability of PLP to modulate the expression of AGT or due to enhancement of residual AGT activity by PLP [29, 30]. Because the primary mode of pyridoxine action is the modulation of AGT expression and/or activity, individual
significant benefits only to a minority of patients, those with

differences in enzyme status render a majority of primary

cybridoxamine is a B6 vitamin, it could also enhance AGT

oxalate precursors can form in vitro [14] (Fig. 6). Since

glyoxylate. The adducts between pyridoxamine and these

intermediates of oxalate biosynthesis glycolaldehyde and

pyridoxamine may act through trapping of carbonyl

mation in rats with experimental hyperoxaluria. The

lower urinary oxalate excretion and kidney crystal for-

mation is about sixfold less efficient as a PLP precursor com-

pared to pyridoxine [38]. Thus, we hypothesize that in the
course of treatment, circulating pyridoxamine is taken up

by the liver where it traps carbonyl intermediates of oxalate
biosynthesis. This trapping by pyridoxamine or by its phos-
phorylated form, pyridoxamine-5′-phosphate (PMP), occurs via adduct formation through the nucle-

ophilic amino group [14]. Pyridoxine, a vitamin B6 pre-
cursor used in the treatment of vitamin B6–dependent

primary hyperoxaluria, cannot trap these intermediates
because it does not possess an amino group. The conver-
sion of pyridoxine to pyridoxamine is a minor pathway of

vitamin B6 metabolism [38]; when rat liver was perfused

with radioactively labeled pyridoxine, less than 1% of

it was converted to pyridoxamine [39]. Conversion of pyri-

doxine to PMP is also limited because of the tight regula-
tion of pyridoxine(pyridoxamine)-5′-phosphate oxidase

by product inhibition [40, 41].

The efficacy of pyridoxamine treatment demonstrated by our work, coupled with a favorable pyridoxamine

safety profile shown in Phase II clinical trials in diabetes

mellitus and controls [abstract; Williams ME, et al, J Am

Soc Nephrol 14:7A, 2003], suggests that pyridoxamine

has potential as a therapeutic agent for primary hyper-

oxaluria or recurrent calcium oxalate stone formation. If

proven effective, pyridoxamine could play an important

role in kidney stone preventive therapies.

ACKNOWLEDGEMENTS

We thank Dr. Agnes Fogo and Ms. Ellen Donnert, Department of
Pathology, Vanderbilt University Medical Center, for invaluable advice
and help with kidney tissue preparations. This work was supported by
the National Institute of Health Research Grants: DK-60251 to P.A.V.
and to BioStratum, Inc. and, in part, DK-18381 and DK-65138 to B.G.H.
This work was presented in part at the Renal Week 2002 and the Renal
Week 2003 sponsored by the American Society of Nephrology.

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