BBA - Molecular Basis of Disease 1867 (2021) 165981

Contents lists available at ScienceDirect

BBA - Molecular Basis of Disease

journal homepage: www.elsevier.com/locate/bbadis

Effect of alanine supplementation on oxalate synthesis

Kyle D. Wood[a], Brian L. Freeman[b], Mary E. Killian[c], Win Shun Lai[d], Dean Assimos[a], John Knight[a], Sonia Fargue[a,*]

[a] University of Alabama at Birmingham, Department of Urology, Birmingham, AL, United States of America
[b] Center for Infectious Disease and Vaccine Research, La Jolla Institute for Immunology, La Jolla, CA, United States of America
[c] University of Tennessee Health Science Center, Department of Urology, Memphis, TN, United States of America
[d] University of Texas Medical Branch, Division of Urology, Galveston, TX, United States of America

ARTICLE INFO

Keywords:
Primary hyperoxaluria
Oxalate
Alanine
Alanine:glyoxylate aminotransferase
Kidney stones

ABSTRACT

The Primary Hyperoxalurias (PH) are rare disorders of metabolism leading to excessive endogenous synthesis of oxalate and recurring calcium oxalate kidney stones. Alanine glyoxylate aminotransferase (AGT), deficient in PH type 1, is a key enzyme in limiting glyoxylate oxidation to oxalate. The affinity of AGT for its co-substrate, alanine, is low suggesting that its metabolic activity could be sub-optimal in vivo. To test this hypothesis, we examined the effect of L-alanine supplementation on oxalate synthesis in cell culture and in mouse models of Primary Hyperoxaluria Type 1 (*Agxt* KO), Type 2 (*Grhpr* KO) and in wild-type mice. Our results demonstrated that increasing L-alanine in cells decreased synthesis of oxalate and increased viability of cells expressing GO and AGT when incubated with glycolate. In both wild type and *Grhpr* KO male and female mice, supplementation with 10% dietary L-alanine significantly decreased urinary oxalate excretion ~30% compared to baseline levels. This study demonstrates that increasing the availability of L-alanine can increase the metabolic efficiency of AGT and reduce oxalate synthesis.

1. Introduction

Primary hyperoxaluria constitutes a group of rare autosomal recessive disorders caused by a defect in glyoxylate metabolism that results in excessive oxalate synthesis and urinary oxalate excretion [1,2]. These disorders may result in the formation of kidney stones, nephrocalcinosis, oxalate deposition in other tissues (oxalosis), and afflicted individuals are at risk for CKD and ESRD [2,3]. Three types of PH have been identified and all three are characterized by an excessive endogenous synthesis of oxalate [2]. Alanine: glyoxylate aminotransferase (AGT), a liver-specific, peroxisomal enzyme, catalyzes the transamination of glyoxylate and alanine to glycine and pyruvate and its deficiency causes PH type 1, the most common and severe form of PH [4]. PH type 2 is caused by a deficiency in glyoxylate reductase (GRHPR), a mitochondrial and cytosolic enzyme highly expressed in liver and kidney [5,6]. In both cases, oxalate synthesis results from the oxidation of glyoxylate by lactate dehydrogenase (LDH). The most recently described form of PH, type 3, is due to a deficiency in the mitochondrial enzyme, 4-hydroxy-2-oxo-glutarate aldolase (HOGA), which also results in the increased production of oxalate [7].

The goals of current treatments in PH are to reduce the urinary excretion of oxalate and the supersaturation of calcium oxalate in urine and plasma. One approach that targets a reduction in oxalate synthesis is the use of therapeutic doses of pyridoxine, the cofactor of AGT, for which a subset of PH1 patients are responsive [2,8]. Liver transplantation in PH patients who reach end-stage renal failure can be considered a form of enzyme replacement therapy, although this comes with significant risks [2,3]. Recently, new therapeutic strategies have been developed and are currently in clinical trials, which aim at decreasing oxalate synthesis by targeting enzymes involved in the glyoxylate and oxalate metabolic pathways, but may not fully normalize excretion [9–14] (glycolate oxidase (GO) for PH1, which targets a reduction in glyoxylate from its substrate, glycolate; and LDHA, which targets the last oxidation step, glyoxylate to oxalate, for PH). Although these approaches result in profound reductions in urinary oxalate excretion in PH1 patients, the effectiveness of these approaches in other forms of PH are not clear. Reducing urinary oxalate synthesis may benefit other cohorts including idiopathic kidney stone formers, and those with chronic kidney disease where it has been shown that oxalate excretion is positively correlated with disease progression [15,16].

Abbreviations: PH1, primary hyperoxaluria Type 1; AGT, alanine:glyoxylate aminotransferase; GO, glycolate oxidase; GR, glyoxylate reductase; LDH, lactate dehydrogenase; CHO, Chinese hamster ovary; ICMS, ion chromatography coupled with mass spectrometry

* Corresponding author.
E-mail address: sfargue@uab.edu (S. Fargue).

https://doi.org/10.1016/j.bbadis.2020.165981
Received 21 July 2020; Received in revised form 10 September 2020; Accepted 23 September 2020
Available online 28 September 2020

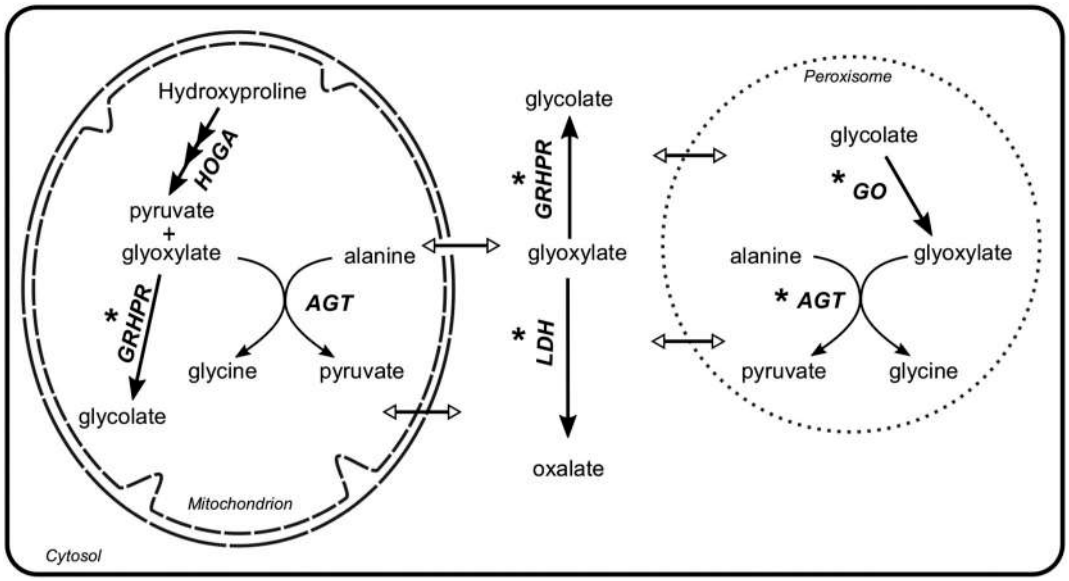

Fig. 1. Metabolism of glyoxylate to oxalate in mouse hepatocytes. AGT: alanine:glyoxylate aminotransferase, GO: glycolate oxidase, GRHPR: glyoxylate reductase, LDH: lactate dehydrogenase. The conversion of hydroxyproline to glyoxylate involves several enzymatic steps: (hydroxyproline dehydrogenase, 1P5C dehydrogenase, aspartate aminotransferase, 4-hydroxy-2-oxo-glutarate aldolase (HOGA)). In mice AGT is expressed both in peroxisomes and in mitochondria, unlike humans in which AGT and GO are only localized in peroxisomes. AGT is the enzyme deficient in primary hyperoxaluria type 1 and GRHPR in type 2. PH3 results from HOGA deficiency. (*) denotes the enzymes expressed (LDH, GRHPR) and overexpressed (AGT, GO) in the Chinese Hamster Ovary cells in their respective subcellular compartments.

A number of precursor molecules are involved in the endogenous synthesis of oxalate including hydroxyproline, glycine, ascorbic acid, glyoxal, glycolate, and glyoxylate [1,17–19]. Studies in PH patients have provided critical insights into this metabolism, but the nature and rarity of these diseases are such that experiments in model systems are still needed to better clarify these pathways (Fig. 1). Rodent models of PH have been established including mouse models of PH1, PH2 and PH3 and most recently a rat model for PH1 [20–22]. An in-vitro model system has been created in which GO and AGT are expressed in Chinese hamster ovary (CHO) cells, allowing the assessment of potential inhibitors of endogenous oxalate synthesis [8,23–27].

L-alanine is the co-substrate of AGT and is necessary for glyoxylate detoxification by AGT. The administration of L-alanine to rats receiving ethylene glycol, an agent that induces hyperoxaluria, attenuated urinary oxalate excretion, ameliorated renal injury from calcium oxalate crystal deposition, and reduced formation of bladder stones. [28,29]. Using isolated peroxisomes from guinea-pigs, Poore and collaborators showed that alanine concentration positively correlated with AGT activity. [30]. Further, the administration of supplemental L-alanine in humans has not been associated with adverse effects. It was shown to be beneficial in the management of those afflicted with glycogen storage disease type II, and in diabetics with nocturnal hypoglycemia [31–33].

In this study, we investigated the impact of L-alanine on the generation of oxalate in a CHO cell culture model in which both GO and AGT where overexpressed, and murine models of PH1 and PH2. We found that L-alanine supplementation resulted in a dose dependent reduction in the generation of oxalate in the CHO cells and urinary oxalate excretion in the PH2 model (*Grhpr* KO) and wild-type mice.

2. Methods

2.1. Cell culture experiments

Chinese Hamster Ovary (CHO) cell lines, either WT or stably transfected with GO and different AGT variants (Table 1) were used as previously reported [8,23,27]. Along with an untransfected CHO wild type cell line, the different cell lines consisted of: CHO cells stably expressing GO; CHO stably expressing GO and an AGT variant. The following AGT variants were used in this study: AGT-MA: normal AGT encoded by the major allele; AGT-mi: normal AGT encoded by the minor allele (P11L; I340M); and a PH1 specific AGT mutant: AGT-170 (P11L, G170R, I340M). For maintenance CHO cells were grown at 37 °C in 5% CO_2 in Ham's F12 with glutamine, 10% FBS, Penicillin/streptomycin and the antibiotic selection appropriate for the plasmid expressed (Zeocin for GO, Geneticin for AGT variants) [23]. The cell-based indirect glycolate toxicity assay has been reported elsewhere [27]. In brief, cells were plated on 96 well plates (6.5×10^3 cells/well) with different concentrations of L-alanine and subsequently incubated with varying doses of glycolate (0, 0.25, 0.5, 0.75 mM) with or without L-alanine, for 24 h. The viability and amount of oxalate synthesized by cells and released in the media after exposure to glycolate depends on the expression of GO and the metabolic efficiency of AGT. Cell viability was assessed by a CCK-8 assay (Dojindo Molecular Technologies, Japan) according to manufacturer's instructions, and normalized to control cells in media without glycolate. For analysis of the oxalate produced, cells were plated on 24 well plates (5×10^4 cells/well) with varying amounts of L-alanine in the media. The following day, media was exchanged with fresh media containing 0.75 mM glycolate with or without L-alanine and incubated for 24 h at 37 °C. The cell culture media was collected, filtered with 10 mM HCl-washed 10 K MWCO filters (Corning, Corning, NY) and the ultrafiltrate was acidified to 60 mM HCl before freezing for future oxalate analysis. Total cell protein content was measured by a Bradford assay, after cell lysis in Hepes 25 mM/Triton X100 0.1% with a Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Grand Island, US). For the effect of L-alanine supplementation on oxalate synthesis and glycolate induced cell toxicity, L-alanine (Sigma-Aldrich, St-Louis, MO) was added to the media (0 - 20 mM) at the time of plating and kept throughout the assay. The same concentration and modality was applied for the effect of glycine and glutamine. The intracellular content of CHO cells after L-alanine loading was assessed in 6-well plates after 2 and 4 h, by extracting intra-cellular content with 0.5 M perchloric acid (PCA). Total cell protein was recovered following PCA extraction by adding 0.2 N NaOH before measurement by a Bradford assay. Stock solutions of

Table 1
Chinese Hamster Ovary cell lines used. The cell line name (abbreviated name), plasmid and protein expressed are given. CHO: Chinese Hamster Ovary, GO: glycolate oxidase, AGT: alanine glyoxylate aminotransferase.

Cell line	Abbreviation	Plasmids expressed and protein changes		
CHO WT	(WT)			
CHO GO	(GO)	GO		
CHO GO AGT-MA	(MA)	GO	Normal AGT, "major allele"	
CHO GO AGT-mi	(mi)	GO	Normal AGT, "minor allele"	p.(Pro11Leu; Ile340Met)
CHO GO AGT-170	(170)	GO	Pathologic AGT variant	p.[(Pro11Leu; Gly170Arg; Ile340Met)]

glycolate were prepared in PBS and the pH adjusted to 7.4. Cell culture reagents were obtained from Invitrogen (Thermo Fisher Scientific, Grand Island, US). Reagent grade chemicals were obtained from Sigma-Aldrich (St Louis, MO).

2.2. Animal experiments

Wild-type, *Grhpr* KO (a model of PH2) or *Agxt* KO (a model of PH1), male and female, adult mice (mean age 17 ± 1 weeks) on a C57BL/6J background were used (6–8 per group). The phenotype of these mice has been previously described [20,21]. Mice were fed ad libitum a custom ultra-low-oxalate diet for the duration of the study to which was added 5 mg calcium chloride/g dry weight (TD, 130032, Harlan Laboratories, Madison, WI). This diet has been previously used in our studies with mouse models of PH and negates the contribution of dietary oxalate to the urinary oxalate pool, thus allowing an assessment of endogenous oxalate synthesis [34]. L-alanine (L-alanine powder, Bulk Supplements, cat # LMOOW3S) at 5 or 10% w/w was added to this purified diet for experiments. For 24-hr urine collections, animals were singly housed in Tecniplast metabolic cages (Buguggiate, Italy) for a total of 7 days. Four baseline 24-hour urine collections were collected on mineral oil following a 3-day acclimation period. At the completion of baseline urine collections, animals were singly housed in regular cages and fed ad libitum the L-alanine diet (either 5 or 10%) for 7 days. Animals were placed back in metabolic cages for urine collections after one week of L-alanine feeding. A chronic L-alanine feeding study was performed to assess the impact on blood glucose. Adult Wt and *Grhpr* KO mice were maintained on the low-oxalate purified diet supplemented with 10% L-alanine for 10–12 weeks and then euthanized after a 6 hour fast. A control group on regular chow diet was also included. Glycemia was measured using a glucometer (Accu-Check Guide, Roche). All animal studies were approved by the University of Alabama at Birmingham Institutional Animal Use and Care Committee and complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

2.3. Analytical methods

Oxalate was measured in acidified urine and cell culture media by ion chromatography coupled with mass spectrometry (ICMS) as described elsewhere using an AS22 anion exchange column and ammonium carbonate mobile phase [18]. $^{12}C_2$-oxalate was measured by a standard $^{13}C_2$-oxalate isotope dilution method. Alanine was measured in perchloric acid extracts by High Performance Liquid Chromatography (HPLC) coupled with UV detection using the Waters AccQ-Tag method (Waters, Milford, MA), with amino butyric acid as internal standard. Urinary creatinine was measured on an EasyRA chemical analyzer. Protein concentration was measured by a Coomassie Plus (Bradford) assay (Thermo Scientific).

2.4. Statistical analysis

Results are expressed as mean ± SEM unless otherwise mentioned. Statistical analyses were performed with GraphPad Prism. All tests were two-sided and a p value ≤ 0.05 was considered statistically significant. Details on tests utilized (1 or 2 way ANOVA, Student *t*-test) are given in the relevant figure legends.

3. Results

3.1. Cell viability is increased by L-alanine addition in the induced glycolate toxicity assay

To assess the metabolic activity of AGT, an indirect glycolate toxicity assay was used, as previously described [23,27]. When glycolate oxidase is expressed in CHO cells and incubated with glycolate, the latter is oxidized to glyoxylate and subsequently to oxalate, leading to cell toxicity and death. The expression of functional AGT diverts glyoxylate to glycine and partially rescues cell viability, in a manner that is linked to the degree of AGT metabolic effectiveness and the amount of glycolate present [8,27].

In order to test whether increased intracellular L-alanine would improve AGT metabolic efficiency in detoxifying glyoxylate, experiments were performed using CHO cell lines expressing glycolate oxidase (GO) with or without AGT variants, either normal variants of AGT (AGT encoded by the so-called "major allele", AGT-MA, and by the "minor allele", AGT-mi) or a common pathological variant of AGT with residual, but reduced, catalytic activity (AGT-170, characterized by mitochondrial mistargeting) [1,8]. Pre-incubations of cells with L-alanine were done at seeding, the day before experiments were started. The relationship between media alanine concentration and intracellular alanine concentration is depicted in Fig. 2.

Following pre-incubation with set L-alanine concentrations, cells were exposed to varying concentrations of glycolate (0–0.75 mM) and fresh set L-alanine concentrations for 24 h and cell viability assessed (Fig. 3). Glycolate did not decrease viability in CHO WT cells (95–104% viability, $p = 0.6$). The viability of CHO GO cells decreased to 28%, 8%

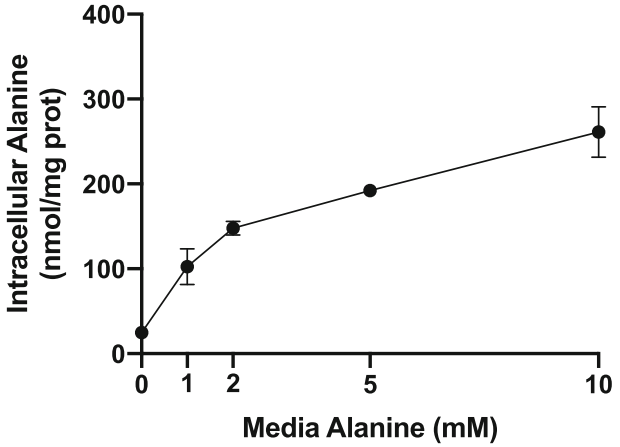

Fig. 2. Alanine intracellular content in cells incubated with L-alanine. CHO cells were incubated with L-alanine 0-10 mM and intracellular alanine content was measured by HPLC and normalized to total cell protein. (results mean ± SEM, n = 3). $p < 0.01$ with one-way ANOVA analysis. L-alanine is present in the Ham's F12 cell culture media at low concentration (0.1 mM).

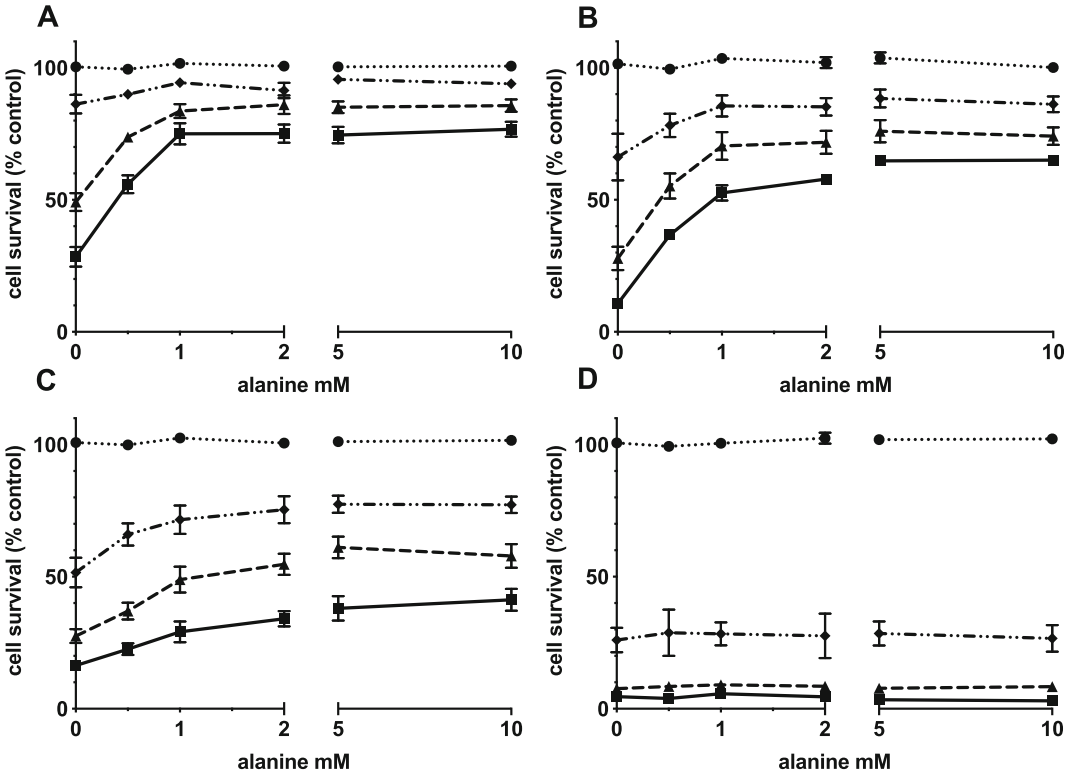

Fig. 3. Effect of L-alanine supplementation on CHO cell viability in a glycolate toxicity assay. CHO cells were preincubated for 24 h with different concentrations of L-alanine before being challenged with glycolate (0–0.75 mM) and L-alanine for 24 h. Cells expressing GO metabolize glycolate to glyoxylate and oxalate, resulting in toxicity that is dependent on the metabolic activity of AGT. A: CHO GO AGT-MA; B: CHO GO AGT-mi; C: CHO GO AGT-170; D: CHO GO. Glycolate 0 mM (dotted line), 0.25 mM (semi dashed line), 0.5 mM (dashed line), 0.75 mM (full line). There is no change in viability at any glycolate and L-alanine level in CHO wt cells (not shown, $p = 0.6$). Results mean ± SEM ($n = 5–9$). Effect of L-alanine and glycolate assessed by 2-way ANOVA, $p < 0.001$ for both factors in CHO GO AGT-MA, AGT-mi, AGT-170 and only for the effect of glycolate for CHO GO.

and 4% after 24 hour incubation with glycolate at 0.25, 0.5 and 0.75 mM, respectively, with no effect of L-alanine supplementation (Fig. 3 D). In the three cell lines co-expressing GO and any of the three AGT variants, increasing glycolate resulted in decreasing cell viability at any given level of alanine (Fig. 3A-C) ($p < 0.001$). There was a significant protective effect of L-alanine supplementation on CHO GO AGT cell viability ($p < 0.001$), with a stronger protective effect when cells co-expressed AGT-MA (Fig. 3A), followed by CHO GO AGT-mi (Fig. 3B), and lastly AGT-170 (Fig. 3C). The effect of L-alanine leveled off above 1 to 2 mM supplementation.

3.2. The effect of L-alanine is specific to AGT

In order to test whether the protective effect of L-alanine supplementation was specific to alanine, and therefore AGT, or was an unspecific protective effect of amino acid supplementation, we assessed the effect of 5 mM glycine or glutamine versus that of alanine (Table 2). Only L-alanine improved cell viability in cells expressing AGT variants ($p < 0.001$).

3.3. Oxalate synthesis is decreased by L-alanine supplementation in CHO cells challenged with glycolate

The effect of L-alanine supplementation on AGT activity was assessed by measuring the amount of oxalate synthesized, and released in the media, by cells exposed to glycolate. Based on the indirect glycolate toxicity experiments reported above, a combination of glycolate 0.75 mM and L-alanine 2 mM supplementation was chosen to optimize detection of changes. Supplementation with L-alanine significantly reduced the amount of oxalate produced in cells expressing all three AGT variants (to 14% of baseline for normal AGT variants and 41% for AGT-170, Fig. 4). In one experiment the oxalate intracellular content of cells was also measured, which showed similar results from that of extracellular media. There was a 40 to 60% decrease of intracellular content with L-alanine supplementation in cells expressing AGT exposed to 0.75 mM glycolate (AGT-MA 1.1 without L-alanine vs 0.5 with 2 mM L-alanine, AGT-170 1.9 vs 1.1 nmol oxalate/mg total cell protein), whereas there was no change in the absence of AGT (WT 0.4 vs 0.4; GO 4.0 vs 3.7 nmol oxalate/mg total cell protein).

Table 2
Effect of L-alanine compared with other amino acids. Cell viability in the indirect glycolate toxicity assay (0.5 mM) was assessed after pre-incubation with 5 mM of either L-alanine, glycine or glutamine. Results % cell survival mean ± SEM ($n = 3–5$) analyzed with one-way ANOVA and Dunnett's multiple comparison test for the effect of amino-acids on viability (**/*** = $p < 0.01/0.001$). The last columns displays which amino acid significantly changed survival.

	Control	Glycine	Glutamine	Alanine		
CHO WT	99 ± 2	104 ± 2	104 ± 1	100 ± 2		
CHO GO	8 ± 1	16 ± 2	17 ± 2	8 ± 1	**	Glycine, glutamine
CHO GO AGT-MA	49 ± 3	50 ± 5	63 ± 2	85 ± 2	***	Alanine
CHO GO AGT-mi	28 ± 4	27 ± 4	37 ± 5	76 ± 4	***	Alanine
CHO GO AGT-170	28 ± 3	32 ± 1	31 ± 1	61 ± 4	***	Alanine

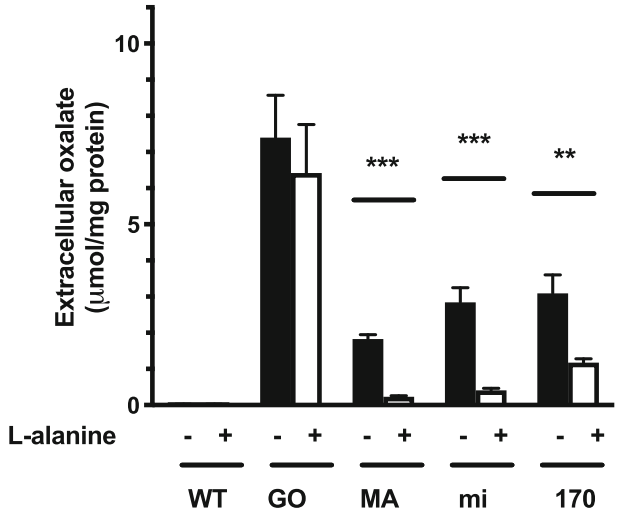

Fig. 4. Effect of L-alanine on oxalate concentration in cell culture media following glycolate metabolism.
CHO cells (wt, GO, AGT-MA [MA], AGT-mi [mi], AGT-170 [170]) were pre-incubated with L-alanine ("−": 0; "+": 2 mM) for 24 h before addition of 0.75 mM glycolate to the media. The content of oxalate in the cell culture media (extracellular oxalate) was normalized to total cell protein. Results expressed as mean ± SEM (n = 2–4). **/***p < 0.01/0.001, with *t*-test.

3.4. Alanine decreases urinary oxalate in mice fed supplementary L-alanine

A low oxalate purified diet was supplemented with L-alanine to test the effect of alanine on urinary oxalate excretion of wild type, *Grhpr* KO (mouse model of PH2), and *Agxt* KO mice (mouse model of PH1). *Grhpr* KO mice express AGT and could therefore benefit from improved metabolic activity of AGT. In contrast, dietary L-alanine supplementation would not be expected to decrease urinary oxalate excretion of *Agxt* KO mice. Mice were fed the purified ultra-low oxalate diet for the duration of the experiments and 24-hr urinary oxalate was collected in metabolic cages without L-alanine during a baseline assessment and after 10 days of equilibration with 5 and 10% L-alanine dietary supplementation.

There was a mean 28% decrease in urinary oxalate excretion compared to baseline in Wt and *Grhpr* KO males (119 ± 15 to 84 ± 2 and 350 ± 83 to 245 ± 40 µg/mg creatinine for Wt and *Grhpr* KO, p = 0.005 and 0.008, respectively), but no significant change in *Agxt* KO male animals (Fig. 5A). A lower dose of L-alanine was administered to confirm the selection of 10% L-alanine supplementation as an effective dose. A reduction in urinary excretion of oxalate was also seen with male mice fed with 5% L-alanine (Fig. 5B), with a more moderate effect (104 ± 11 to 88 ± 13 and 280 ± 46 to 252 ± 51 µg/mg creatinine in Wt and *Grhpr* KO, p = 0.0108 for wt and p = 0.0067 for *Gr* ko).

In order to test whether there was an influence of sex on the response of oxalate synthesis with L-alanine, female mice were treated with the dose of L-alanine that was found to be effective. A similar reduction in oxalate excretion was seen in Wt and *Grhpr* KO females treated with 10% L-alanine (120 ± 5 to 83 ± 8, ↓ 30.5%, and 242 ± 13 to 172 ± 30 µg/mg creatinine, ↓28.9% in Wt and *Grhpr* KO, respectively, p < 0.001, Fig. 5C). An increase in hepatic alanine content was not detected with supplementation, which we attribute to a rapid metabolism of L-alanine given tissue was collected after a 6 hour fast.

To assess the impact of dietary L-alanine supplementation on blood glucose levels, mice (4 male Wt, 8 male *Grhpr* KO, 6 female Wt, 7 female *Grhpr* KO) were fed 10% L-alanine, for 10–12 weeks. The control group were maintained on a regular chow diet (males, n = 8 *Grhpr* KO, n = 10 *Agxt* KO). There was no difference in change of body weight with L-alanine supplementation compared to the rodent chow fed control group (data not shown). There was no hyperglycemia or glycosuria with L-alanine chronic feeding (blood glucose 157 ± 40, 162 ± 44, 152 ± 37 and 186 ± 45 mg/dl, for Wt and *Grhpr* KO, males and females, respectively). The blood glucose in all alanine treated mice was not significantly different from that of a control group (blood glucose 166 ± 43 in L-alanine fed group versus 181 ± 18 mg/dl in control group).

4. Discussion

The results of our in-vitro and in-vivo studies support the concept that increasing availability of L-alanine to AGT results in increased glyoxylate transamination and decreased oxalate synthesis. The specificity to AGT is suggested by the absence of protection in the cell experiment with other amino acids and the lack of effect in *Agxt* KO mice. In experiments in CHO cells expressing GO and AGT the magnitude of the effect of L-alanine was inversely correlated with the basal catalytic

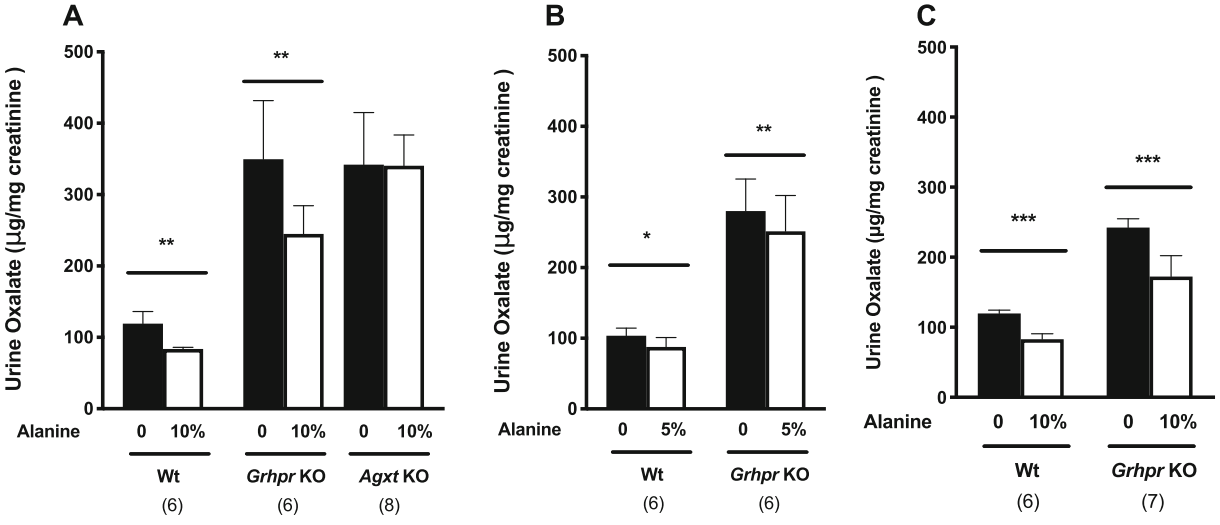

Fig. 5. Effect of L-alanine on urinary oxalate excretion in mice. Male (A, B) and female (C) mice from Wt, Grhpr (Gr) KO and Agxt KO strains were fed a controlled low oxalate diet with 0 (baseline) followed by 10% (A, C) or 5% (B) additional L-alanine (treatment) for 10 days. Urinary oxalate excretions were measured on 24 h urine collections in metabolic cages and expressed as µg oxalate/mg creatinine. Results expressed as mean ± SD. (*/**/***): data analyzed with paired *t*-test, p < 0.05/0.01/0.001 compared to pre-treatment baseline.

activity of the AGT variant expressed by the cells: highest in cells expressing the normal AGT-MA, lowest in cells expressing AGT-170 [1,35].

The dynamics and function of AGT provide an explanation for our results. This enzyme catalyzes the transamination of alanine to pyruvate and glyoxylate to glycine [1,35]. Studies on purified AGT variants have defined the structure and biochemical characteristics of normal AGT, both the prevalent variant encoded by the "major allele" and the normal variant encoded by the "minor allele", as well as pathological variants found in PH1 patients [35,36]. Whereas the Km of AGT for glyoxylate is low (0.2–0.4 mM), the Km for alanine is high, ranging between 28 and 41 mM for the most common variants [35]. The normal range of concentration of alanine in human liver is not well known. One study reported an alanine concentration of 5.97 ± 3.11 mM in the liver of patients with cholecystectomy, others 1.8–3.7 mM in muscle, with a normal range of plasma concentrations between 200 and 579 μM in adults [37–39]. In rodents, hepatic alanine concentrations is reported to be 0.38 mM in guinea-pig liver and 0.8–3.4 μmol/g dry weight in rats [40,41]. Together, these data suggest that the low tissue alanine concentrations do not provide the catalytic support for optimal AGT function.

The results of our in-vitro study demonstrate that a plateau effect of alanine on AGT function exists, which probably reflects the concentration at which L-alanine was no longer rate limiting in the conditions tested. This level depended on the AGT variant studied, with increased L-alanine requirements for those variants with lower catalytic activity. The AGT-170 variant, which is the mutation most commonly found in PH1 patients (25–30%) retains some catalytic activity in vitro, but not exceeding 50% of the normal range in human liver [1,2]. This mutant was less responsive to alanine than the other variants assessed. The reduction in oxalate synthesis we observed in the cell model appeared greater than that seen in mouse models. Considering the short half-life of alanine, reported as 30 min in human plasma [42], it is possible that the rapid metabolism of alanine in the liver prevented alanine reaching the levels obtained in the cell experiments. It is also possible that the difference might be due to the low baseline alanine concentration in cell culture media.

A dose dependent reduction in urinary oxalate excretion was demonstrated in wild type and PH2 *Grhpr* KO mice but not in the *Agxt* KO cohort. These results are expected as AGT activity in the former two cohorts is normal. The more profound reduction in the *Grhpr* KO mice as compared to the wild type is expected due to the larger glyoxylate pool and urinary oxalate excretion. The male *Grhpr* KO mice had higher baseline oxalate levels than the female group which is consistent with hormonal effects on oxalate production in rodents that has been previously reported [21]. However, the relative reduction in oxalate excretion was similar in both sexes.

In PH1, some patients respond clinically to pharmacological doses of pyridoxine, the cofactor of AGT, with decreased urinary excretion of oxalate and slower kidney function decline. The threshold of this pyridoxine-responsiveness is defined as a minimum 30% decrease in urinary oxalate [2,15]. The risk of kidney stones formation has also been shown to increase with the level of urinary oxalate excretion in subjects without PH, with relative risk increasing to approximately 1.6–2.5 fold when urinary oxalate excretion increased from 20 mg/day to 30 and 40 mg/day [43]. In such a context, the 28 to 30% decrease in urinary oxalate that we observed in mice seems promising if translatable in human subjects with elevated baseline urinary oxalate excretions.

The administration of L-alanine supplements to humans with certain diseases has been demonstrated to be safe and efficacious. Bodamer and collaborators have reported an improvement of energy expenditures in patients with glycogen storage diseased type II and myopathy [31]. In diabetic patients, high doses of supplemental L-alanine has been demonstrated to prevent nocturnal hypoglycemia [33]. While a theoretic potential for higher glucose levels in normal populations exists, we found no changes in glucose levels in our mouse studies. We did not assess glucose tolerance or insulin resistance, which future studies would need to address. Our study suggests that assessing the impact of supplemental L-alanine on urinary oxalate excretion as an adjuvant therapy in idiopathic calcium oxalate kidney stone formers as well as those afflicted with types 2 and 3 PH is warranted. If effective it could provide a safe and relatively low-cost method of attenuating urinary oxalate excretion.

In conclusion, we demonstrated that L-alanine supplementation reduces oxalate synthesis in in-vitro cellular model of oxalate synthesis and reduces urinary oxalate excretion in wild type and *Grhpr* KO mice. The clinical utility of this approach for lowering urinary oxalate excretion in relevant cohorts needs to be determined.

Transparency document

Some of this work was presented in part as an abstract in Journal of Urology, AUA and a poster at the ASN Kidney Week annual meeting.

CRediT authorship contribution statement

Kyle D. Wood: Formal analysis, Writing - review & editing. **Brian L. Freeman:** Investigation. **Mary E. Killian:** Investigation. **Win Shun Lai:** Investigation. **Dean Assimos:** Conceptualization, Writing - review & editing. **John Knight:** Resources, Writing - review & editing. **Sonia Fargue:** Conceptualization, Investigation, Validation, Formal analysis, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no conflict of interest with the content of the article.

KW is consultant for Synlogic Therapeutics, Oxidien Therapeutics, Novome Therapeutics, and BioBridge Therapeutics. JK is a consultant for Synlogic, Novome and Chinook Therapeutics. The other authors have no disclosures.

Acknowledgments

This work was supported by the National Institutes of Health (DK114332, DK087967, DK115833, NIH NIDDK grants P20DK119788, P30DK079337) and by a grant from the Urology Care Foundation (to SF).

The authors thank Dr. CJ. Danpure for the gift of the transformed CHO cells, which were developed thanks to the Oxalosis and Hyperoxaluria Foundation (grant to CJ Danpure). The technical assistance of Songlian Zhou, Michelle Bui, Tamara Keenum was greatly appreciated.

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