Primary hyperoxaluria type 1: clinical, genetic and biochemical studies

van Woerden, C.S.

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CHAPTER 08. Oxalate production in \textit{Agxt} \textsuperscript{-/-} mice Hepatocytes during incubation with potential sources of glyoxylate metabolism suggests additional routes for glyoxylate detoxification

\textit{Christiaan S van Woerden, Jos P Ruiter, Lia EM van Lint, Jaap W Groothoff, Frits A Wijburg, Marinus Duran, Ronald JA Wanders, Eduardo C Salido}

\textbf{ABSTRACT} \textbf{Introduction.} Excess oxalate production results in renal sequelae in patients with primary hyperoxaluria type 1 (PH1), due to deficiency of the liver-specific peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT). Oxalate is produced from glyoxylate. To date, the absolute contribution of potential precursors of glyoxylate has been investigated in various models, among which HepG2 cells and wild type animal hepatocytes but not in an \textit{in vitro} model for PH1. Recently, a mouse model, deficient in peroxisomal AGT activity, has been generated. It is biochemically comparable to human PH1, exhibiting elevated urine oxalate excretion. We used this model to investigate the \textit{in vitro} oxalate production upon incubation with different potential precursors.

\textbf{Methods.} Metabolic incubation studies were performed in isolated hepatocytes of \textit{Agxt} \textsuperscript{-/-} and wild type mice. Experiments were performed in alanine depleted and alanine enriched media (10mM). Seven different potential precursors were used, including glyoxylate, glycolate, glycine, L-serine, hydroxyproline, xylitol and fructose. Production of oxalate and glycolate was measured by gas chromatography-mass spectrometry (GC-MS) and glycine production by high-pressure liquid chromatography (HPLC).

\textbf{Results.} Incubation of wild type hepatocytes with glyoxylate resulted in a production of 4\% oxalate and 8\% glycolate. Incubation of \textit{Agxt} \textsuperscript{-/-} hepatocytes with glyoxylate resulted in a production of 2\% oxalate and 4 \% glycolate of the initial total amount of the precursor. None of the other precursors resulted in the formation of oxalate or glycolate. Production rate was decreased in wild type cells upon addition of 10mM alanine but not in \textit{Agxt} \textsuperscript{-/-} cells. In alanine-enriched media, glycine synthesis was lower in \textit{Agxt} \textsuperscript{-/-} cells compared to wild type cells, but not zero. Depletion of alanine resulted in complete inhibition of glycine synthesis in both cell types.

\textbf{Conclusion.} Except for glyoxylate and glycolate, none of the proposed glyoxylate precursors contributes to \textit{in vitro} oxalate production. Incubation studies in \textit{Agxt} \textsuperscript{-/-} hepatocytes show modest fluxes through the glyoxylate pathway. In view of sustained glycine synthesis upon alanine addition and moderate oxalate excretion, our studies support a potential role for a second, potentially mitochondrial, AGT mediated glyoxylate metabolism, at least in mice.
INTRODUCTION

Oxalate overproduction is the biochemical hallmark of primary hyperoxalurias. Oxalic acid complexes with calcium ions, resulting in the deposition of insoluble calcium oxalate crystals, notably in the kidneys. Primary hyperoxaluria type 1 (PH1) results from the deficiency of alanine:glyoxylate aminotransferase, AGT (FIGURE 08.1). The liver specific peroxisomal enzyme alanine:glyoxylate aminotransferase (AGT) normally converts toxic glyoxylate into glycine. Mutations in the AGXT gene cause AGT deficiency in PH1 after which glyoxylate is diverted in two ways: (1) the peroxisomal enzyme glycolate oxidase (GO, Km 0.2 mM. as studied by Schwam et al (1) oxidizes glyoxylate into oxalate as does the cytosolic enzyme lactate dehydrogenase (LDH) and (2) glyoxylate reductase (GR) reduces glyoxylate into glycolate. For a genetically specified subgroup of patients with primary hyperoxaluria type 1, a reduction of urinary oxalate by suppletion of pharmacological doses of pyridoxine, which is a co-factor of AGT, results in a decreased urinary excretion of oxalate and a clinically relevant longer preservation of kidney function (2). Unfortunately, pyridoxine responsiveness is only observed in 35-40% of the patients. Correction of the enzymatic defect is otherwise only achieved by a liver transplantation, subject to compatible organ supply, with its major drawback of life long requirement of immunosuppressive therapy that causes transplantation related morbidity. Therefore, there is an urgent need for better therapy.

One of the potential therapeutic approaches might be by dietary modifications or pharmacological interventions that divert or alter metabolic pathways. Ideally, these interventions will decrease availability of substrates that result in the formation of disease-causing metabolites. For the primary hyperoxalurias however, the pathways leading to oxalate synthesis are only partly understood. A clear understanding of these metabolic pathways is a prerequisite for the design of alternative therapies. Studies providing insight into the biochemistry of oxalate metabolism are limited especially because oxalate metabolism only occurs in hepatocytes, and hepatocytes cell lines of patients with primary hyperoxaluria are not available for research. Recently, an Agxt−/− mouse, deficient for the peroxisomal AGT enzyme, has been developed. We initiated the current research project to investigate the contribution of potential precursors to oxalate production by means of incubation studies in isolated hepatocytes of Agxt−/− mice.

FIGURE 08.1 Fluxes through the glycolate pathway with its relevant enzymes.
MATERIALS AND METHODS

Hepatocytes  Agxt /- mice were generated as described previously (3). Genetic analysis of the AGXT gene was performed prior to the experiments to ascertain the desired genotype was obtained.

Mice were anaesthetized using pentobarbital, and subjected to laparotomy. A 24 Gauge canula was inserted in the portal vein, and the liver was first perfused with Hepes buffer at pH 7.4 and afterwards with collagenase (50mg/100ml) in Hepes buffer. Liver cells were harvested in Williams medium and washed for three consecutive times. After each wash step, cells were spun for five minutes at 300 rpm and the pellet was resuspended in 50 ml Williams medium. After the last washing step, cell numbers were calculated manually and using a Coulter Counter (Beckman Coulter, Inc. 2007). Viable cells were resuspended in Krebs solution and enough buffer was added to generate a concentration of one million cells per milliliter prior to the start of the incubation studies.

Experiments were performed in Eppendorf tubes (2 ml) at 0ºC until addition of the precursors. Incubation of the samples was performed for one hour at 37ºC, and gently shaken during incubation. The incubations were stopped by adding 20 μl of HCl 30%. Samples were stored at -20ºC until analysis.

For every precursor, an extra sample was taken separately and acidified immediately upon its preparation, to measure background levels of oxalate, glycolate and glycine. In the same way, two separate samples, one containing Agxt /- and one containing wild type hepatocytes were acidified before incubation to measure background activity. Seven potential precursors were chosen and prepared in five different concentrations (0.25, 0.5, 1.0, 5.0 and 10.0 mM) for incubation, including glyoxylate, glycolate, glycine, L-serine, hydroxyproline, xylitol and fructose. Experiments were performed in alanine depleted and alanine enriched (10mM) media.
**Product analysis**

**Oxalate, glycolate**

Concentrations of oxalate and glycolate were measured by gas chromatography-mass spectrometry (GC-MS) in one run.

**Chemicals**

[1,2-13C]oxalate and D2-glycolate were from Cambridge Isotope Laboratories. MTBSTFA and ethyl acetate were from Sigma Aldrich.

**Preparation and GC-MS analysis**

All samples were thawed and centrifuged at 4°C for five minutes at 14000 rpm. With slight modifications of the procedure, solvent extraction with ethyl acetate and trimethylsilylation was performed as described previously (4). For derivatization, 50 μl sample was used. Of a saturated solution of NaCl 50 μl was added to enhance extraction. Internal standards for oxalate and glycolate determination were combined in 100 μl distilled water and added to the medium. Extraction was performed two times using 2.5 ml ethyl acetate. The pooled ethyl acetate fractions were evaporated under N2 at room temperature. Derivatization was performed using 80 μl MTBSTFA at 80°C for 30 minutes. After cooling, 1 μl of sample was injected into the gas chromatograph (Hewlett Packard). Runtime was 30 minutes.

**Gas chromatography-Mass spectrometry**

A MSD 5973N mass spectrometer, coupled to a GC6890N gas chromatograph (Hewlett Packard) was used according to the manufacturer's protocol. A CPsil-19CB column, (25mtr*0.25mm*0.20μm) was used.

The gas chromatographic conditions were as follows: carrier gas helium, injection temperature: 300 °C, split ratio 1:10 column flow rate: 1.8 ml/min. Oven program: 80 °C for 1.5 min, followed by 10°C/min until 200 °C is reached. Then 30 °C/min until 250°C for five minutes. Quantization was performed by SIM. The ions at m/z 261 and 263 were used for oxalate and its internal standard, and the ions at m/z 247 and m/z 249 were used for glycolate and its internal standard. A correction of 10% was made for the quantitation of oxalate and glycolate, because endogenous and labelled compounds both contribute significantly to the other ions.

For validation, the same sample was measured 10 consecutive times, yielding a standard deviation of 0.05 μM for oxalate and 0.14 μM for glycolate. Variation coefficients were 1.02% for oxalate and 1.32% for glycolate. Linearity was observed at least until a spiked concentration of 100 μM for oxalate and glycolate (R2 = 1). Recovery was 113% for oxalate and 91% for glycolate.
Glycine

Glycine was analyzed by derivatization using a HPLC procedure, as described previously. Samples were thawed and centrifuged at 4°C for five minutes at 14000 rpm. 25 μl of supernatants was diluted with 140 μl of distilled water and neutralized with 10 μl 2M KOH/ 0.6 M MOPS. This sample was used for HPLC analysis.

RESULTS

We were able to detect low amounts of oxalate, with a detection limit of 0.5 μM by using GC-MS. Background levels of oxalate and glycolate in wild type and Agxt−/− cells were similar. For glycine measurements, the established HPLC procedure was used and proved highly reliable under the current conditions, with excellent calibration curves.

Glyoxylate incubation

Up to 8% of added glyoxylate was converted into glycolate and 4% into oxalate for wild type isolated hepatocytes, whereas the production of glycolate and oxalate was reduced in Agxt−/− knockout hepatocytes with 3% (glycolate) and 2% (oxalate) produced from glyoxylate. Addition of alanine resulted in a decreased production of oxalate and glycolate in both wild-type and AGT deficient hepatocytes, though reduction was more pronounced in the wild type hepatocytes (FIGURE 08.2 and 08.3). Glycine was formed in wild type hepatocytes and to a lesser extent in Agxt−/− hepatocytes. Addition of alanine strongly enhanced glycine production in both cell types (FIGURE 08.4).

FIGURE 08.2 Oxalate production during incubation of wild type and Agxt−/− hepatocytes with glyoxylate.

<table>
<thead>
<tr>
<th>Glyoxylate μM</th>
<th>Oxalate μM</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>300</td>
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</tbody>
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Highest glycolate concentration for wild type cells is 398 μM and for Agxt−/− cells is 227 μM.
**FIGURE 08.3** Glycolate production during incubation of wild type and Agxt⁻/⁻ hepatocytes with glyoxylate.

Highest glycolate concentration for wild type cells is 791 μM and for Agxt⁻/⁻ cells is 292 μM. Open bars: wild type hepatocytes Dashed bars: Agxt⁻/⁻ hepatocytes

**FIGURE 08.4** Glycine production during incubation of wild type and Agxt⁻/⁻ hepatocytes with glyoxylate, in alanine depleted and alanine enriched media.

Open bars: wild type hepatocytes Dashed bars: Agxt⁻/⁻ hepatocytes

**FIGURE 08.5** Glycine production during incubation with different precursors. The first bar in each category represents experiments with 10 mM alanine, and the second bar during alanine depletion.
A low amount of oxalate was produced during glycolate and 4-hydroxyproline incubation. Only for wild type cells, the amount was lower after addition of alanine, but not for Agxt -/- cells. Glycolate production was also raised upon loading with 4-hydroxyproline. Comparable amounts of glycine were formed in glycolate incubated cells, and alanine increased its synthesis to the same extent (concentrations 25 μM in alanine depleted and 45 μM in alanine enriched experiments). For 4-hydroxyproline loading, elevated concentrations of glycine were observed in wild type cells, that did only slightly respond to alanine addition (61 and 70 μM, respectively). A low concentration of glycine was observed in Agxt -/- cells without any influence of alanine (15 μM).

For the other compounds, slightly raised glycolate production was observed, but not at all for oxalate. For glycine and fructose incubation, addition of alanine resulted in lower glycolate concentrations in wild type cells but not in Agxt -/- cells. For L-serine, glycolate production was higher in wild type cells that were stimulated with alanine. For xylitol incubations, addition of alanine resulted in higher glycolate concentrations in both types of cells. Lowest concentrations of glycine were seen in wild type cells upon incubation with xylitol and fructose. Agxt -/- cells did not show glycine production with any of these two precursors. Serine incubation resulted in the second highest glycine concentration (112 μM for wild type and 79 μM for Agxt -/- cells) with only small change after alanine addition (123 μM for wild type and 55 μM for Agxt -/- cells).

**DISCUSSION**

We investigated the production of oxalate, glycolate and glycine in hepatocytes of wild type mice and compared them with their production in Agxt -/- mice, a recently developed genetically modified animal model for primary hyperoxaluria type 1, during incubation with various naturally occurring potential precursors of glyoxylate, oxalate and glycolate. Because oxalate and glycolate are both end products of glyoxylate metabolism, which is defective in primary hyperoxaluria type 1, the measurement of oxalate and glycolate serves as an indicator of glyoxylate metabolism. The quantification of glycine was performed to analyze any persistent normal flux through the glyoxylate pathway.

Concerning the detection of oxalate and glycolate, we were able to measure small concentrations by means of gas chromatography-mass spectrometry in the same run. Stable isotopes of oxalate and glycolate were used as internal standards, which makes the procedure very specific and accurate. Linearity was excellent (R2 = 0.99), Recovery for oxalate was 133%, variation coefficient 5.2%, and recovery for glycolate was 90%, with a variation coefficient of 3.2%. Limit of detection was 0.5 μM.
for oxalate and 1.5 μM for glycolate. In patients with primary hyperoxaluria type 1, plasma concentrations of oxalate and glycolate are usually in the same range (5). With the current method we were able to discriminate between low variations in oxalate and glycolate production rates.

In Agxt⁻/⁻ mice and in human primary hyperoxaluria type 1, levels of urinary oxalate excretion are three to four times the upper limit of the reference range (3). Surprisingly, only a modest production of oxalate was observed in hepatocytes of Agxt⁻/⁻ mice after exposure with any of the precursors. Even wild type hepatocytes produced more oxalate and especially more glycolate, if depleted for the co-substrate alanine. This difference between wild type cells and Agxt⁻/⁻ hepatocytes might be due to differences in viability of the cells. However, in previous experiments with wild type mice hepatocytes, we assessed viability of the cells prior to our experiments (results not shown). Since our procedures always resulted in good viability of the cells, we did not test for viability in the knockout mice hepatocytes, in order not to lose any cells. Before proceeding to the metabolic studies, we analyzed the cells under the microscope for manual counting. During this procedure, there was no evidence of potential loss of viability of cells. Moreover, addition of alanine during the incubation, which is the co-substrate of the enzyme alanine:glyoxylate aminotransferase (AGT), resulted in a change in production of metabolites, suggesting that viability was not decreased.

Upon addition of alanine a pronounced decrease of oxalate and glycolate production was seen in wild type cells, but to a much less extent in Agxt⁻/⁻ cells. On the other hand, depletion of alanine resulted in almost absent glycine production. The strong influence of alanine in these experiments points towards the existence of residual AGT activity in the livers of Agxt⁻/⁻ mice. As demonstrated before, no AGT activity is present in peroxisomal fractions of these mice (3). However, additional AGT2 activity in mitochondria of the mice may be found, like in HepG2 cells (6). Therefore, the absence of excess oxalate and glycolate production in Agxt⁻/⁻ hepatocytes, and the sustained glycine synthesis upon addition of its co-substrate alanine, would suggest that mitochondrial AGT2 contributes significantly and is capable of detoxifying precursors of glyoxylate metabolism, at least in the experimental system used in this study. These precursors may not have reached the peroxisomal matrix, therefore not leading to oxalate production. The biochemical phenotype of primary hyperoxaluria with excess production of oxalate, results from exclusively peroxisomal oxalate generation, and any precursor of glycolate, glyoxylate or oxalate must be imported or synthesized in the peroxisomal compartment to contribute to this biochemical phenotype.
Whether the addition of alanine also results in decreased oxalate production *in vivo* may be addressed by adding alanine to the diet of *Agxt*−/− mice. We did not yet perform such experiments.

Glycolate production was much higher in wild type cells. Since glyoxylate is reduced to glycolate by the enzyme glycolate reductase, the production of glycolate in wild type cells was not expected to be higher than in *Agxt*−/− hepatocytes. Alternatively, the cytosolic enzyme glutamate:glyoxylate aminotransferase (GGT) may also catalyze the conversion of glyoxylate into glycine. However, a clear response upon alanine was found, suggesting that this enzyme did not contribute to the production of glycine or decreased oxalate formation.

If mitochondria significantly contribute to glyoxylate detoxification, this may also explain the high amount of glycine as was found in *Agxt*−/− hepatocytes, almost equaling glycine production in wild type hepatocytes. Wild type cells metabolized approximately 140% of glycine found in *Agxt*−/− hepatocytes. This likely reflects the synthesis of glycine in both mitochondria as well as peroxisomes, whereas *Agxt*−/− hepatocytes only metabolize glyoxylate via mitochondria.

The clinical importance of this pathway has been discussed by Baker et al, who proposed an important role for mitochondrial glyoxylate metabolism regarding the breakdown of collagen during the turnover of various tissues (6). The results of our experiments are in line with this hypothesis.

During incubation with L-serine, knockout hepatocytes showed a glycine production which was 50% of glycine synthesis in wild type cells. This may be the result of absence of peroxisomal AGT activity. Apart from glyoxylate detoxification, the AGT enzyme has a dual function as it also demonstrates serine:pyruvate transaminase (SPT) activity. As an SPT, the enzyme converts serine into hydroxypyruvate in peroxisomes and mitochondria (7). This could lead to glycine production (FIGURE 08.6), as proposed by Holmes and Assimos (8). In the absence of peroxisomal AGT/ SPT activity, serine can only be metabolized via mitochondrial AGT/SPT mediated pathways. Therefore, wild type cells produce more glycine, as was demonstrated in our experiments. On the other hand, there is also a higher production of oxalate and glycolate in cells that were exposed to L-serine. This implies that the flux through this AGT/SPT mediated pathway is high enough to generate glycine, glycolate and oxalate. The low oxalate and glycolate concentrations in *Agxt*−/− hepatocytes illustrates a generally lower flux of serine metabolism, since the peroxisomal route is not functional.

For glycine as a precursor, the absence of oxalate production in wild type and knockout hepatocytes is in line with the reported lack of D/amino acid oxidase expression in the mouse liver (9).
With the current experiments, we observed only small fluxes in glyoxylate metabolism in cells if they were abundantly fed with various precursors of oxalate synthesis. Whether these low fluxes are sufficient to generate the high urinary excretion of oxalate in Agxt-/- mice or in patients with primary hyperoxaluria type 1, remains to be established. An estimation of the internal flux in the glyoxylate metabolism should therefore be performed by other studies, such as isotope dilution techniques in patients.

The limitations of this study concern the difficulty to establish the point of entry of the applied metabolic precursors of glyoxylate. Since intact hepatocytes have been used and not separated organelles, the observed product synthesis can only reflect what occurs in the entire hepatocytic metabolome. Furthermore, extrapolation of results from animal studies to the human analogue should be undertaken with caution, especially since additional enzymes such as glycolate oxidase, glyoxylate reductase/hydroxypyruvate reductase can modulate oxalate metabolism in a critical way. Regardless of this drawback, this is the first time a knockout model has been used in the metabolic studies of primary hyperoxaluria. Previously, incubations have been carried out in different models, including in humans in vivo, rats in vivo, perfused rat livers, isolated hepatocytes, subcellular fractions of liver tissue (as discussed in Chapter 01). Other studies have drawn conclusions from extrapolations from the properties of isolated enzymes. Therefore, the type of research presented here may be of value since it creates new ways to study oxalate metabolism, more closely related to human primary hyperoxaluria than before. Further experiments may investigate if a change in the permeability of peroxisomes results in different rates of oxalate and glycolate production, in order to assess if transport of substrates across the peroxisomal membrane limits glyoxylate synthesis. Alternatively, isolated peroxisomes may be used for this purpose. For research aiming to identify potential sources of glyoxylate, intermediate metabolites, notably alfa-keto-gamma-hydroxyglutarate (produced in the catabolism of hydroxyproline), and indolepyruvate (produced in the catabolism of tryptophan) may be studied (10). Furthermore, an additional knockout mouse model in which both mitochondrial and peroxisomal AGT activities are absent might be constructed. This model should demonstrate strongly decreased glycine synthesis and increased oxalate and glycolate production, if mitochondria or other compartments have indeed an important role in the detoxification of glyoxylate. Eventually, elucidation of oxalate metabolism, its involved precursors and enzymes, may facilitate development of rational therapies, such as substrate deprivation, metabolic route blockade via enzyme modification, or pathway diversion through enzyme induction (with GR/HPR, or LDH Figure 08.1). This might be an important step in studying potential therapeutic goals for PH1.
The glyoxylate pathway, as proposed by Holmes and Assimos (8).

REFERENCES


