Glyoxylate is a substrate of the sulfate-oxalate exchanger, sat-1, and increases its expression in HepG2 cells

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Background & Aims: Hyperoxaluria is a major problem causing nephrolithiasis. Little is known about the regulation of oxalate transport from the liver, the main organ for oxalate synthesis, into the circulation. Since the sulfate anion transporter-1, sat-1, is present in the sinusoidal membrane of hepatocytes and translocates oxalate, its impact on increased oxalate synthesis was studied.

Methods: Sat-1 expressing oocytes were used for cis-inhibition, trans-stimulation, and efflux experiments with labelled sulfate and oxalate to demonstrate the interactions of oxalate, glyoxylate, and glycine with sat-1. HepG2 cells were incubated with oxalate and its precursors (glycine, hydroxyproline, glyoxylate, and glycinate). Changes in endogenous sat-1 mRNA-expression were examined using real-time PCR. After incubation of HepG2 cells in glyoxylate, sat-1 protein-expression was analysed by Western blotting, and oxalate uptake into HepG2 cells was measured. RT-PCR was used to screen for mRNA of other transporters.

Results: While oxalate and glyoxylate inhibited sulfate uptake, glycolate did not. Sulfate and oxalate uptake were trans-stimulated by glyoxylate but not by glycolate. Glyoxylate enhanced sulfate efflux. Glyoxylate was the only oxalate precursor stimulating sat-1 mRNA-expression. After incubation of HepG2 cells in glyoxylate, both sat-1 protein-expression and sulfate uptake into the cells increased. mRNA-expression of other transporters in HepG2 cells was not affected by glyoxylate treatment.

Conclusions: The oxalate precursor glyoxylate was identified as a substrate of sat-1. Upregulated expression of sat-1 mRNA and of a functional sat-1 protein indicates that glyoxylate may be responsible for the elevated oxalate release from hepatocytes observed in hyperoxaluria.

Keywords: Sat-1; Glyoxylate; Oxalate; Hyperoxaluria; HepG2 cells.

Introduction

Nephrolithiasis is a major public health problem of largely unknown cause in industrialized nations [1–3]. The formation of stones in the urinary tract is based on a wide range of underlying disorders. Eighty percent of the kidney stones consist of calcium salts and usually occur as calcium oxalate [4]. Hyperoxaluria is caused by an excess of dietary oxalate, bowel pathology, or increased production of endogenous oxalate in the liver, e.g. in primary hyperoxaluria [5].

In the gut, oxalate is absorbed from food [6] by members of the SLC 26 gene family [7]. Aliments rich in oxalate are cacao, spinach, or rhubarb [8]. Endogenous oxalate production mainly occurs in hepatocytes [9,10] where oxalate’s main precursor glyoxylate [11–13] can be converted to oxalate by peroxisomal glycolate oxidase (GO) and by cytoplasmatic lactate dehydrogenase (LDH, Fig. 1). Under normal conditions, glyoxylate is metabolized to glycolate and glycine by alanine:glyoxylate aminotransferase (AGT) and glyoxylate reductase (GR), respectively. Both enzymes, AGT and GR, are necessary to avoid oxalate formation from glyoxy-

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In the following study, the impact of oxalate and its precursors on sat-1 was analysed. Sat-1 transport modes were examined using *X. laevis* oocytes. After identifying sat-1 in HepG2 cells, an established model for oxalate metabolism [14], we studied the influence of different compounds from oxalate metabolism [11–13] to sat-1 mRNA-expression, and glyoxylate’s impact on sat-1 protein-expression and transport function.

**Methods**

**Chemicals**

Oxalate and glycolate were purchased as free acids, glyoxylate as a sodium salt from Sigma–Aldrich (Taufkirchen, Germany), sulfate (sodium salt) and glycine from Merck (Darmstadt, Germany), and hydroxyproline from Applichem (Darmstadt, Germany). All chemicals used were of analytical grade.

**Transport experiments with oocytes**

*In vitro* transcription of sat-1 mRNA, solutions for oocyte experiments (ORi), oocyte preparation and storage were described previously [18]. Uptake of $^{35}$S sulfate (1200 Ci/mmol; Hartmann, Braunschweig, Germany) or $^{14}$C oxalate (100 mCi/mmol; Biotrend, Köln) in oocytes was assayed at room temperature.

All uptake and efflux studies were performed in calcium-free ORi. For *cis*-inhibition experiments, oxalate, glyoxylate, or glycolate of various concentrations were added to the uptake medium containing 20 μM sulfate and 0.01 μM $^{35}$S sulfate. To perform trans-stimulation experiments, 23 nl ORi, ORi containing 50 mM glyoxylate or 50 mM glycolate were injected into oocytes which were subsequently incubated in calcium-free ORi containing 50 μM sulfate and 0.01 μM $^{35}$S sulfate or 20 μM $^{14}$C oxalate. After incubation in the respective solutions for 5 min, radioactivity was aspirated and the oocytes were washed twice in ice-cold ORi to stop substrate uptake.

To carry out sulfate efflux in the presence and absence of glyoxylate, 23 nl of an 8 μM $^{35}$S sulfate solution was injected into the oocytes. Oocytes were incubated for 30 min in 150 μl calcium-free ORi in the presence or absence of 50 μM glyoxylate. Oocytes were washed with 150 μl incubation medium and the $^{35}$S efflux was assayed by measuring the $^{35}$S content of each medium united with the washing medium. The $^{35}$S efflux was expressed in % of the sum of $^{35}$S efflux and $^{35}$S content of the oocyte was set to 100%.

For every experimental condition, sat-1 expressing oocytes and mocks were treated in a similar manner. Independent of whether *cis*-inhibition, *trans*-stimulation, or efflux experiments were performed, oocytes were dissolved by gently shaking for 2 h in 250 μl 1 N NaOH, neutralized with 250 μl 1 N HCl, and their $^{35}$S or $^{36}$C contents were determined by liquid scintillation counting (Packard Instrument Co., Meriden, CT, USA).

**Cell culture**

HepG2 cells were grown in Dulbecco’s Modified Eagle Medium high glucose (25 mM D-glucose) supplemented with 2 mM glutamine, 0.625 mM pyruvate, 10% fetal bovine serum, and 1% sodium pyruvate.

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Fig. 1. Oxalate metabolism in hepatocytes (A) and chemical structures of oxalate, glyoxylate, and glycolate (B). Abbreviations: AGT1 and 2, alanine:glyoxylate aminotransferase 1 and 2; DAO, d-amino acid oxidase; LDH, lactate dehydrogenase; GO, glycolate oxidase; GR, glyoxylate reductase; HKGA, 4-hydroxy-2-ketoglutarate lyase; sat-1, sulfate anion transporter-1 (modified from [14]).
RNA isolation, RT-PCR, and real-time PCR

RNA was isolated using TRIZOL®-Reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer’s instructions. After denaturation at 70 °C for 10 min, RNA was reverse transcribed using oligoDT primer and MuLV reverse transcriptase (AppliChem, Darmstadt, Germany). cDNA was used for PCR and real-time PCR.

For PCR analysis, reaction mixtures (50 µl) contained 2 µl (different transporters) or 5 µl (GAPDH) of the RT product, 10 nmol dNTPs, 20 pmol of the gene-specific primers, and 5 units of Taq polymerase in 1× buffer. Amplification of GAPDH was carried out using an initial denaturation of 3 min at 94 °C, and 22 cycles of the following protocol: 94 °C for 40 s, 58 °C for 50 s, and 72 °C for 1 min followed by a final elongation of 5 min at 72 °C. For PCR analysis of different transporters, the same protocol was used with changing annealing temperatures (Table 1).

PCR products were separated on agarose gels and visualized with ethidium bromide. Plasmids containing transporter or GAPDH DNA were used as positive controls. Confluent cells were incubated with 1 mM glyoxylate for 4 days, others served as controls. Confluent cells were washed twice with prewarmed PBS and sulfate-free mammalian Ringer (130 mM NaCl, 4 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, 1 mM TMAPo4, 1 mM NaH₂PO₄, 18 mM glucose). The two groups of cells were incubated in mammalian Ringer containing an addition of 50 µM sulfate and 0.01 µM [³⁵S]sulfate. After an incubation of 5 min, cells were washed three times with ice-cold PBS and dissolved in 500 µl of 1 N NaOH by gently shaking. After 2 h the solution was neutralized by addition of 500 µl 1 N HCl. A total of 50 µl of the cell solution was used for a Bradford protein assay, the ³⁵S content of the remaining solutions was determined by liquid scintillation counting.

Membrane preparation

Cells were washed twice with ice-cold phosphate buffered saline (PBS; pH 7.4) and incubated in membrane buffer (150 mM NaCl, 50 mM Tris–HCl, 5 mM ethylenediaminetetraacetic acid, 100 µl/ml phenylmethylsulfonylfluoride, 4 µl/ml aprotonine, 4 µl/ml leupeptine) for 5 min for dissolution. The cell solution was homogenized and centrifuged for 10 min at 13,000 g. The supernatant was ultracentrifuged for 30 min at 50,000 rpm (OTD65B, rotor TFF6513, Thermo Fisher Scientific, Waltham, MA, USA); the pellet contained the membranes and the supernatant contained the cytosol. For sat-1 protein determination, proteins were denatured in 5 mM Tris–HCl (pH 6.8) containing 1% sodium dodecyl sulfate (SDS), 10% glycerol, and 1% β-mercaptoethanol. Proteins were electrophoresed on 10% SDS-polyacrylamide gels and the samples were transferred onto polyvinylidene fluoride membranes. After blocking the membrane with PBS containing 5% skimmed dried milk and 0.05% Tween 20, membranes were incubated overnight at 4 °C with a 1:1,000 dilution of the rabbit polyclonal antibody specific for human sat-1 (AIVAVA, San Diego, CA, USA). The membrane was washed three times with PBS containing 0.05% Tween 20 and incubated for 2 h with a 1:1,000 dilution of anti-rabbit antibody from goat linked to horseradish peroxidase (Calbiochem, Darmstadt, Germany). Antibody binding was detected by enhanced chemiluminescence (ECL) using LAS 3000 Imager (Fujifilm, Düsseldorf, Germany).

Results

Impact of glyoxylate and glycolate on sulfate and oxalate transport in sat-1-expressing oocytes

Experiments performed with sat-1 expressing X. laevis oocytes revealed different effects of oxalate and its precursors on [³⁵S]
sulfate uptake. An IC50 value of 55 \(\mu\)M \((n = 3)\) for the inhibition of sulfate uptake by oxalate was determined (Fig. 2A). A glyoxylate concentration of 5 mM inhibited \[^{35}S\] sulfate uptake significantly by 42 ± 10% (Fig. 2B), and an IC50 value of 5.5 mM \((n = 3)\) was calculated. Glycolate concentrations up to 5 mM did not inhibit \[^{35}S\] sulfate uptake (Fig. 2C). To test whether glyoxylate or glycolate exchange against sulfate or oxalate, trans-stimulation experiments by injecting either glyoxylate or glycolate with subsequent measurement of \[^{35}S\] sulfate or \[^{14}C\]oxalate uptake were performed. These experiments exhibited a two-times higher \[^{35}S\] sulfate (Fig. 3A) and \[^{14}C\]oxalate (Fig. 3B) uptake after glyoxylate injection. Injection of glycolate had no impact on either sulfate (Fig. 3A) or oxalate uptake (Fig. 3B). To demonstrate that enhanced uptake was not an effect of increased osmolarity within the oocytes, oocytes were injected with ORi containing 135 mM NaCl and \[^{35}S\] sulfate uptake was measured subsequently. Increased osmolarity did not influence sulfate uptake (data not shown). To demonstrate bidirectional sulfate-glyoxylate exchange, sat-1 expressing oocytes preloaded with \[^{35}S\] sulfate were incubated in glyoxylate containing medium. The 50 \(\mu\)M glyoxylate in the medium enhanced \[^{35}S\] sulfate efflux by 14 ± 1% (Fig. 4). Mocks did not show any change at different experimental conditions (Figs. 2–4).

**Change of sat-1 mRNA-expression upon incubation of HepG2 cells with oxalate or its precursors**

RT-PCR analysis detected expression of sat-1 mRNA in HepG2 cells (Fig. 5). PCR using sat-1 primers and separation of the PCR product on an agarose gel revealed a band at 254 bp. GAPDH was used as a housekeeping gene.

Sat-1 mRNA-expression was determined in HepG2 cells, which were incubated in different compounds, in varying incubation conditions. The results are shown in Figs. 2–4.

**Fig. 2. Sat-1 mediated sulfate transport in the presence of oxalate, glyoxylate, and glycolate.** cis-Inhibition of sulfate (20 \(\mu\)M, 0.01 \(\mu\)M \[^{35}S\] sulfate) uptake by increasing concentrations of (A) oxalate, (B) glyoxylate, and (C) glycolate. Data are mean values from two or three experiments with 5–12 oocytes per condition.

**Fig. 3. trans-Stimulation of sulfate and oxalate uptake into sat-1 expressing oocytes.** Oocytes were injected with either 23 nl ORi, ORi containing 50 mM glyoxylate or 50 mM glycolate and (A) sulfate (50 \(\mu\)M sulfate, 0.01 \(\mu\)M \[^{35}S\] sulfate) and (B) \[^{14}C\]oxalate (20 \(\mu\)M) uptake was determined. Data are mean values from four experiments with 4–12 oocytes per condition.
Kidney stones are a common disease due mostly to the supersaturation of the urine with calcium oxalate [4]. To reduce oxalate excretion it is necessary to understand oxalate transport and metabolism in the body. We recently identified sat-1 as a sulfate-anion exchanger being able to translocate oxalate in the in- and efflux mode [18]. Now we extended our investigations on the impact of oxalate’s precursors on sat-1 transport, mRNA, and protein-expression.

**Discussion**

Glycolate and glycylate are oxalate’s direct precursors. Since the molecular structures are very similar, we assumed that sat-1 not only translocates oxalate [16,18,20,26] but also its precursors glycylate and glycolate. In the present study, an IC_{50} value of 55 μM for the inhibition of sulfate uptake by oxalate was determined. This value is of similar range as the previously determined K_i for inhibition of sulfate uptake by oxalate (53 ± 5 μM [18]). In sat-1-expressing oocytes, glycylate concentrations as high as 5 mM were necessary to inhibit sulfate uptake significantly, glycylate concentrations of the same range did not influence sulfate uptake at all, indicating that both compounds exhibit only a low affinity or are not substrates of sat-1. As indicated by Baker [14], both compounds were taken up into HepG2 cells. It was suggested that glycylate was probably transported by the ubiquitously present MCT-1 that we identified in HepG2 cells. MCT-1, however, transports glycylate and glycylate with low affinity, MCT-1 does not seem to be the most prominent transporter, at least not for glycylate, in HepG2 cells.

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To exclude that enhanced sulfate uptake after incubation with glycylate is the effect of another transporter and to show that exclusively sat-1 is upregulated, RT-PCR analysis was performed with primers for a chloride-formate exchanger (CFEX), monocarboxylate transporter-1 and -2 (MCT-1 and -2), and sodium dependent sulfate transporter-1 (Na-Si-1). CFEX and MCT-1 mRNA was detected in HepG2 cells. PCR analysis ruled out that the enhanced sulfate uptake was mediated by CFEX (Fig. 8A and B).

**Additional transporters in HepG2 cells possibly mediating sulfate or oxalate transport**

To demonstrate that not only sat-1 mRNA and protein-expression is upregulated but also the upregulated protein is functional, sulfate uptake into HepG2 cells was tested. [35S] sulfate uptake was significantly enhanced by 34 ± 3% (n = 3) after incubation in 1 mM glyoxylate for 4 days (Fig. 7B).
Fig. 6. Relative sat-1 mRNA-expression in HepG2 cells. (A and B) HepG2 cells incubated for 4 days in 1 mM glycine, hydroxyproline, glyoxylate, glycolate, sulfate or 100 μM oxalate. (C and D) HepG2 cells incubated in 1 mM glyoxylate for 1–6 days. (E and F) HepG2 cells incubated in different concentrations of glyoxylate for 4 days. Expression of sat-1 in HepG2 cells was determined by TaqMan real-time PCR, using TaqMan assays directed against sat-1 splice variants 1 and 3 (A, C, and E) and against splice variant 2 (B, D, and F). Sat-1 mRNA-expression was normalized to GAPDH. The ΔCt shows the difference between the expression in non-treated and treated cells. Means calculated from three to five independent experiments from different passages of HepG2 cells with two repeats each.
When HepG2 cells were incubated in glyoxylate, intracellular concentrations of glyoxylate and glycolate and extracellular concentrations of oxalate and glycolate rose with increasing glyoxylate concentrations in the medium [14]. Since the intracellular oxalate concentration remained unchanged, while the oxalate concentration in the medium increased, transport of oxalate into the medium probably occurred. Since accumulation of oxalate would lead to intracellular damages [28], a rapid outward directed transport is necessary. Incubation of HepG2 cells in glyoxylate led to an upregulation of sat-1-expression, and sat-1 probably mediates this outward directed transport of oxalate.

We identified human sat-1 as a possible candidate responsible for enhanced oxalate release from HepG2 cells for three reasons. First, sat-1 mRNA-expression increased upon incubation of HepG2 cells for 4 days in glyoxylate containing medium [14]. Since the intracellular oxalate concentration remained unchanged, while the oxalate concentration in the medium increased, transport of oxalate into the medium probably occurred. Since accumulation of oxalate would lead to intracellular damages [28], a rapid outward directed transport is necessary. Incubation of HepG2 cells in glyoxylate led to an upregulation of sat-1-expression, and sat-1 probably mediates this outward directed transport of oxalate.

Fig. 7. Upregulation of sat-1 protein-expression by glyoxylate and functional analysis. (A) Protein-expression in the membrane and cytosol of HepG2 cells incubated for 4 days with or without 1 mM glyoxylate. Proteins were separated by SDS-polyacrylamide gel electrophoresis and detected by Western blotting. A representative experiment is shown and similar results were obtained with the protein isolated from two independent passages of HepG2 cells. (B) [35S] sulfate (50 μM sulfate, 0.01 μM [35S] sulfate) uptake into HepG2 cells incubated for 4 days in the presence and absence of 1 mM glyoxylate. Data are mean values from three experiments with six repeats per condition.

Fig. 8. mRNA-expression of different transporters in HepG2 cells. HepG2 cells were incubated for 4 days in 1 mM glyoxylate. Total RNA was isolated and reverse transcribed. (A) Expression of CFEX, MCT-1, MCT-2, NaSi-1, and GAPDH in HepG2 cells was determined by PCR using specific primers (Table 1). PCR products were electrophoresed on agarose gels and visualized with ethidium bromide. Similar results were obtained with mRNA isolated from three independent passages of HepG2 cells. (B) Expression of CFEX in HepG2 cells was determined by TaqMan real-time PCR, using TaqMan assays directed against CFEX. CFEX mRNA-expression was normalized to GAPDH. ΔCt shows the difference between the expression in non-treated and treated cells. Means calculated from three independent experiments from different passages of HepG2 cells with two repeats each.

for Biotechnology Information). Although the physiological significance of these isoforms is not clear up to now, all primer sets identified comparable changes of sat-1 mRNA due to different experimental conditions.

Third, we could show enhanced expression of a functional sat-1 protein. Western Blot analysis using anti-sat-1 antibody revealed a band at 70 kDa in HepG2 plasma membrane and cytosol. A second faint band with a molecular weight of approximately 100 kDa occurred after incubation of HepG2 cells with glyoxylate. This is in agreement with results from Karniski et al. [20] who identified a band at 68 kDa and one at 97 kDa which...
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is the glycosylated sat-1 protein. Sat-1 was glycosylated under native conditions when isolated from the basolateral membrane from rat or rabbit kidneys but has incomplete or absent glycosylation when sat-1 was expressed in insect cells (Sf9 cells). Since sat-1 expressing Sf9 cells mediated sulfate and oxalate transport, glycosylation is not necessary for transport mechanisms [20]. The hsat-1 protein has two putative extracellular N-glycosylation sites [16]. The glycosylated as well as the non-glycosylated occurred in HepG2 cells after glyoxylate treatment. The increase in sulfate uptake detected in HepG2 cells incubated with glyoxylate could be due to an increased protein synthesis revealed by the more pronounced band at 70 kDa. Enhanced sulfate uptake into HepG2 cells after glyoxylate incubation demonstrated upregulation of a functional sat-1 protein. To rule out that this effect was mediated by other transporters being able to transport sulfate affected by glyoxylate incubation, we examined mRNA-expression of different transporters in HepG2 cells. None of the transporters showed upregulation after glyoxylate incubation. CFEX and NaS⁻/1 are well-known sulfate transporters [25,29], but NaS⁻/1 was not expressed in HepG2 cells, and CFEX was not upregulated after glyoxylate incubation.

There is only little known about the regulation of sat-1 expression. Patel et al. [30] demonstrated that glyoxylate changes vitamin D induced expression by binding to the vitamin D receptor. Regions showing similarities with vitamin D and thyroid hormone responsive elements were identified in the sat-1 promoter region but their functional analysis did not show a regulation of sat-1 expression by vitamin D [16]. How glyoxylate induces sat-1 expression still needs to be elucidated.

In conclusion, we identified glyoxylate as a substrate of sat-1 and an inducer of sat-1 mRNA and protein-expression in HepG2 cells and may be responsible for the elevation of the outward directed oxalate transport observed under hyperoxaluria. Whether this substrate induction occurs also in healthy hepatocytes, awaits further investigations.

Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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