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Reduction in urinary oxalate excretion in mouse models of Primary Hyperoxaluria by RNA interference inhibition of liver lactate dehydrogenase activity

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A B S T R A C T

The Primary Hyperoxaluria's (PH) are rare autosomal recessive disorders characterized by elevated oxalate production. PH patients suffer recurrent calcium oxalate kidney stone disease, and in severe cases end stage renal disease. Recent evidence has shown that RNA interference may be a suitable approach to reduce oxalate production in PH patients by knocking down key enzymes involved in hepatic oxalate synthesis. In the current study, wild type mice and mouse models of PH1 (AGT KO) and PH2 (GR KO) were treated with siRNA that targets hepatic LDHA. Although siRNA treatment substantially reduced urinary oxalate excretion [75%] in AGT KO animals, there was a relatively modest reduction [32%] in GR KO animals. Plasma and liver pyruvate levels significantly increased with siRNA treatment and liver organic acid analysis indicated significant changes in a number of glycolytic and TCA cycle metabolites, consistent with the known role of LDHA in metabolism. However, siRNA dosing data suggest that it may be possible to identify a dose that limits changes in liver organic acid levels, while maintaining a desired effect of reducing glyoxylate to oxalate synthesis. These results suggest that RNAi mediated reduction of hepatic LDHA may be an effective strategy to reduce oxalate synthesis in PH, and further analysis of its metabolic effects should be explored. Additional studies should also clarify in GR KO animals whether there are alternate enzymatic pathways in the liver to create oxalate and whether tissues other than liver contribute significantly to oxalate production.

1. Introduction

Endogenous synthesis of oxalate occurs primarily in the liver and contributes to calcium oxalate kidney stone disease [1]. The Primary Hyperoxalurias (PH) are inherited rare diseases of glyoxylate metabolism that result in an increased endogenous oxalate synthesis [2,3]. Disease progression can lead to recurrent calcium oxalate kidney stone formation, and in some cases, end stage renal disease. Despite an increased understanding of metabolic pathways associated with PH, treatment options are limited. Type 1 (PH1), arises from mutations in the enzyme alanine-glyoxylate aminotransferase (AGT). The resulting deficiency in this enzyme leads to the inability of hepatocytes to convert glyoxylate to glycine, resulting in an increased flux of glyoxylate to glycolate and oxalate (Fig. 1). Primary Hyperoxaluria type 2 (PH2) involves a mutation in glyoxylate reductase (GR) which prevents the conversion of glyoxylate to glycolate [4]. Patients with PH type 3 have a deficiency in the aldolase that normally cleaves 4-hydroxy-2-oxoglutarate (HOG), a metabolite of hydroxyproline, into pyruvate and glyoxylate [5,6]. Inhibition or modulation of the metabolic pathways that contribute to endogenous oxalate production may be beneficial to these patients.

One of the final steps of endogenous oxalate synthesis in the liver is the conversion of glyoxylate to oxalate by lactate dehydrogenase (LDH) (Fig. 1). LDH is composed of four subunits with the two most common forms being the homotetramers, LDH-M and LDH-H [7]. These proteins are encoded by the LDHA and LDHB genes, respectively, with only LDHA expressed and LDH-M formed in hepatocytes. LDHA is also highly expressed in muscle tissue and plays an essential role in the Cori cycle and the inter-conversion of lactate to pyruvate. In human cases of LDHA deficiency, muscle weakness and breakdown can occur with high intensity activity [8–12]. Yanagawa et al. [13] reported that oxalate excretions in random urine samples from individuals with LDH deficiency were normal, not reduced, but dietary oxalate and calcium intake were not controlled. We had previously questioned whether blocking hepatic LDH activity would drastically alter liver metabolism due to its interface between glycolysis, gluconeogenesis and energy production [1]. While the studies reported here were in progress, Lai et al. described experiments showing that decreasing LDH activity using siRNA targeting liver LDHA reduced oxalate excretion in mouse models of PH [14]. The present studies also show substantial reductions in oxalate production in PH1 mice (AGT KO), while demonstrating more modest reductions in oxalate production in the mouse model of PH2 (GR KO). Hepatic LDHA knock down does not impact urinary oxalate excretions in wild type mice, but results in significant changes in plasma pyruvate and liver tricarboxylic acid (TCA) organic acids, consistent with the central role of LDH in metabolism.

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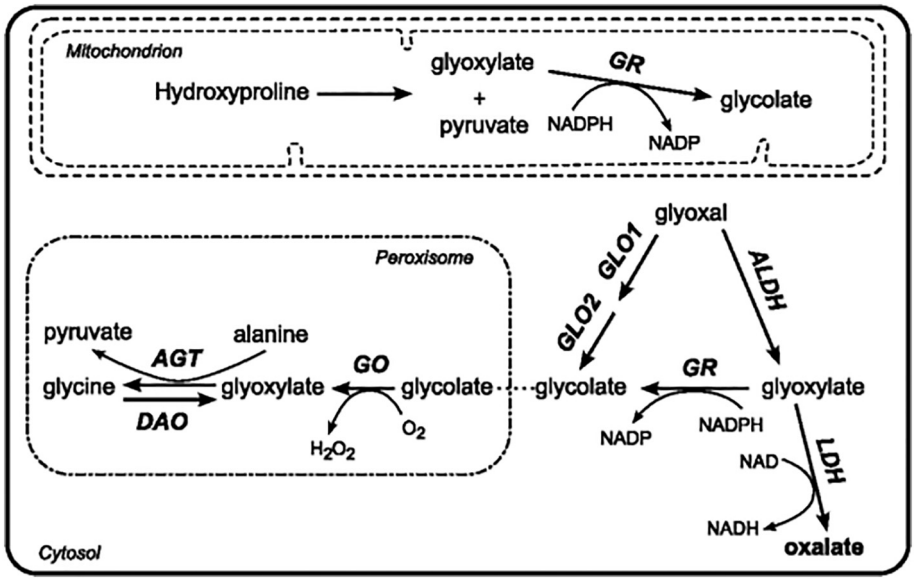

Fig. 1. Endogenous oxalate synthesis pathway in hepatocytes. The last step in oxalate production involves lactate dehydrogenase (LDH). GR, glyoxylate reductase; AGT, alanine glyoxylate aminotransferase; DAO, D-amino oxidase; GO, glycolate oxidase; GLO1 and GLO2, glyoxalase system; ALDH, aldehyde dehydrogenase.

2. Materials and methods

2.1. Chemicals

Reagent grade chemicals were obtained from either Sigma-Aldrich Chemicals (St Louis, MO) or Fisher Scientific (Pittsburgh, PA). Carbon 13 isotopes were purchased from Cambridge Isotopes Laboratories, Inc., MA. The design and synthesis of siRNA with conjugation to *N*-acetyl galactosamine (GalNAc) and specific to LDHA (GalNAc-LDHA siRNA) were done as previously described for glycolate oxidase [15].

2.2. Animals

All studies were conducted using protocols consistent with local, state and federal regulations as applicable, and with adherence to the NIH Guide for the Care and Use of Laboratory Animals. All studies were approved by the Institutional Animal Care and Use Committees at University of Alabama at Birmingham (UAB), AL. The phenotypes of AGT KO and GR KO mice have been previously described [16,17]. Wild type animals were generated from breeding pairs of Agxt heterozygous animals. Experimental animals were male and 12–15 weeks old. Mice were maintained in a barrier facility with a 12-h light/dark cycle, an ambient temperature of 23 ± 1 °C, and had free access to food and water. They were fed a custom purified diet TD.130032 (Envigo, Madison, WI) to which calcium chloride was added at 5 mg per gram dry diet. The custom purified diet has a low oxalate content (12.9 ± 1.1 μg oxalate per gram diet), which limited the contribution of dietary oxalate to urinary oxalate excretion.

2.3. Urinary collections

Animals were injected subcutaneously with GalNAc-LDHA siRNA and 24 hour urines collected over time. Animals were singly housed in Techniplast mouse metabolic cages for collection of three consecutive 24-hour urines. 24-hour urines were collected on 1 ml mineral oil to prevent evaporation and 50 μl 2% sodium azide to prevent bacterial growth.

2.4. Necropsy and tissue harvest

After completion of experiments, mice were anesthetized with isoflurane, and necropsy was performed. A terminal bleed was performed by left ventricle aspiration and blood collected in heparinized tubes. Plasma was stored at −80 °C. Liver, muscle, and cardiac tissue were harvested immediately and stored in liquid nitrogen. One lobe of each liver was fixed in 4% buffered paraformaldehyde and embedded in paraffin and sectioned with a cryostat. All fixed liver tissues were stained with hematoxylin and eosin for histopathology examination by the UAB Comparative Pathology Lab and Gnotobiotic Core.

2.5. RNA extraction and quantitative assay

Total RNA was isolated from frozen liver tissues using the RNAqueous-96 Total RNA Isolation Kit from Thermo Fisher Scientific in Waltham, MA 02451 (Catalog No. AM1812) as per the manufacturer's protocol. Briefly, liver tissue was lysed and after a series of centrifugation steps (1900 ×*g*, 2 min) samples were washed, treated with DNase reagents, and eluted into 100ul nuclease free water (Qiagen, Cat# 129115). RNA concentration was determined with the NanoDrop 8000 Spectrophotometer (ThermoFisher, Cat# ND-8000-GL). Total RNA was reverse transcribed using High Capacity cDNA synthesis kit (ABI, Cat# 4374967). Quantitative PCR was performed on cDNA using the Roche LightCycler480 Probes Master Mix in the Roche 480 LightCycler Real Time PCR System to determine the relative abundance of LDHA mRNA normalized to the housekeeping gene GAPDH. The Taqman probes used for mouse LDHA and GAPDH mRNA detection were Life Technologies Cat# Mm01612132_g1 and 4351309 respectively.

2.6. Sample preparation and analytical measurements

For oxalate determination, part of the urine collection was acidified to pH between 1 and 2 with HCl prior to storage at −80 °C to prevent any possible oxalate crystallization that could occur with cold storage and/or oxalogenesis associated with alkalinization. The remaining non-acidified urine was frozen at −80 °C for the measurement of glycolate and creatinine. Plasma preparations were filtered through Nano-sep centrifugal filters (VWR International, Batavia, IL) with a 10,000 nominal molecular weight limit to remove macromolecules prior to ion chromatography coupled with mass spectrometry or ICMS (Thermo Fisher Scientific Inc., Waltham, MA). Centrifugal filters were washed with 10 mM HCl prior to sample filtration to remove any contaminating

trace organic acids trapped in the filter device. Liver tissue was extracted with 10% (wt/vol) trichloroacetic acid (TCA) for organic acid analysis. Liver TCA extracts were directly used for glyoxylate measurement by reversed phase HPLC, as previously described [18]. Organic acids were measured by ICMS following removal of TCA by vigorous vortexing with an equal volume of 1,1,2-trichlorotrifluoroethane (Freon)-trioctylamine (3:1, vol/vol; Aldrich, Milwaukee, WI), centrifuging at 4 °C to promote phase separation, and collecting the upper aqueous layer for analysis, as previously described [19]. The NAD:NADH ratio in liver tissues was determined using the NAD:NADH Glo Assay kit from Promega Corp, Madison, MI, according to the manufacturer's instructions. Urinary creatinine was measured on a chemical analyzer, and urinary oxalate by ICMS, as previously described [18]. Glycolate and lactate were determined by $^{13}C_2$-glycolate and $^{13}C_3$-lactate isotope dilution ICMS, respectively, with an AS15, 2 × 150 mm, anion exchange column, using IC and MS settings previously described [18]. Selected-ion monitoring (SIM) at the following mass/charge ratios and cone voltages were used to quantify lactate (SIM 89.0, 35 V), $^{13}C_3$-lactate (SIM 92.0, 35 V), glycolate (SIM 75.0, 35 V) and $^{13}C_2$-glycolate (SIM 77.0, 35 V). Other organic acids, including pyruvate and TCA cycle metabolites were measured by IC/MS with an AS11-HC 4 μm, 2 × 150 mm, anion exchange column at a controlled temperature of 30 °C and a Dionex™ ERS™ 500 anion electrolytically regenerated suppressor. A gradient of KOH from 0.5 to 80 mM over 60 min at a flow rate of 0.38 ml/min was used to separate sample anions. The mass spectrometer (MSQ-PLUS) was operated in ESI negative mode, needle voltage 1.5 V, 500 °C source temperature, and column eluent was mixed with 50% acetonitrile at 0.38 ml/min using a zero dead volume mixing tee prior to entry into the MSQ. Selected-ion monitoring (SIM) at the following mass/charge ratios and cone voltages were used to quantify citrate and isocitrate (SIM 191.0, 40 V), pyruvate (SIM 87.0, 30 V), ATP (SIM505.8, 40 V), succinate (SIM177.0, 35 V), fumarate (SIM115.0, 35 V), alpha-keto glutarate (SIM 145.0, 30 V), malate (SIM 133, 30 V), oxaloacetate (SIM 131 and 87.0, 40 V), and glycerol-3-phosphate (SIM 171, 35 V). Liver alanine was measured in liver TCA extracts by the Waters (Milford, MA) AccQ.Tag method according to the manufacturer's instructions.

2.7. Enzyme assays

Tissue was homogenized in iced cold lysis buffer (25 mM HEPES, pH 7.3, 0.1% Triton-X-100) with probe sonication to give a 10% wt/vol lysate. LDH activity was measured by the increases in absorbance at 340 nm with the reduction of NAD to NADH in the presence of either lactate or glyoxylate, as both substrates are oxidized by LDH [20]. Lactate to pyruvate activity of LDH was measured with 20 mM lactate, 100 mM Tris-HCl, pH 9.0, 2 mM NAD^+, 0.01% liver lysate. Glyoxylate to oxalate activity of LDH was measured by the increase in absorbance at 340 nm with the reduction of NAD to NADH in the presence of 20 mM glyoxylate, 100 mM Tris-HCl, pH 9.0, 2 mM NAD^+, 0.01% liver lysate. A Coomassie Plus protein assay kit (Pierce, Rockford, IL), with bovine serum albumin (BSA) as the standard, was used to determine protein concentrations in tissue lysates. For determination of glycogen in mouse liver, approximately 500 μg of mouse liver tissue was analyzed as described by Lo et al. [21].

2.8. Western blot of phosphorylated AMP-activated protein kinase

Mouse liver 10% wt/vol lysates were prepared by probe sonication in lysis buffer (25 mM HEPES, 0.1% Triton X-100). Protein content was assessed via Bradford assay. Twenty–five micrograms of protein were electrophoresed under reducing conditions on a precast 12% mini-PROTEAN TGX polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA), transferred to PVDF membrane (Bio-Rad Laboratories), blocked with 5% w/v bovine serum albumin in Tris Buffered Saline with Tween 20, before overnight incubations at 4 °C with primary antibodies. Immunoblotting against phospho-AMPKα, was done with phospho-AMPKα (Thr172) antibody (1:2000, catalog # 07-626; Millipore, Billerica, MA), immunoblotting against actin was done with anti-beta-actin (1:5000, catalog # A2228, Sigma-Aldrich, ST-Louis, MO) after stripping the membrane. Visualization was done by chemiluminescence, using anti-rabbit HRP conjugated secondary antibody (Abcam, Cambridge, UK) and Luminata Crescendo Western HRP Substrate (Millipore). Signal acquisition was done on ImageQuant LAS4000 (GE Life Sciences; Marlborough, MA). Densitometry analysis was done with Fiji (Image J) for phospho-AMPKα and actin and expressed as their ratio.

2.9. Statistical analyses

All graphing and statistical analyses were conducted in GraphPad Prism v.6 (GraphPad Software Inc., San Diego, CA). A Student's *t*-test was used to compare data between saline and siRNA treated animals. The mean of at least two 24-h urine analyte determinations was used to characterize excretions in each mouse. Data are expressed as mean ± SD, unless otherwise indicated. The criterion for statistical significance was $P < 0.05$.

3. Results

3.1. Dose dependent silencing of LDHA mRNA and effects on LDH activity and body composition

Wild type mice received a single subcutaneous injection of GalNAc-LDHA siRNA at doses ranging between 0 and 10 mg/kg, and then were sacrificed after 10 days. Prior studies with HAO1 siRNA indicated that mRNA synthesis is suppressed for > 6 weeks [22]. Maximal LDHA mRNA reduction of approximately 95% was observed at 10 mg/kg, with dose dependent reductions observed at the other doses evaluated (Fig. 2). Dose dependent effects of a single injection of siRNA on liver lactate to pyruvate activity and glyoxylate to oxalate activity of AGT KO mice after 4 weeks are shown in Fig. 3. Wild type and GR KO animals treated with 10 mg/kg GalNAc-LDHA siRNA showed a similar decrease in liver lactate to pyruvate activity and glyoxylate to oxalate activity (data not shown). No effects were observed on LDH activity in muscle or heart tissue in AGT KO mice receiving weekly injections of 10 mg/kg siRNA for 4 weeks, consistent with the liver-specific targeting of the GalNAc-LDHA siRNA (data not shown). Following monthly injections for 5 months with saline or 10 mg/kg LDHA siRNA, body weights, lean body mass, % body fat, and bone mineral density in treated and untreated wild type mice were not significantly different (Table 1). Apart from 2 saline treated and 2 siRNA treated animals, which showed patchy, moderate steatosis with no inflammation, there were no significant histopathologic findings observed in any other saline or siRNA treated mice.

3.2. Effects on urinary oxalate excretion

To test the potential for lowering oxalate excretion following a reduction in LDH activity, wild type, AGT KO, and GR KO mice were injected with GalNAc-LDHA siRNA (10 mg/kg) and 24 h urine samples were collected 4 weeks post dose. While oxalate excretion was not significantly different in wild type mice following treatment, excretions in AGT KO and GR KO mice were significantly decreased by 75% ($P < 0.0001$) and 32% ($P < 0.0001$) respectively, relative to saline-treated mice (Fig. 4). Notably, while siRNA treatment nearly normalized urinary excretion in the AGT KO mice, urinary oxalate excretion in GR KO mice remained ~2 fold higher than urinary excretions in wild type mice following treatment. To establish the sensitivity and duration of this decrease in oxalate excretion, AGT KO mice were treated with a single injection of varying doses of GalNAc-LDHA siRNA. Maximal urinary oxalate lowering was observed at 4 weeks with doses of 3 and

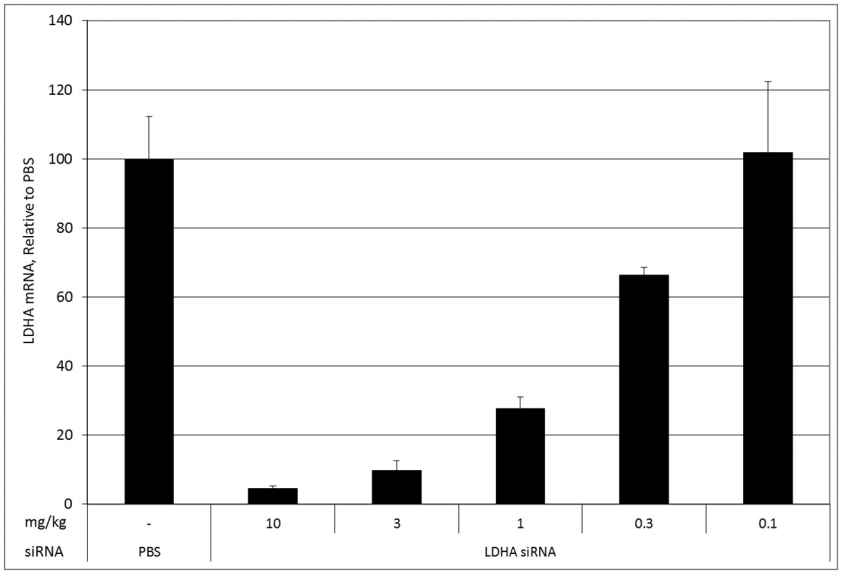

Fig. 2. Liver LDHA mRNA levels after single administration of various doses of LDHA-GalNAc siRNA in female adult wild type mice (n = 3 for each dose). Liver tissue was harvested 10 days after dosing.

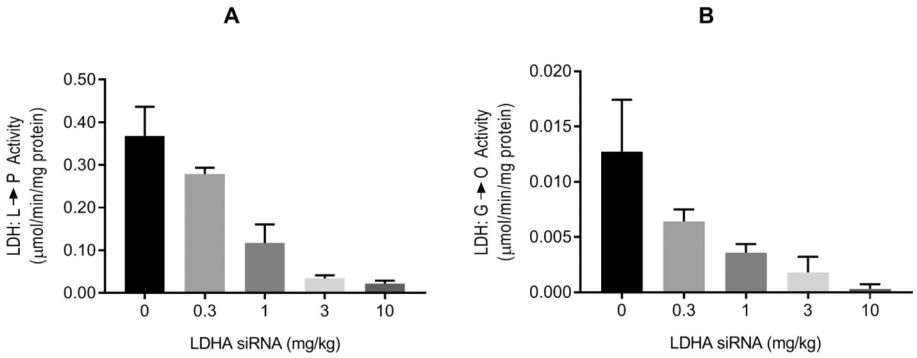

Fig. 3. Liver lactate dehydrogenase (LDH) activity after single administration of various doses of LDHA-GalNAc siRNA in AGT KO animals (n = 6). Specific activity was measured with either glyoxylate (glyoxylate to oxalate activity or G to O) or lactate (lactate to pyruvate activity or L to P) as substrate. Liver tissue was harvested 28 days after dosing. Panel A: L to P activity was measured by the increases in absorbance at 340 nm with the reduction of NAD to NADH in the presence of 20 mM lactate, 2 mM NAD^+, pH 9.0. Panel B: G to O activity was measured by the increases in absorbance at 340 nm with the reduction of NAD to NADH in the presence of 20 mM glyoxylate, 2 mM NAD^+, pH 9.0.

Table 1
Body composition as determined by dual energy X-ray absorptiometry (DXA) scan of wild type mice (n = 6) following treatment with GalNAc-LDHA siRNA (10 mg/kg) or saline monthly for five months.

	Saline		GalNAc-LDHA siRNA		P value
	Mean	SD	Mean	SD	
Weight (g)	34.44	7.24	38.18	5.66	0.36
BMD (g/cm^2)	0.0494	0.0016	0.0503	0.0004	0.23
BMC (g)	0.45	0.03	0.45	0.04	0.85
Area (cm^2)	9.04	0.40	8.97	0.76	0.87
Lean (g)	22.40	2.55	23.80	1.20	0.26
Fat (g)	8.88	4.99	10.87	4.48	0.50
Total (g)	31.28	7.10	34.67	5.39	0.39
%Fat	26.62	10.23	30.30	8.00	0.52

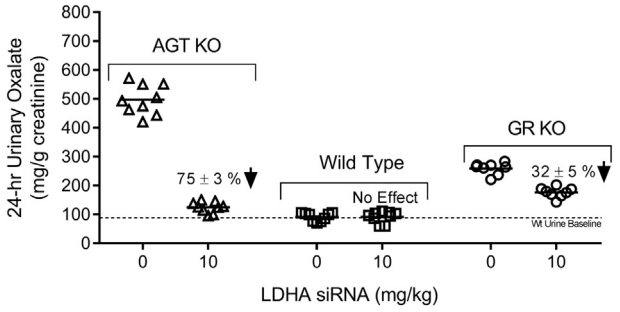

Fig. 4. 24-hour urinary oxalate excretion of AGT KO, wild type, and GR KO mice before and 4 weeks after GalNAc-LDHA siRNA (10 mg/kg) treatment. Horizontal line through symbols for each group represents the mean. The dotted horizontal line represents mean wild type baseline 24-hour urinary oxalate excretion.

10 mg/kg, while oxalate lowering was sustained for longer in the 10 mg/kg group (Fig. 5).

3.3. Effects on plasma and liver metabolites

Reduction of liver LDHA could potentially increase tissue glyoxylate levels, which could lead to an increase in glycolate following reduction of glyoxylate by GR (Fig. 1). However, siRNA treatment had no impact on liver glyoxylate levels in AGT KO animals (Table 2). Furthermore, although there was a trend for an increase in glycolate levels in plasma and liver in AGT KO animals with siRNA treatment, these changes were not significant (Table 2). 24-hour urinary glycolate excretion was also not significantly different (P = 0.89) in siRNA treated animals (275 ± 14 µg glycolate/mg creatinine) compared to saline treated animals (282 ± 78 µg glycolate/mg creatinine). Although siRNA treated animals had similar plasma lactate levels to untreated animals, they had significantly higher pyruvate (57%) compared to control mice (Table 2). These data would suggest that measuring plasma pyruvate may be one approach to monitor LDHA siRNA treatment. Liver lactate decreased by 37%, although this was not significant (P = 0.11), and liver pyruvate increased 3 fold (P < 0.001) in treated animals

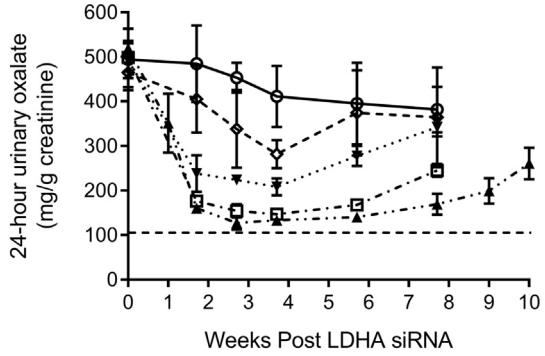

Fig. 5. 24-hour urinary oxalate excretion over time of AGT KO mice (n = 4 for each dose) after a single administration of various doses of GalNAc-LDHA siRNA. The dotted horizontal line represents mean wild type baseline 24-hour urinary oxalate excretion. Data expressed as mean ± SD. ○, untreated; ◊, 0.3 mg/kg; ▼, 1 mg/kg; □, 3 mg/kg; ▲, 10 mg/kg.

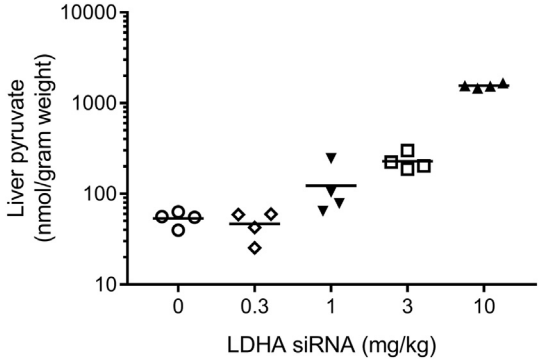

Fig. 6. Liver pyruvate levels in AGT KO mice after treatment with varying doses of GalNAc-LDHA siRNA. Horizontal line through symbols for each group represents the mean. ○, untreated; ◊, 0.3 mg/kg; ▼, 1 mg/kg; □, 3 mg/kg; ▲, 10 mg/kg.

Table 2
Metabolite analysis of plasma and liver extracts of AGT KO animals (n = 4) four weeks after a single dose of 3 mg/kg GalNAc-LDHA siRNA or saline. For reference, liver glycolate and glyoxylate levels in wild type mice at baseline are 77 ± 2 nmol/g tissue (P = 0.03 when compared with AGT KO at baseline) and 2.0 ± 0.1 nmol/g tissue (P = 0.09 when compared with AGT KO at baseline), respectively, and plasma glycolate in wild type animals is 26 ± 0.2 μM (P < 0.0001 when compared with AGT KO at baseline).

	Saline control		GalNAc-LDHA siRNA		P value
	Mean	SEM	Mean	SEM	
Plasma (μM)					
Lactate	5396	290	5202	180	0.590
Pyruvate	**87**	**9**	**137**	**17**	**0.040**
Lac:Pyr ratio	62	7	42	6	0.070
Glycolate	116	6	196	43	0.110
Liver glycolytic intermediates (nmol/g)					
Lactate	4575	777	2898	470	0.110
Pyruvate	**54**	**5**	**228**	**25**	**0.0005**
Lac:Pyr ratio	**93**	**27**	**13**	**3**	**0.030**
Liver TCA cycle intermediates (nmol/g)					
Citrate	**169**	**7**	**230**	**17**	**0.015**
Isocitrate	**75**	**2**	**84**	**2**	**0.017**
2-Oxoglutarate	58	6	67	6	0.323
Succinate	388	87	387	79	0.996
Fumarate	**127**	**8**	**218**	**17**	**0.003**
Malate	**350**	**19**	**534**	**30**	**0.001**
Oxaloacetate	**26**	**0**	**29**	**1**	**0.009**
Other liver metabolites (nmol/g)					
NAD:NADH ratio	2.00	0.35	1.90	0.05	0.950
Glycerol-3-phosphate	1145	253	1800	295	0.070
Glycolate	144	26	231	45	0.140
Glyoxylate	2.50	0.05	2.61	0.13	0.540
Alanine	**1836**	**218**	**2657**	**247**	**0.047**
ATP	**2692**	**45**	**1955**	**271**	**0.036**
AMP	1645	153	2061	388	0.320
AMP:ATP ratio	0.61	0.06	1.21	0.39	0.180
Glycogen	45.7	4.6	43.7	4.7	0.758

The bolded values are those metabolites that have changed significantly (P < 0.05) with siRNA treatment.

compared to livers from control animals (Table 2). In fact, as LDHA inhibition increased with increasing doses of siRNA, liver pyruvate levels increased remarkably in AGT KO animals (Fig. 6). Given the findings of altered liver lactate and pyruvate levels, changes in other liver metabolites were measured. Significant increases following GalNAc-LDHA siRNA treatment were seen in all TCA cycle intermediates, except succinate and 2-oxoglutarate (Table 2), suggesting the higher level of pyruvate brought about by siRNA treatment increased

Fig. 7. Relative expression of hepatic phosphorylated AMP-activated protein kinase (Phospho-AMPK) of control and 3 mg/kg LDHA siRNA treated animals.

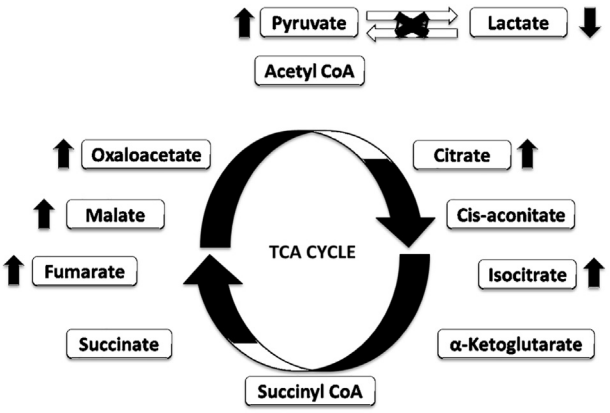

Fig. 8. Changes in liver TCA metabolites following GalNAc LDHA siRNA treatment.

TCA cycle flux. Alanine was increased in liver with siRNA treatment, which may be due to increased transamination of pyruvate via alanine transaminase activity. A loss of LDH activity could potentially alter the NAD:NADH ratio; however, the NAD:NADH ratio was not significantly impacted with siRNA treatment. The glycerol-3-phosphate shuttle is a mechanism that regenerates NAD^+. Glycerol-3-phosphate levels in liver were increased (Table 2), approaching significance (P = 0.07), suggesting this system may play a role in maintaining the NAD:NADH ratio following siRNA treatment. Such a response has been reported in muscle tissue of individuals with LDHA deficiency [23,24]. ATP liver levels were significantly lower (~30%) and the AMP:ATP ratio was higher suggesting ATP synthesis/consumption was impacted with siRNA treatment. However, the glycogen liver content (Table 2) and

Table 3
Percent decrease in liver parameters and urinary oxalate excretion in AGT KO mice 4 weeks after various doses of GalNAc-LDHA siRNA treatment.

GalNAc-LDHA siRNA dose (mg/kg)	% decrease in liver LDH Lac to Pyr activity	% decrease in liver LDH Glx to Ox activity	% decrease in liver lactate:pyruvate ratio	% decrease in urinary oxalate
0.3	24 ± 4^{A}	47 ± 9^{A}	5 ± 19^{A}	32 ± 8^{A}
1	68 ± 13^{B}	70 ± 6^{BC}	51 ± 15^{B}	49 ± 5^{B}
3	91 ± 2^{C}	85 ± 12^{C}	92 ± 7^{C}	64 ± 3^{C}

To determine the percent decrease in each siRNA treated animal, the mean value of non-treated mice was used. One-Way Anova was used for statistical analysis and all parameters were found to significantly change with treatment ($P < 0.05$). Tukey's post hoc multiple comparison's test was performed and alphabet letters show doses significantly different from each other. LDH, lactate dehydrogenase; Lac, lactate; Pyr, pyruvate; Glx, glyoxylate; Ox, oxalate. Data expressed as mean ± SD.

relative expression levels of phosphorylated AMPK (Fig. 7) were not altered.

4. Discussion

For individuals with PH, there are few treatment options and a great need exists for therapies that decrease endogenous oxalate synthesis. In the current study, subcutaneous injections of GalNAc-LDHA siRNA significantly decreased urinary oxalate excretion in AGT KO mice compared to saline injections, a result similar to that recently reported by Lai et al. [14]. We also observed a significant reduction in GR KO mice treated with siRNA targeting LDHA, although these animals still excreted ~2 fold more urinary oxalate than wild type mice. Of note, there was no effect of siRNA treatment on urinary oxalate excretion in wild type mice. Based on our current understanding of glyoxylate metabolic pathways, this finding in wild type mice was unexpected as the liver is believed to be the major source of endogenous oxalate synthesis with LDH catalyzing the oxidation of glyoxylate to oxalate [1]. This observation in wild type mice was further supported by the lack of change in liver glyoxylate levels, suggesting that these levels are tightly regulated and maintained at very low levels through the actions of AGT and GR activities. Other potential sources of oxalate production in the liver that now require scrutiny include ascorbate breakdown [25], which may increase in PH due to oxidative stress, and xanthine oxidase (XO) activity [13,26]. Additionally, further studies are needed to determine whether tissues other than liver can contribute significantly to oxalate production. For example, renal endogenous oxalate synthesis in GR KO animals may be elevated and could explain the more modest response to liver-specific siRNA treatment in these animals.

There was no change in liver glyoxylate levels in AGT KO animals following LDHA siRNA treatment. Furthermore, liver glycolate levels did not increase significantly with siRNA treatment. These data together could suggest that glyoxylate levels do not build up significantly in the AGT KO animals with siRNA treatment because glyoxylate can act as an amino-acceptor for aminotransferases other than AGT [27]. This hypothesis is further supported by the observation that liver glyoxylate levels were similar in both wild type and AGT KO animals at baseline (Table 2).

Patients with hereditary LDHA deficiency may present with exertional myopathy, skin lesions, and other clinical findings [12,28–31]. However, with the liver-specific targeting siRNA approach used here, no changes in LDH activity in heart and muscle tissue were seen. Consistent with this, Lai et al. did not observe any signs of exertional myopathy in mice receiving high dose therapy for 4 weeks [14]. Chronically siRNA treated mice also did not demonstrate any weight loss or any significant changes in body composition, as measured by DXA, and no liver histopathology was observed. Another potential concern with LDH inhibition is an alteration in the lactate/pyruvate ratio. Our data demonstrate that alterations in the lactate/pyruvate ratio do occur within the liver following treatment. Despite these liver changes, however, there were no alterations in the plasma ratio, although pyruvate levels did significantly increase. Other significant metabolic changes were noted in the liver, specifically in organic acids that function in the TCA cycle (Fig. 8). The long-term consequences of lactate/pyruvate ratios and TCA cycle changes in the liver require further investigation following chronic dosing. Despite these findings, our dosing data suggest that LDH inhibition could have a greater impact on glyoxylate to oxalate production compared to lactate/pyruvate changes. It may be possible to identify an intermediate dose that limits changes in liver organic acid levels, but maintains a desired effect of reducing glyoxylate to oxalate synthesis. For example, when the relationship between siRNA treatment and changes in liver LDH activity, liver lactate:pyruvate ratio and urinary oxalate excretion was examined in this study (Table 3), the analysis highlighted that a dose between 0.3 and 1 mg/kg siRNA may achieve a substantial decrease in urinary oxalate excretion, but with a more limited impact on liver metabolism.

This study questions the current understanding of the sources, tissues, and their respective contributions to endogenous oxalate production. Further studies are needed to clarify these findings and also clarify the effects of LDH inhibition on liver metabolism, including levels of TCA cycle organic acids. Nonetheless, this study demonstrates the therapeutic potential of GalNAc-LDHA siRNA in PH1 and to a lesser extent, PH2.

Disclosures

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Transparency document

The Transparency document associated with this article can be found, in online version.

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