Generation of a GLO-2 deficient mouse reveals its effects on liver carbonyl and glutathione levels

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ABSTRACT

Objective: Hydroxyacylglutathione hydrolase (aka as GLO-2) is a component of the glyoxalase pathway involved in the detoxification of the reactive oxoaldehydes, glyoxal and methylglyoxal. These reactive metabolites have been linked to a variety of pathological conditions, including diabetes, cancer and heart disease and may be involved in the aging process. The objective of this study was to generate a mouse model deficient in GLO-2 to provide insight into the function of GLO-2 and to determine if it is potentially linked to endogenous oxalate synthesis which could influence urinary oxalate excretion.
Methods: A GLO-2 knock out mouse was generated using CRISPR/Cas 9 techniques. Tissue and 24-h urine samples were collected under baseline conditions from adult male and female animals for biochemical analyses, including chromatographic measurement of glycolate, oxalate, glyoxal, methylglyoxal, D-lactate, ascorbic acid and glutathione levels.
Results: The GLO-2 KO animals developed normally and there were no changes in 24-h urinary oxalate excretion, liver levels of methylglyoxal, glyoxal, ascorbic acid and glutathione, or plasma D-lactate levels. GLO-2 deficient males had lower plasma glycolate levels than wild type males while this relationship was not observed in females.
Conclusions: The lack of a unique phenotype in a GLO-2 KO mouse model under baseline conditions is consistent with recent evidence, suggesting a functional glyoxalase pathway is not required for optimal health. A lower plasma glycolate in male GLO-2 KO animals suggests glyoxal production may be a significant contributor to circulating glycolate levels, but not to endogenous oxalate synthesis.

1. Introduction

Enzymes of the glyoxalase pathway are ubiquitously expressed in cells and tissues and utilize reduced glutathione (GSH) to detoxify reactive carbonyl species, including methylglyoxal and glyoxal. Methylgyoxal is produced primarily from triose phosphate isomerase activity during glycolysis [1,2]. Glyoxal, the simplest dialdehyde, and methylglyoxal can also be produced via autoxidation of carbohydrates and lipids. Both glyoxal and methylglyoxal can react with amino groups in proteins, nucleic acids and lipids to form advanced glycation end-products leading to carbonyl stress. Excessive glycation is believed to be associated with ageing and several chronic diseases including diabetes, cancer, and neurodegenerative and cardiovascular diseases [1, 3–6]. Compartmentation of the enzymes in the glyoxalase system may

be important and appears to be linked to their function. Distinct mitochondrial and cytosolic forms of GLO-2 are generated from a single gene [7]. Glutathione which is synthesized in the cytosol is essential for limiting free radical damage in mitochondria. Two potentially important processes include the transport of GSH into mitochondria and the S-glutathionylation of proteins in the mitochondrial compartment. GLO-2 may influence both of these processes. Armeni et al. have shown that S-D-Lactoylglutathione is transported into mitochondria where GLO-2 converts it to D-lactate and GSH [8]. In addition, GLO-2 glutathionylation significantly augments the activity of malate dehydrogenase, an important component of the citric acid cycle which occurs in the matrix of the mitochondrion [9].

Oxalate, the simplest dicarboxylic acid, is an important component of calcium oxalate kidney stones, a condition afflicting ~10% of the U.S.

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population [10]. We have previously shown that glyoxal can be converted to oxalate, in erythrocytes and cultured liver cells [11,12], and thus proposed that a possible important function of the glyoxalase pathway is to limit endogenous oxalate synthesis [3]. To help determine if GLO-2 metabolic pathways are linked to the endogenous production of oxalate, we undertook studies in a GLO-2 deficient mouse that we have created.

2. Materials and methods

2.1. Chemicals

Analytical grade chemicals were obtained from either Sigma-Aldrich Chemicals (St Louis, MO) or Fisher Scientific (Pittsburgh, PA).

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 Committee at University of Alabama at Birmingham, AL. Mice were maintained in a barrier facility with a 12-h light/dark cycle and an ambient temperature of $23 \pm 1\ ^\circ C$ and had free access to food (NIH-31 open formula mouse/ rat sterilizable diet, 7917) and water.

2.3. Generation of GLO-2 knockout mice using CRISPR/Cas9 methodology

With a goal of disrupting the early part of the gene by frame-shift mutations, we identified five CRISPR targets in three exons (1, 2 and 4) of the mouse *GLO-2* gene (Gene ID: ENSMUSG00000024158; Transcript ID: ENSMUST00000118788.7) using a web-based design tool (Benchling, Biology Software, 2017; retrieved from https://benchling. com) (Table 1). Single guide RNA (sgRNA) molecules were generated by modifying a cloning-free method previously described [13], and

recombinant *S.py* Cas9 protein was obtained from QB3 MacroLab at UC Berkeley. An injection solution with a final concentration of 50 ng/uL for each sgRNA and 250 ng/uL of Cas9 protein was used in pronuclear injections of C57BL/6J mouse zygotes followed by their transfer into CD1 pseudo-pregnant mice, as described previously [14]. Genotyping of G0/founder and F1 animals was done using PCR-heteroduplex mobility assay (HMA), as previously described [14]. DNA oligonucleotide primers used for PCR and the expected amplicon sizes are provided in Table 1. The PCR amplicons were analyzed by polyacrylamide gel electrophoresis and products with heteroduplexes were cloned and sequenced using the Sanger method to obtain the mutant allele information (previously described in detail [15]). Pronuclear injections of zygotes and their transfer into pseudo-pregnant female mice resulted in a litter of 19 pups. Genotyping by PCR-HMA showed that $10/19$ G_0 pups (52%; 6 males and 4 females) had indels in either one, two or all three targeted exons. We were able to obtain information of 9 mutant alleles carried by the 6 males using standard Sanger sequencing and found that the mutant alleles were predominantly deletions ranging from 5 bp to 45 bp; one animal carried three independent deletion alleles in exon 1 and two independent deletion alleles in exon 4. One exception to these deletion alleles is an indel resulting from a 23 bp deletion and insertion of 6 bp likely because of a 7 bp tandem duplicated sequence (GGGTCGC) at the beginning of exon 1. Table 1 summarizes the various deletions that were unambiguously identified.Upon breeding of the 6 males carrying indels with wildtype C57BL/6J females, we obtained G_1 litters from 5/6 breeding pairs. Based upon the type of allele transmitted and availability of male and female animals, we selected three G_1 heterozygote pairs carrying the same mutant allele ($\Delta45$ bp in exon 1 and $\Delta5$ bp in exon 4) to cross and obtain F2 litters. The heterozygote animals thus obtained were used for further breeding to generate GLO-2 KO and WT littermates for phenotyping.

2.4. Metabolic cage urine collections

Adult animals (3–10 months of age), were singly housed in Techniplast mouse metabolic cages for 24-h urine collections, as previously described [16]. Animals had free access to water and an ultra-low oxalate containing diet (TD.130032) designed by Envigo (Madison, WI), and thus urine produced by animals on this diet contains oxalate predominantly generated from endogenous synthesis.

2.5. Plasma creatinine and D-Lactate

D-lactate was quantified using a fluorometric D-lactate assay kit from LSBio (LSBio #LS-K884-100). Plasma creatinine was quantified using liquid chromatography tandem mass spectrometry (Waters 2795 LC-MS/MS, Waters Corporation, Milford, MA) by the UAB-UCSD O'Brien Center.

2.6. Liver function analysis

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzyme activity was measured by the following reaction conditons (AST, 100 mM L-Asparate, 2 mM 2-Oxoglutarate, 100 mM phosphate buffered saline, pH 7.4; ALT, 200 mM L-Alanine, 2 mM 2-Oxoglutarate, 100 mM phosphate buffered saline, pH 7.4). Briefly, 20 µl plasma was added to reaction mixture (final volume 100 µl) and incubated at 37 °C for 1 h. The reaction was stopped with trichloroacetic acid (10% v/v final). Production of oxaloacetate (AST activity) or pyruvate (ALT activity) was measured by ion chromatography with conductivity detection using a hydroxide based AS11-HC 4 µm column operating at 0.38 ml/min at 30 °C (Thermo Fisher Scientific Inc.).

2.7. Histology

Embedded liver tissues were sectioned at 5-µm thickness and stained

Table 1
Generation of GLO-2 knockout mice using CRISPR/Cas9 methodology.

CRISPR guides with their target sequences and off-target scores		
Guide	Target sequence (PAM)	Benchling off-target score
MmGLO-2Ex1-C1	GTCGCGGGTCGCTGTGTCTC (CGG)	83.4
MmGLO-2Ex1-C2	CACCTGCGCGCGCCGTGGTC (TGG)	95.7
MmGLO-2Ex2-C1	CACCCAGGAGGCCGCCATCG (TGG)	87.3
MmGLO-2Ex2-C2	TGGTTGCACTGGGTCCACGA (TGG)	85.8
MmGLO-2Ex4-C1	TATGGAGGTGATGACCGCAT (CGG)	85.2
PCR primer sequences and expected amplicon sizes		
Exon1-F1	TAAAGAGCAGGAGTTGGTTGG	500 bp
Exon1-R1	TCGCCACATCAGACGTTAAG	
Exon2-F1	GACTTACGTGGTCTCTGCTTTC	582 bp
Exon2-R1	CTCTGTGTAGCCTTGATTGTCC	
Exon4-F1	GCAGGTAAACAGTGGTTCTAGG	419 bp
Exon4-R1	ACACAGCAGCTGACTTTCTC	

Summary of mutant alleles found in G_0 animals				
Ear tag	Sex	Exon 1	Exon 2	Exon 4
28,913	M			Δ26 bp
28,915	M		Δ13 bp	Δ17 bp
28,917	M	Δ23 bp; +6 bp		
28,929	M	Δ6 bp	Δ20 bp	
28,930	M	Δ11 bp		Δ5 bp
		Δ45 bp (x2)		Δ18 bp

using hematoxylin and eosin (H&E), performed by the UAB Comparative Pathology Laboratory. Stained sections were imaged and analyzed at 40x magnification by light microscopy (Model BZ-×800, Keyence).

2.8. Analytical measurements

Oxalate and glycolate were measured by Ion Chromatography/Mass Spectrometry (ICMS), as previously described [17]. Liver glyoxal and methylglyoxal were measured by HPLC with UV detection following derivatization with o-phenylenediamine, as previously described [11]. Liver glyoxylate was measured by HPLC with UV detection following derivatization with phenylhydrazine, as previously described [18]. Liver glutathione and ascorbic acid were measured by HPLC with coulometric electrochemical detection as previously described [19].

2.9. Tissue collection

Tissue was collected at the same time of day (± 2 h), and animals fasted for 4 h prior to tissue collection. Animals were anesthetized with isoflurane, and blood collected from the heart followed by immediate freeze clamping of liver, kidney and brain tissue (in that order) in liquid nitrogen to limit tissue metabolite changes. Liver mitochondria were purified using fresh liver and Percoll density gradient ultracentrifugation, as previously described [20]. Tissues, including liver mitochondria, were stored in liquid nitrogen.

2.10. Western blot

Tissues were probe sonicated in 25 mM HEPES with 0.1% Triton X-100 (pH7.4) that contained Halt™ Protease Inhibitor Cocktail (Thermo Fisher Scientific, MA, USA). Protein extracts (25 μg) were boiled for 10 min in Laemmli buffer and separated by 8–16% SDS-PAGE (Invitrogen), before electrophoretic transfer onto Immobilon-FL membranes (# IPFL00010, Millipore Inc., Temecula, CA). The membranes were blocked in ODYSSEY blocking buffer (#927–5000), and then probed individually with a rabbit polyclonal anti-GLO-2 (1:1000 dilution, abcam #ab154108), and mouse monoclonal anti-GAPDH (1:7500 dilution, Abcam #ab8245). After washes, the blots were incubated for 1 h at room temperature with IRDye680RD goat anti-rabbit IgG or IRDye800CW goat anti-mouse IgG (1:20,000 dilution). Bands were detected using Odyssey Infrared Imaging System (Li-Cor Biosciences Lincoln NE).

2.11. GLO-2 enzyme activity

GLO-2 tissue activities were measured at 240 nm in a 96 well UV transparent plate, as previously described [21]. Tissues, including purified liver mitochondria, were homogenized by probe sonication in 10 mM phosphate buffered saline (PBS), pH7.4, at a ratio of 1 wt tissue to 9 vol PBS, and the assay performed with 1% tissue lysate in 50 mM Tris-HCl, pH 7.4 and 0.3 mM S-Lactoylglutathione.

2.12. Statistical analysis

All graphing and statistical analyses were conducted using GraphPad Prism v.8 (GraphPad Software Inc., San Diego, CA). The mean of three 24-hr urine analyte determinations was used to characterize excretions in each mouse. Data are expressed as mean ± SD. Independent student's *t*-test was used to compare sex and strain differences. The criterion for statistical significance was P < 0.05.

3. Results

3.1. GLO-2 KO mice are healthy

When heterozygous animals were mated, homozygous pups (GLO-2 KO) were born at the expected frequency. The absence of GLO-2 was confirmed by Western blots of liver, kidney, and brain tissues (Fig. 1A), and GLO-2 enzymatic activity (Fig. 1B). Purified liver mitochondria of wild type animals, but not GLO-2 KO mice, exhibited GLO-2 enzymatic activity (Fig. 1B). There was no detectable change in overall health of the animals as assessed by weight gain. Kidney function, as measured by fasting plasma creatinine, 24hr urinary creatinine and 24hr urinary volume excretion, was similar between GLO-2 KO and wild type animals (Table 2). Liver histology was normal in GLO-2 KO animals (data not shown). Plasma AST and ALT activities were not significantly different between strains or sexes, suggesting normal liver function in GLO-2 KO animals.

3.2. Biochemical analysis of GLO-2 KO

Glyoxal and methylglyoxal are converted by the glyoxalase system to glycolate and methylglyoxal, respectively. Glycolate levels in 24-h urine collections, liver tissue and plasma of GLO-2 KO mice and wild type litter mates showed no differences, except male GLO-2 KO animals had 35% significantly lower plasma glycolate compared with male wild type controls (Table 2). Female mice had higher excretions of urinary glycolate compared with males (Table 2), a sex difference we have previously reported [16]. Plasma D-Lactate levels were similar between strains and sexes (Table 2). We have previously shown that glyoxal can be a source of oxalate [11,12]; however measurements of 24-h urinary oxalate excretion, which reflects endogenous oxalate synthesis, showed no differences between GLO-2 KO mice and wild type controls (Table 2).

The liver is a significant source of glyoxal and methylglyoxal due to its high metabolic activity and the known generation of methylglyoxal from triose phosphate isomerase activity during glycolysis [1,2]. Furthermore, the liver exhibited the highest GLO-2 activity of the tissues tested (Fig. 1B), further supporting the liver as a significance source of glyoxal and methylglyoxal. However, measurements of liver concentrations of GSH, glyoxal, and methylglyoxal showed no differences between GLO-2 KO and wild type animals (Table 2). Glyoxal is a source of glyoxylate [3], and in the absence of GLO-2 activity glyoxylate levels might be expected to increase. However, there was no difference in liver glyoxylate concentration between male GLO-2 KO and wild type animals (3.1 ± 0.3 vs. 3.3 ± 0.3 nmol/g wet weight, P = 0.560), which is consistent with a lack of effect of GLO-2 deficiency on liver glyoxal levels (Table 2). Changes in GSH metabolism can impact levels of ascorbic acid, another major antioxidant in cells [22,23]. No difference in liver AA was observed between GLO-2 KO mice and wild type controls, which is consistent with a lack of effect of GLO-2 deficiency on GSH levels (Table 2).

4. Discussion

To better understand the functional role of GLO-2, and specifically its role in glycolate and oxalate synthesis, we generated a global GLO-2 KO using CRISPR/Cas9 techniques. This mouse developed normally and levels of glutathione, ascorbic acid, methylglyoxal and glyoxal in the liver were unchanged under baseline conditions. This lack of a unique phenotype in GLO-2 KO mice is consistent with mounting evidence that a functioning glyoxalase system may not be required for optimal health. The evidence was recently reviewed by Morganstern and colleagues [24]. They identified studies showing that GLO-1 can be knocked out without visible repercussions for cells and tissues, similar to what we found for GLO-2 in this study. Furthermore, these results are consistent with those observed in Schwann cells modified not to express GLO-1 [25]. Morgenstern and associates observed no change in methylglyoxal concentration nor an increased glycation of susceptible protein residues [25]. They also provided evidence that aldose reductase, which has been shown to convert methylglyoxal to non-toxic compounds [26], can compensate for the loss of GLO-1 activity. We speculate that the results we obtained in mice lacking GLO-2 activity are also due to
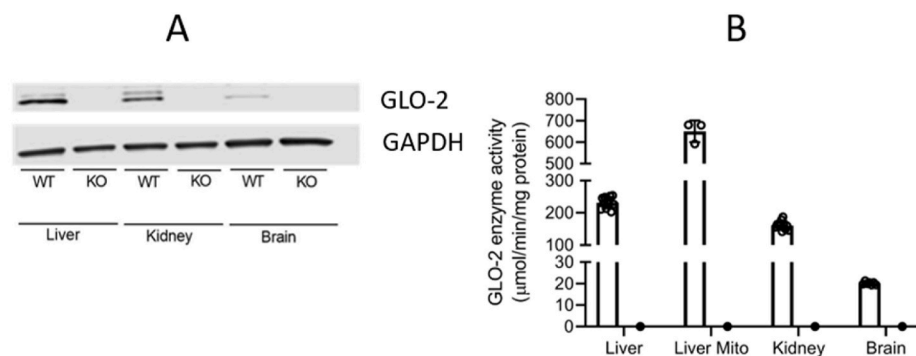
Fig. 1. Western blot (A) and GLO-2 enzymatic activity (B) of mouse tissues. Panel A. WT, wild-type; KO, GLO-2 knockout. Panel B,. ○, wild type animals; ●, GLO-2 KO animals. Male and female wild type animals showed no difference in GLO-2 activity in liver, kidney and brain lysates, and thus data were combined (n = 7–11). Liver Mito, purified male liver mitochondria (n = 3).

Table 2
Biochemical characteristics of GLO-2 deficient mice.

	Male		Female	
	Wild Type	GLO-2 KO	Wild Type	GLO-2 KO
Age (weeks)	26.5 ± 13.9	25.5 ± 14.0	24.6 ± 13.9	25.3 ± 14.1
Body weight (g)	34.5 ± 5.8	32.2 ± 5.2	23.5 ± 4.3*	21.9 ± 3.1*
24-hr Urine volume (ml)	2.1 ± 0.4	2.0 ± 0.6	1.7 ± 0.4*	1.6 ± 0.4
24-hr Creatinine (mg)	0.52 ± 0.07	0.50 ± 0.06	0.36 ± 0.03*	0.35 ± 0.05*
24-hr Urine Oxalate (μg/ mg Creatinine)	162.5 ± 19.5	151.9 ± 22.6	168.9 ± 22.8	159.1 ± 20.2
24-hr Urine Glycolate (μg/ mg creatinine)	105.5 ± 18.4	115.3 ± 26.1	441.0 ± 130.6*	441.1 ± 123.0*
Liver glycolate (nmol/g)	60.2 ± 12.4	57.8 ± 12.7	68.0 ± 11.9	59.6 ± 10.9
Plasma glycolate (μM)	**46.4 ± 5.8**	30.1 ± 2.9#	48.7 ± 2.9	41.1 ± 3.6
Liver ascorbic acid (μmol/ g)	1.2 ± 0.2	1.3 ± 0.3	1.3 ± 0.2	1.2 ± 0.2
Liver GSH (μmol/g)	5.0 ± 0.1	5.4 ± 0.5	5.2 ± 1.0	5.4 ± 0.7
Liver Glyoxal (nmoles/g)	3.7 ± 1.0	4.6 ± 0.6	4.8 ± 0.6	4.5 ± 0.6
Liver Methylglyoxal (nmoles/g)	3.1 ± 0.4	3.7 ± 0.5	3.4 ± 0.2	3.5 ± 0.3
Plasma Creatinine (mg/dL)	0.048 ± 0.016	0.055 ± 0.010	0.053 ± 0.010	0.048 ± 0.013
Plasma D-Lactate (μM)	4.70 ± 0.76	4.73 ± 0.94	4.43 ± 0.73	4.36 ± 1.24
Plasma AST (μmol/min/L)	97.3 ± 33.6	64.3 ± 13.9	95.0 ± 35.6	74.4 ± 18.7
Plasma ALT (μmol/min/L)	7.8 ± 8.1	3.5 ± 1.0	7.7 ± 6.1	4.0 ± 1.1

Data expressed as mean ± SD, n = 5–16 per group. Liver tissue metabolites are expressed per gram wet weight tissue. #, strain difference between GLO-2 KO and wild type animals (P < 0.05); * Sex difference (P < 0.05). GSH, reduced glutathione.

compensatory mechanisms. Two studies in mice deficient in GLO-1 have also revealed a lack of major changes [27,28]. Studies addressing the concentration effects of methylglyoxal may also be informative. One study demonstrated in breast cancer and glioblastoma cell lines that the maintenance of sub-toxic levels of methylglyoxal inhibited tumor growth, but low concentrations were pro-tumorigenic [29]. Another study showed increased formation of methylglyoxal extended the life-span of C.elegans [30]. These and other studies suggest that sub-toxic doses of methylglyoxal can have beneficial effects, a process called "hormesis" [24,31]. This effect offers an explanation for why the loss of GLO-1 and GLO-2 activities do not create pathological responses.

Measurements of glycolate, the product of glyoxal metabolism by the glyoxalase pathway, revealed lower plasma glycolate in GLO-2 KO animals, although this was only significant in males. These data confirm glyoxal detoxification as a function of the glyoxalase system *in vivo*, and indicate glyoxal production as a significant contributor to endogenous glycolate synthesis. Of note, despite a lower plasma glycolate, differences in liver and urinary glycolate excretion were not observed between GLO-2 KO and wild type controls (Table 1). This could be partly explained by the presence of glycolate oxidase in liver which efficiently oxidizes glycolate to glyoxylate [32], and reabsorption of >90% of the filtered glycolate in rodent kidney [33]. The reasons for the sex dependent decrease in plasma glycolate with GLO-2 deficiency are not clear, but may be due to the higher expression of hepatic glycolate oxidase activity in male mice compared with females [34]. Differences in renal and gastrointestinal (secretion) glycolate handling, and non-hepatic tissue stores may also be contributory.

Plasma D-lactate levels were similar between GLO-2 KO and wild type controls (Table 2). This finding would suggest other sources, including diet and gut bacteria [35], play a more important role in influencing host D-lactate levels than GLO-2 activity. Liver methylglyoxal levels were not different between GLO-2 KO and wild type animals, and thus further examination of whether methylglyoxal can be metabolized to D-lactate in the absence of GLO-2 is warranted. D-lactate dehydrogenase, a mitochondrial protein expressed in a wide range of tissues, maintains D-lactate levels at low levels [35]. D-lactate dehydrogenase expression in GLO-2 KO also warrants further investigation.

There may be differences in glyoxal synthesis between male and female animals. Glyoxal can be produced via autoxidation of carbohydrates and ascorbate, degradation of glycated proteins, and lipid peroxidation [36–38], although the contributions of these various processes to glyoxal synthesis have not yet been fully defined. This appears to be due to its high reactivity and low concentration in tissues. Studies have suggested oxidative stress levels are higher in males compared with females [39–42], and thus it is possible glyoxal synthesis is also higher in males.

Glycolate is potentially an important source of oxalate synthesis, and thus lowering glycolate synthesis may have relevance in individuals with calcium oxalate kidney stone disease [43,44]. In this study, there was no difference in 24-h urinary oxalate excretion between GLO-2 KO and wild type mice, despite 35% lower plasma glycolate in GLO-2 KO male mice. These data suggest glycolate and glyoxal are not important sources of oxalate in healthy mice. Our previous in vitro studies in human erythrocytes and cultured human hepatoma cells showed glyoxal can be a source of oxalate; however, these studies were conducted with supraphysiological concentrations of glyoxal, where other enzymes, such as aldehyde dehydrogenase [45], can oxidize glyoxal to glyoxylate, a major precursor of oxalate synthesis [46]. It is possible that this pathway could be augmented under certain conditions that we did not assess in this study such as heightened oxidative stress.

There are several limitations to our study. It is possible that more serious consequences of the lack of GLO-2 activity will emerge with the

stresses of aging or conditions such as diabetes and obesity. In addition, the exploratory scope of this study did not permit an assessment of whether absence of GLO-2 activity influences cancer susceptibility or progression which may be relevant since GLO-2 activity has been shown to be regulated by tumor suppressor genes [24,47,48]. Our biochemical analysis did not include S-D-glycolylglutathione or S-D-lactoylglutathione, which would be expected to increase in the absence of GLO-2. A limitation of this study is our biochemical analysis did not include S-D-glycolylglutathione or S-D-lactoylglutathione. It is also possible that S-D-Lactoylglutathione is detoxified by an alternative enzyme and warrants further examination.

4.1. Conclusion

Metabolite levels in animals with a global deficiency in GLO-2 activity are otherwise normal and showed a gender dependent decrease in plasma glycolate. This suggests that the glyoxylase pathway may not be essential under normal or non-stressful states.

Declaration of competing interest

The authors have no conflict of interests.

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101138.

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