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Hepatic metabolic adaptation in a murine model of glutathione deficiency

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Abstract

Glutathione (GSH), the most abundant cellular non-protein thiol, plays a pivotal role in hepatic defense mechanisms against oxidative damage. Despite a strong association between disrupted GSH homeostasis and liver diseases of various etiologies, it was shown that GSH-deficient glutamate-cysteine ligase modifier subunit (*Gclm*)-null mice are protected against fatty liver development induced by a variety of dietary and environmental insults. The biochemical mechanisms underpinning this protective phenotype have not been clearly defined. The purpose of the current study was to characterize the intrinsic metabolic signature in the livers from GSH deficient *Gclm*-null mice. Global profiling of hepatic polar metabolites revealed a spectrum of changes in amino acids and metabolites derived from fatty acids, glucose and nucleic acids due to the loss of GCLM. Overall, the observed low GSH-driven metabolic changes represent metabolic adaptations, including elevations in glutamate, aspartate, acetyl-CoA and gluconate, which are beneficial for the maintenance of cellular redox and metabolic homeostasis.

Keywords

Glutathione; Glutamate cysteine ligase; Fatty liver disease; Steatosis; Metabolomics

1. Introduction

Glutathione (GSH) is the most abundant cellular non-protein thiol, attaining concentrations in the high millimolar range in liver. It is involved in a variety of cellular functions,

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Appendix A. Supplementary data

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including serving as a direct scavenger of free radicals and as a cofactor for antioxidant and xenobiotic metabolizing enzymes, forming mixed disulfide redox couples, mediating post-translational protein modification, and regulating nitric oxide homeostasis [1,2]. Because of its abundance, GSH plays a pivotal role in hepatic defense mechanism against toxicities arising from exposure to excessive amounts of endogenous and exogenous electrophiles [3]. As such, studies in human subjects and animal models have implicated an important role for disrupted GSH homeostasis in the pathogenesis of liver diseases of various etiologies, including non-alcoholic fatty liver disease [4], alcoholic liver disease [5], and drug-induced liver injury [6].

GSH is synthesized by the sequential actions of glutamate-cysteine ligase (GCL) and glutathione synthase. GCL is the rate-limiting enzyme in GSH biosynthesis and, in higher eukaryotes, is a heterodimer comprising a catalytic (GCLC) and a modifier subunit (GCLM) [7–9]. GCLC possesses all the catalytic activity, whereas GCLM serves to optimize the catalytic properties of the GCL holoenzyme [7]. Global disruption of the mouse *Gclc* gene is incompatible with life [10], whereas global disruption of the mouse *Gclm* gene generates a mouse model (*Gclm*-null) that exhibits no overt phenotype despite having only 10–40% of normal tissue GSH levels. Thus, the *Gclm*-null mice represent a useful model for studying chronic GSH deficiency [9].

We and other groups have used *Gclm*-null mice to elucidate the role of GSH in hepatic responses to numerous insults. *Gclm*-null mice, although having 15% of the wild-type (WT) GSH level in the liver, are unexpectedly resistant to the development of hepatic steatosis induced by high fat diet [11], methionine-choline deficient diet [12], 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [13] and chronic alcohol consumption [14]. Biochemical and gene microarray studies indicate that this resistance is generally accompanied by hepatic oxidative stress, enhanced metabolic capacity and antioxidant responses, and inhibition of lipid and cholesterol biosynthesis [11–14]. These findings indicate that chronic GSH deficiency in the *Gclm*-null mouse liver is associated with beneficial metabolic and stress response, the molecular details of which are yet to be clearly defined. The aim of the current study is to utilize the *Gclm*-null mouse model to characterize the intrinsic metabolic signature in the liver as they relate to chronic GSH deficiency. To achieve this, global profiling of hepatic polar metabolome was performed in the absence of any dietary or chemical exposures.

2. Materials and methods

2.1. Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.2. Animals

Gclm-null and WT littermates were bred in-house from a line previously characterized [9] and backcrossed onto a C57BL/6J background. Mice were group-housed, maintained on a 12-h light-dark cycle, with free access to regular chow and water *ad libitum*. Ten-week old

male mice were euthanized by CO₂ asphyxiation. Their livers were harvested, flash frozen in liquid nitrogen and stored at -80 °C for latter metabolomics and gene expression analyses. All animal procedures were approved by and conducted in compliance with the Institutional Animal Care and Use Committee (IACUC) of Yale University and of the University of Colorado Anschutz Medical Campus.

2.3. Extraction of hepatic polar metabolites

Frozen liver tissues were homogenized and extracted for polar and non-polar metabolites using modified Bligh-Dyer method [15]. Before extraction, α -aminopimelic acid (10 μ M) was spiked into each sample to normalize variations in extraction efficiency. The aqueous layer containing polar metabolites was vacuum-dried and reconstituted in buffer acetonitrile/water/methanol (65:30:5) mixture containing difluoromethylornithine (5 μ M).

2.4. Untargeted metabolic profiling

Metabolic signature in liver extracts was analyzed by electrospray ionization mass spectrometry (ESI-MS) coupled with hydrophilic interaction liquid chromatography (HILIC) using a HILIC BEH amide column (Waters Corp, Milford, MA) as described previously [16]. A data matrix comprising m/z values, retention times and normalized peak areas was generated following the normalization of individual ion intensities by total ion counts or liver weights (gm). Unsupervised and supervised analyses of metabolomic signatures were performed using the SIMCA-P12 + software (Umetrics, Kinnelon, NJ) as described previously [16]. A list of ions showing significant ($P < 0.05$) difference in abundance between genotypes (N = 4 per genotype) was generated and used for further identification and quantitation. Data mining for metabolite identification was performed using MassTRIX (<http://metabolomics.helmholtz-muenchen.de/masstrix/>) as previously described [16].

2.5. Targeted metabolite quantitation

Selected metabolites in the liver extract were quantitated by multiple reaction monitoring using ESI triple-quad platform coupled with 2.1 $\times$ 50 mm Acquity UPLC HILIC BEH amide column as described previously [16]. α -Aminopimelic acid (5 μ M) was used as an internal standard to normalize area under the peak (response). The concentration of each metabolite was determined from a calibration curve (derived from serially-diluted solutions of an authentic standard) and normalized to liver weight (gm). Results are expressed as mole per gm liver weight (N = 4 per genotype).

2.6. Quantitative real-time PCR (Q-PCR)

Total RNA was isolated from frozen liver tissues using TRIzol Reagent™ according to the manufacturer's protocol. cDNA was synthesized using the iScript cDNA synthesis kit (BioRad, Hercules, CA) according to the manufacturer's instructions using 1 μ g total RNA in a 20 μ l reaction volume. Q-PCR reaction mixtures contained 0.5 μ l cDNA, SYBR Green Supermix (BioRad, Hercules, CA), and 0.15 μ M gene-specific primer sets in a total volume of 10 μ l. Sequences of Q-PCR primers can be found in Table S1. Expression of β 2-microglobulin (*B2m*) was used for normalization of cycle threshold (CT) data according to

the CT method [17]. Relative mRNA levels of individual genes in *Gclm*-null mice were reported as fold of the expression in WT mice (N = 5–6 per genotype).

2.7. Statistic analysis

Qlucore Omics Explorer program version 3.4 (Qlucore AB, Sweden) was used to generate the hierarchy clustering heat map of differential abundance of metabolites between WT and *Gclm*-null mice; $P < 0.05$ by two-tailed Student's unpaired *t*-test was used as the significance cut-off criteria. Pearson correlation analyses of GSH versus other metabolites were analyzed using Graphpad Prism software (San Diego, CA) by two-tailed Student's *t*-test; Pearson *r* values and 95% confidence intervals were presented for each correlation pair; $P < 0.05$ was considered significant. Group differences in individual metabolite quantitation (N = 4 per genotype) and gene expression (N = 5–6 per genotype) were analyzed using Graphpad Prism software (San Diego, CA) by two-tailed Student's unpaired *t*-test; $P < 0.05$ was considered significant.

3. Results

3.1. Overview of global metabolic profiling of the liver

Untargeted profiling of liver polar compounds by HILIC-ESI-MS revealed significant difference between WT and *Gclm*-null mice. Ions showing a significant difference in abundance were selected and the corresponding metabolites were identified. Using authentic standards, a panel of 72 compounds were quantitated, including those identified by profiling and others belonging to related biochemical pathways. UPLC/MS quantitation confirmed 14 metabolites (including GSH and GSSG) in the liver that showed differential abundance between WT and *Gclm*-null mice (Fig. 1A). Pearson correlation analysis of these molecules revealed two clusters of metabolites that were either positively (Pearson $r > 0.7$; $n = 5$) or inversely (Pearson $r < -0.7$; $n = 7$) correlated with GSH concentrations (Fig. 1B) in this sample pool (N = 8 in total).

3.2. Low GSH-driven changes in amino acids and metabolites derived from fatty acids, glucose and nucleic acids

By UPLC quantitation, GSH and GSSG concentrations in the *Gclm*-null liver were $< 5\%$ and $\sim 24\%$, respectively, of those in the WT liver (Fig. 2A); the resultant GSH/GSSG ratio was reduced > 6 -fold in the *Gclm*-null liver (Fig. 2A). Relative to WT mice, *Gclm*-null mice had lower hepatic levels of butyryl-carnitine ($\sim 52\%$), thiamine ($\sim 63\%$), lactate ($\sim 67\%$) and S-adenosylmethionine ($\sim 75\%$) (Fig. 2B). Eight metabolites were found to be more abundant in *Gclm*-null livers than WT livers (Fig. 2C), including glutamate (~ 1.7 -fold), aspartate (~ 1.3 -fold), choline (~ 1.8 -fold), gluconate (~ 2.2 -fold), deoxyuridine (~ 2.3 -fold), cytidine (~ 1.6 -fold), acetyl-CoA (~ 1.6 -fold), and N ϵ -acetyllysine (~ 1.5 -fold). Collectively, these compounds represented metabolites of multiple metabolic pathways involved in the metabolism of amino acids, glucose, fatty acids, and nucleic acids.

3.3. Gene expression alterations linked to metabolic changes

To explore the molecular mechanisms underlying the above-noted metabolic changes, the expression of selective genes that are directly or indirectly involved in related pathways were

examined (Fig. 3). These genes were also selected based on our previous gene microarray analysis [11,13]. In the *Gclm*-null liver, mRNAs of genes involved in lipogenesis, including sterol regulatory element-binding protein 1 (*Srebfl*), fatty acid synthase 1 (*Fasn1*), fatty acid desaturase 2 (*Fads2*) and acetyl-CoA carboxylase β (*Acacb*), were decreased; whereas mRNAs of genes involved in fatty acid transport and oxidation, including carnitine palmitoyltransferase 1 (*Cpt1*), uncoupling protein 1 (*Ucp*), cytochrome P450 4A14 (*Cyp4a14*) and solute carrier family 22 member 5 (*Slc22a5*), were increased. The expressions of genes encoding two components of the pyruvate dehydrogenase complex, namely pyruvate dehydrogenase α -1 (*Pdha1*) and dihydrolipoamide dehydrogenase (*Dld*), were not altered in the *Gclm*-null liver. We examined three glutamate metabolizing genes and the glutaminase 2 (liver isoform, *Gls2*) mRNA was reduced. Among genes involved in amino sugar and nucleotide sugar metabolism, the phosphoglucomutase 3 (*Pgm3*) gene was suppressed and UDP-glucose 6-dehydrogenase (*Ugdh*) gene was induced. No genes involved in nucleic acids metabolism were found altered in the *Gclm*-null liver (data not shown).

4. Discussion

Despite the strong association between oxidative stress and liver diseases of various etiologies, the GSH-deficient *Gclm*-null mice are resistant to fatty liver development induced by several environmental and dietary insults [18]. Such protection against steatosis development is likely to be due to the suppression of lipogenesis genes [11,13,14], enhanced basal metabolic rates [11], and/or activation of nuclear-factor-erythroid 2-related-factor 2 (NRF2) and AMP-activated protein kinase (AMPK) pathways [14]. At the level of cellular metabolism, a previous lipidomics study demonstrated that total hepatic contents of triglycerides and cholesterol esters in naïve *Gclm*-null mice are approximately 50% of the levels in WT mice [14]. This current study focused on characterizing changes in the hepatic polar metabolome of naïve *Gclm*-null mice. Our results show an overall small spectrum of changes in amino acids and metabolites derived from fatty acids, glucose and nucleic acids to be associated with chronic GSH deficiency in the liver.

Glutamate and aspartate are two amino acids found to exist in higher concentrations in the liver of *Gclm*-null mice. Glutamate is a precursor for GSH *de novo* biosynthesis. Compromised capacity of this pathway may contribute to accumulation of glutamate. Hepatic enrichment of glutamate is of great relevance to the *Gclm*-null mouse model because it may compensate for the loss of the GCLM subunit by meeting the higher demand for this substrate by the GCLC catalysis [7]. It is important to appreciate that both glutamate and aspartate are crucial components of the malate-aspartate shuttle (MAS), a system that transports glycolysis-derived electrons from the cytoplasm to the mitochondrial matrix for oxidative phosphorylation [19,20]. This shuttle system has been proposed to act as an important regulatory mechanism in glycolysis and lactate metabolism [21]. Interestingly, the lactate concentration in the *Gclm*-null liver was about 33% lower than that in the WT liver. Based on these data, one may speculate that the concomitant increase of glutamate and aspartate levels in the *Gclm*-null liver may lead to accelerated MAS flux and thereby promote pyruvate oxidation while inhibiting lactate production. The combination of the above-noted metabolic changes would be expected to result in enhanced mitochondrial

respiration, which was implicated in protecting against high-fat diet induced steatosis and insulin resistance [11].

Acetyl-CoA represents a central metabolic node that connects cellular metabolism of amino acids, glucose and fatty acids [22]. In mitochondria, acetyl-CoA is a product of fatty acid β -oxidation, pyruvate oxidation and catabolism of branched-chain amino acids [22]. Mitochondrial acetyl-CoA can feed the tricarboxylic acid (TCA) cycle for ATP generation or can be shuttled to the cytosol to serve as a substrate for biosynthesis of lipids, cholesterol, steroids, or amino acids [22]. In *Gclm*-null mice, a 60% increase in hepatic acetyl-CoA levels was observed relative to the WT mice. This may be partially attributed to the suppression of lipogenesis genes and simultaneous induction of fatty acid oxidation genes seen in the *Gclm*-null liver. Enhanced pyruvate oxidation may as well contribute to a net increase in acetyl-CoA levels in the *Gclm*-null liver. The elevated N ϵ -acetyllysine levels observed in the *Gclm*-null liver in the present study would also be expected, given that acetylation of lysine can serve as an important metabolic sink of acetyl-CoA [22]. Importantly, a potential increase of acetyl-CoA entering the TCA cycle would be metabolically compatible with a robust mitochondrial respiratory activity in the *Gclm*-null liver as reported previously [11].

Gluconate is considered a human orphan metabolite since its metabolism and physiological functions in humans are largely unknown [23]. Recent computational metabolic modeling studies suggest that gluconate may have a significant impact on cellular metabolism and GSH recycling due to its metabolic connection with the pentose phosphate pathway [24]. The increased levels of gluconate in the livers of *Gclm*-null mice may therefore reflect an additional metabolic adaptation that helps to maintain GSH redox homeostasis in response to chronic GSH deficiency.

When compared with WT mice, *Gclm*-null mice showed a reduction in S-adenosylmethionine (SAM) levels and an elevation in choline levels in the liver. SAM is the primary methyl donor for cellular methylation process. In the liver, SAM metabolism is biochemically linked to GSH biosynthesis through the methionine cycle and the transsulfuration pathway [25]. Choline also serves as an important methyl donor, via its oxidation to betaine, to the methionine cycle for SAM regeneration [26]. The observed changes in the hepatic methyl donor pool in *Gclm*-null mice suggest that chronic GSH deficiency may modulate cellular transmethylation process. Differential abundance was also noted for thiamine, butyrlcarnitine, deoxyuridine and cytidine in the liver between WT and *Gclm*-null mice. It appeared that low GSH was associated with decreased concentrations of thiamine and butyrlcarnitine, but increased concentrations of Deoxyuridine and cytidine. Thiamine, in its diphosphate form, serves as an essential cofactor for several key enzyme complexes in glycolysis and the TCA cycle. Butyrlcarnitine is a short-chain acylcarnitine that can be derived from both fatty acids oxidation and amino acids catabolism. Deoxyuridine and cytidine are pyrimidine nucleosides. Whether alterations in these metabolites have any impact on the overall metabolic phenotype of the *Gclm*-null mice is unclear.

Taken together, using global metabolic profiling and subsequent targeted quantitation, we herein report changes in the polar metabolome of the liver associated with chronic GSH deficiency. The observed metabolic changes represent beneficial adaptations at two layers. First, elevations in glutamate (that can boost GSH biosynthesis) and gluconate (that may promote a faster recycling of GSH) are beneficial for the maintenance of cellular redox homeostasis. Second, the concomitant increase of glutamate and aspartate (that can facilitate electron transfer into mitochondria through MAS) and a net increase in acetyl-CoA (resulting from suppression of lipogenesis and inductions of fatty acid oxidation and pyruvate oxidation) are beneficial for efficient mitochondrial biogenesis and metabolism. It is well established that mitochondrial oxidative dysfunction plays a key pathogenic role in the development of hepatic steatosis [27]. Thus, it is a testable hypothesis that above-noted metabolic adaptations are mechanistically involved in protecting *Gclm*-null mice against hepatic steatosis induced by environmental or dietary insults [18]. Future metabolomics studies under conditions of particular exposures are warranted to investigate this hypothesis with greater molecular details. The current study also showed that majority of observed metabolic changes could not be explained by gene expression changes in related biochemical pathways, suggesting that post-transcriptional mechanisms may play an important role in mediating these metabolic changes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

<i>Acacb</i>	acetyl-CoA carboxylase β subunit
AMPK	AMP-activated protein kinase
<i>B2m</i>	β 2-microglobulin
<i>Cpt1</i>	carnitine palmitoyltransferase 1
CT	cycle threshold
<i>Cyp4a14</i>	cytochrome P450 4A14
<i>Dld</i>	dihydrolipoamide dehydrogenase
ESI-MS	electrospray ionization mass spectrometry
<i>Fads2</i>	fatty acid desaturase 2
<i>Fasn1</i>	fatty acid synthase 1

GCL	glutamate-cysteine ligase
GCLC	glutamate-cysteine ligase catalytic subunit
GCLM	glutamate-cysteine ligase modifier subunit
Gls	glutaminase (kidney isoform)
Gls2	glutaminase 2 (liver isoform)
Glud1	glutamate dehydrogenase 1
GSH	reduced glutathione
GSSG	oxidized glutathione
HILIC	hydrophilic interaction liquid chromatography
IACUC	Institutional Animal Care and Use Committee
MAS	malate-aspartate shuttle
NRF2	nuclear factor erythroid 2-related factor 2
Pdha1	pyruvate dehydrogenase α 1 subunit
Pgm3	phosphoglucomutase 3
Q-PCR	quantitative real-time PCR
SAM	S-adenosylmethionine
Slc22a5	solute carrier family 22 member 5
Srebf1	sterol regulatory element-binding protein 1
TCA	tricarboxylic acid
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Ucp	uncoupling protein 1
Ugdh	UDP-glucose 6-dehydrogenase
Ugp2	UDP-glucose pyrophosphorylase 2
UPLC	ultra-performance liquid chromatography
WT	wild-type

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Fig. 1. Overview of metabolic profiling of liver polar metabolites.

Untargeted metabolic profiling was conducted in the aqueous phase of liver extracts from wildtype (WT) and *Gclm*-null mice (male, 10 wk old, N = 4/genotype) using HILIC/ESI-MS. Metabolites showing a significant difference in abundance were selected for subsequent targeted quantitation by UPLC-MS/MS. (A) Hierarchy clustering heat map of 14 metabolites showing differential abundance ($P < 0.05$, two-tailed Student's unpaired *t*-test) between WT and *Gclm*-null mice. The color key represents values of relative abundance. (B) Pearson correlation analysis of hepatic concentrations of GSH versus other 71 metabolites revealed that concentrations of 12 metabolites were highly correlated with GSH levels ($P < 0.05$, two-tailed Student's *t*-test).

Fig. 2. Differential abundance of 14 polar metabolites in WT and *Gclm*-null livers.

Hepatic concentrations of (A) GSH and GSSG, and metabolites that were less (B) or more (C) abundant in the *Gclm*-null liver than in the WT liver. Metabolite concentrations were normalized to liver weight. Data are presented as floating bars (min to max, line at mean; N = 4/genotype) showing individual data points from WT (closed circles) and KO (open circles) mice. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by Student's unpaired *t*-test, compared to WT mice.

Fig. 3. Alterations in gene expressions involved in related biochemical pathways.

Relative mRNA abundance was measured by Q-PCR and reported as fold of expression in the livers of WT mice normalized to 1 (horizontal dashed line). Results are expressed as mean $\pm$ S.D. (N = 5–6/genotype). *: $P < 0.05$ by Student's unpaired t -test, compared to the abundance in WT mice. *Srebf1*, sterol regulatory element-binding protein 1; *Fasn1*, fatty acid synthase 1; *Fads2*, fatty acid desaturase 2; *Acacb*, acetyl-CoA carboxylase beta; *Cpt1*, carnitine palmitoyltransferase 1 (Liver); *Ucp*, uncoupling protein 1; *Cyp4a14*, cytochrome p450 4A14; *Slc22a5*, solute carrier family 22 member 5; *Pdha1*, pyruvate dehydrogenase alpha-1; *Dld*, dihydrolipoamide dehydrogenase; *Glc*, glutaminase (Kidney isoform); *Glc2*, glutaminase (liver isoform); *Glud1*, glutamate dehydrogenase 1; *Gnpda2*, glucosamine-6-phosphate deaminase 2; *Pgm3*, phosphoglucomutase 3; *Ugp2*, UDP-glucose pyrophosphorylase 2; *Ugdh*, UDP-glucose 6-dehydrogenase.