J Biomol NMR (2011) 49:165–173
DOI 10.1007/s10858-011-9485-5

ARTICLE

Metabolic profiling of vitamin C deficiency in *Gulo*−/− mice using proton NMR spectroscopy

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Received: 15 October 2010 / Accepted: 26 November 2010 / Published online: 1 March 2011

Abstract Nutrient deficiencies are an ongoing problem in many populations and ascorbic acid is a key vitamin whose mild or acute absence leads to a number of conditions including the famously debilitating scurvy. As such, the biochemical effects of ascorbate deficiency merit ongoing scrutiny, and the *Gulo* knockout mouse provides a useful model for the metabolomic examination of vitamin C deficiency. Like humans, these animals are incapable of synthesizing ascorbic acid but with dietary supplements are otherwise healthy and grow normally. In this study, all vitamin C sources were removed after weaning from the diet of *Gulo*−/− mice (n = 7) and wild type controls (n = 7) for 12 weeks before collection of serum. A replicate study was performed with similar parameters but animals were harvested pre-symptomatically after 2–3 weeks. The serum concentration of 50 metabolites was determined by quantitative profiling of 1D proton NMR spectra. Multivariate statistical models were used to describe metabolic changes as compared to control animals; replicate study animals were used for external validation of the resulting models. The results of the study highlight the metabolites and pathways known to require ascorbate for proper flux.

Keywords Metabolomics · Scurvy · Ascorbic acid · Glutathione · Vitamin C · Vitamin deficiency

Introduction

Vitamin C deficiency in humans results in scurvy, a debilitating condition that is well known historically as it affected sailors that were circumnavigating the globe. In 1747, in what was probably the first clinical trial, the Scottish naval surgeon James Lind discovered that lemon and orange juice could be used to prevent or reverse the effects of scurvy. It took until the 1800s however for this cure to become widely accepted (Harvie 2005). The chemical structure of the active ingredient vitamin C was conclusively established in 1932 (Svirbely and Szent-Györgyi 1932). Most animals, including mice, are capable of synthesizing ascorbic acid to satisfy their endogenous needs; it is produced through a well-defined metabolic pathway from D-glucose and D-galactose. Guinea pigs, apes and humans however have lost the ability to synthesize ascorbic acid, because they do not have a functional L-gulonolactone oxidase gene (*GULO*), which codes for the enzyme that catalyses the final step in the biosynthesis of vitamin C (Chatterjee et al. 1961). Fortunately many fresh fruits and vegetables contain high concentrations of ascorbic acid and hence humans and simians can acquire a sufficient supply of this vitamin through regular consumption of appropriate foods (Nishikimi and Yagi 1991; Naidu 2003). It is noteworthy that humans still possess a nonfunctional *GULO* pseudogene on chromosome 8p21.1, with several introns missing and numerous insertions, and

Electronic supplementary material The online version of this article (doi:10.1007/s10858-011-9485-5) contains supplementary material, which is available to authorized users.

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it has been estimated that most primates lost the ability to synthesize their own vitamin C over 40 million years ago (Nishikimi et al. 1994). Similarly, guinea pigs still contain the highly mutated remnants of an ancestral *GULO* gene (Nishikimi et al. 1992).

Without access to environmental sources of vitamin C, humans suffer system-wide failures in health; long term, severe shortages lead to scurvy, which is invariably fatal if untreated. Some researchers have referred to this 'hypoascorbemia' as a 'public inborn error of metabolism' (Stone 1979). Interestingly several studies have shown that gene therapy can restore the ability of human cells to produce ascorbic acid endogenously (Ha et al. 2004; Li et al. 2008).

High levels of vitamin C are thought to have beneficial effects on various diseases ranging from cancer, all the way to the common cold (for a review see Naidu 2003). In order to facilitate studies of the effects of vitamin C on the progression of various diseases in mouse models, a *Gulo*−/− knockout strain has been developed (Maeda et al. 2000). Like humans, this mouse strain needs to obtain vitamin C from its food or drinking water. As a result, they suffer several deficiencies, including partial loss of neutrophil function and increased oxidative stress during development (Vissers and Wilkie 2007; Harrison et al. 2010). By crossing *Gulo* knockout mice with other disease model strains, the protective effects of ascorbate can be investigated in a controlled environment. In this work we have used the *Gulo*−/− strain to determine the effects of vitamin C deficiency on the metabolic profile of these mice.

Proton Nuclear Magnetic Resonance (^{1}H NMR) analysis of serum has the advantage of quantitative, simultaneous measurements for many metabolites (Weljie et al. 2007; Shearer et al. 2008; Schicho et al. 2010; Dunn et al. 2011). This approach facilitates interpretation and visualization of the resulting metabolic snapshot and is widely used for mouse model studies (Griffin 2006). The spectral area derived from each metabolite can be segregated and attributed to individual metabolites. Targeted profiling provides a significant advantage in metabolite identification by virtue of correlated and corroborated grouping in peaks based on latent biochemical knowledge. We have used the Chenomx Profiler approach (Weljie et al. 2006) to identify specific metabolites and translate peak intensities into metabolite concentrations using a library of spectral profiles. The resulting metabolite concentrations provide a simplified dataset on which to perform multivariate statistical analysis, which is easier to interpret and assess for significance.

These data were further analyzed using Orthogonal Projection to Latent Structures (OPLS), a multivariate analysis technique that can separate the metabolic variation in samples into noise and two classes of structured variation: patterns of change which are correlated to the onset of vitamin deficiency, and coherent patterns of change which are however not correlated to a particular phenomenon (Trygg and Wold 2002). As such, OPLS can be used to identify and remove variation between sample batches and highlight metabolic changes of interest.

Experimental procedures

Mouse maintenance

Procedures were approved by the University of Calgary Animal Care and Use Committee and abided by the Canadian Association for Laboratory Animal Science guidelines for animal experimentation. Animals were maintained in a humidity-controlled room with a 12-h light:dark cycle. Following weaning (3 week of age), male *Gulo*−/− (KO) and *Gulo* +/+ (WT) mice (on a C57BL/6 background) were maintained in microisolator cages for one week. Following this acclimation period, all animals were switched to a vitamin C deficient diet (58R3—Test-Diet, Purina, Richmond, IN). The diet met all other nutritional requirements of adult mice. Food and water were provided ad libitum throughout the experiment.

Animal experimentation

Animal mass was recorded on the date of vitamin C restriction and weekly thereafter. Following 12 weeks of experimental observation, WT animals (n = 7) in study 1 (model training) were healthy and KO animals (n = 7) showed signs of vitamin C deficiency. WT animals in study group 2 (model testing) were harvested after 11 (n = 4) or 19 days (n = 4). KO animals in group 2 were harvested after 19 days (n = 4). Final mass was recorded for all animals before euthanasia by carbon dioxide inhalation (Weljie et al. 2007). Whole blood (~1 mL) was obtained by a cardiac puncture and placed on ice and allowed to clot for 30 min. Samples were then centrifuged for 10 min (3,000 rpm) and sera collected and divided into two aliquots. Serum was stored at −80 °C until ready for data acquisition.

Metabolite sample preparation

Serum samples (0.2–0.3 mL) were thawed and filtered twice using 3 kDa NanoSep microcentrifuge filters, prewashed to reduce preservative contamination. Filtrates were then transferred to clean microfuge tubes; final sample volume ranged from 150 to 250 μL. Samples were brought to 650 μL by addition of 140 μL of phosphate buffer containing dimethyl-silapentane-sulfonate (DSS, final concentration 0.5 mM), 40 μL of sodium azide to limit bacterial growth, and D_20. Sample pH was then adjusted to a range of 7.0 ± 0.01.

Spectrum acquisition

One-dimensional ^{1}H NMR spectra with optimal water suppression were acquired using a standard pulse sequence (Bruker pr1d_noesy pulse definition) (Nicholson et al. 1995). Spectra were acquired in two batches, using an automated NMR Case sample changer on a 600 MHz Bruker Ultrashield Plus spectrometer. The pulse sequence had a mixing time of 100 ms, a Z-gradient water presaturation pulse, and a 3 s acquisition time. Samples were individually shimmed to ensure half-height line width of approximately 0.8 Hz for the major DSS peak calibrated to 0.0 ppm. Spectra were acquired with 512 scans, zero-filled and Fourier transformed to 64 k points. Standard post-processing of the spectra included deletion of the water region, B-spline baseline correction, reference deconvolution and calibration of the DSS peak. ^{1}H,^{13}C heteronuclear single quantum coherence (HSQC) spectra of two samples, chosen at random, were acquired for peak assignment and verification.

Metabolite concentration profiling

Processed spectra were imported into Chenomx Profiler version 5 (Edmonton, Alberta, Canada) for quantification. A standard set of fifty (50) compounds were profiled based on chemical shift assignments verified by the ^{1}H,^{13}C HSQC results. These metabolites represent an average of 84% of the total spectral area, excluding the water, lactate and glucose areas. Spectra were randomly ordered for fitting in Chenomx Profiler to avoid progressive bias. Compounds were fit from highest initial concentration to lowest, with an iterative reinspection. Each measurement was normalized to the mean sample concentration, dividing profiled spectral concentrations by the total concentration of all profiled metabolites in that sample (Weljie et al. 2006).

Multivariate statistical analysis

Normalized concentration values were imported into SIMCA-P software (Umetrics, Sweden) for multivariate pattern analysis. Orthogonal Projection to Latent Structures (OPLS) was used to capture artifactual differences between Batch 1 and 2 (irrespective of strain) and to compensate for batch variation. Hierarchical OPLS was then applied to the residuals of Batch 1 samples to isolate changes between KO and WT animals. 50 metabolite concentrations were used as X variables; animal body mass at time of harvesting was used as the supervisory (Y) variable (Schicho et al. 2010).

Cross validation

A seven-fold rotating cross-validation (CV) was used to estimate the robustness of the OPLS model. In each iteration, a model based on ~85% of Batch 1 samples was used to predict the response (final body mass) for the other 15%. As a measure of model strength (Eastment and Krzanowski 1982), the average ratio of total sum of squared errors (Q^2Y) was compared to the percentage of Y variance captured in the total model (R^2Y). The distribution of predicted body-mass was compared to that of actual training sample body masses.

External validation

The model constructed from Batch 1 samples were used to predict body-mass for batch two samples. Predicted body-mass is compared to the actual strain and actual mass of animals to determine the predictive ability of the model on external samples not used in the model's construction.

Model interpretaton

Primary component OPLS coefficients for the Batch 1 model, multiplied by the weighting, were used to interpret the response pattern in affected animals (Trygg et al. 2007). 95% confidence intervals were determined from CV jackknifing and t-value distributions (Efron and Tibshirani 1993).

Results

Animal characteristics

Wild-type animals in Batch 1 (training samples) showed no visible signs of vitamin C deficiency after 12 wks. In contrast, *Gulo*−/− animals exhibited significant signs of scurvy, including joint bruising and osteodegeneration (Hirschmann and Raugi 1999). Batch 2 (testing) animals were harvested prior to the onset of symptoms, for external validation purposes.

Animal body mass was reasonably consistent within strains, with a significant difference ($P < 0.05$) induced between them when vitamin C was removed from the diet (Fig. 1). The body mass of wild-type animals in Batch 1 increased steadily after the weaning period finished. The mass of KO mice lagged in the first month, tailed off in the second and fell steeply in the last 4 weeks. Batch 2 KO mice already had significantly lower body mass than WT mice when harvested after 3 weeks.

Statistical validation

The multivariate OPLS model of Batch 1 animals was supervised using the animals' body mass after 12 weeks as the Y variable. Seven-fold cross validation was used, with

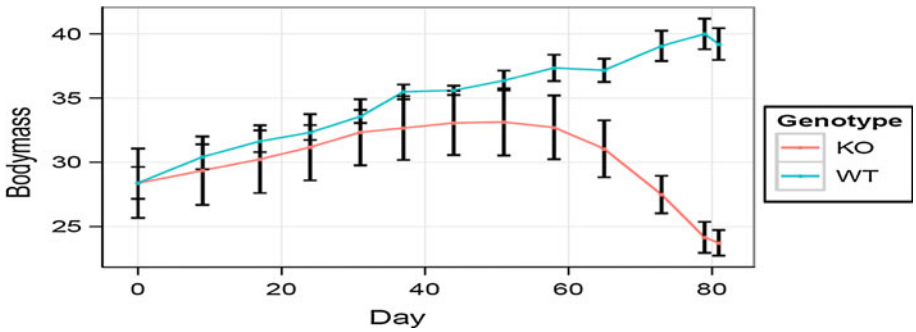

Fig. 1 Body mass of WT (*light*) and KO (*dark*) animals over time. Animal mass was used as the supervisory variable to indicate the degree of affectedness in each sample

Table 1 Cross validation metrics for model training

Model	Samples	R^2Y (Coverage)	Q^2Y (Quality)
KO vs WT (body mass)	Batch 1	96.8%	71% (very good)
Batch differences	All	96.5%	96% (excellent)

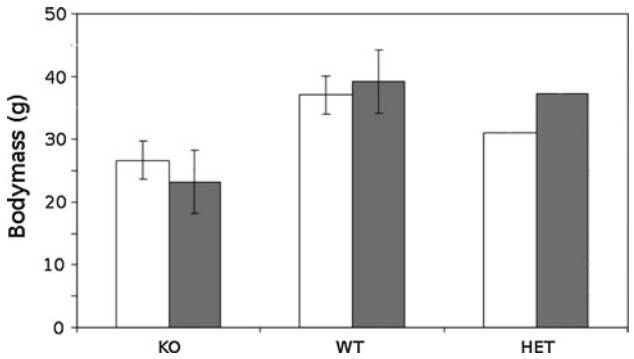

Fig. 2 Average cross-validation predicted (*light*) vs actual (*dark*) body mass for batch 1 mice. Predictions were made for samples left out of each CV model. Intraclass overlap and interclass separation are a measure of model quality. One sample, believed to be a knockout, fell between the expected range for both classes. Re-typing showed it to be an unexpected heterozygote

CV predictions of each training sample's mass when left out of the model construction. Overall 96.8% of the variance in body mass (Table 1) could be accounted for, with 71% accuracy (mean normalized sum of squares across cross validation iterations). Given the relatively small number of samples in the training data set, good agreement between predicted and actual body mass (Fig. 2) indicated a strong model of the metabolic changes between WT and KO mice when vitamin C was withheld.

External validation

A principle components analysis (PCA) model of all samples showed that the differences between Batches 1 and 2 were more significant than differences in KO and WT mice (Supplementary Figure S1). This is quite common in metabolomics studies, where sensitive techniques such as serum NMR-profiling can detect differences in animal environment (e.g. Bollard et al. 2005), but in this case is caused by differences in animal age. An OPLS model of inter-batch differences showed excellent quality measures (Table 1), indicating the model's ability to put samples on a common basis for comparison, independent of age (Fig. 3). Based on these analyses, the Batch 1 model was able to significantly ($P < 0.05$, Fig. 4) differentiate WT and KO mice in Batch 2—pre-symptomatic early harvesting of Batch 2 samples notwithstanding.

Model interpretation

OPLS coefficients were multiplied by the scaling weights (Trygg et al. 2007) for interpretation of the metabolite changes between strains. Jackknifing was used to calculate 95% confidence intervals, and only metabolites with significantly increased or decreased concentrations are reported (Fig. 5). Several groups of metabolites with similar biochemical functions (Kanehisa et al. 2010) were seen to respond in concert.

Discussion

An estimated 15–30% of low-income North Americans suffer from varying degrees of vitamin C deficiency (Frikke-Schmidt and Lykkesfeldt 2009), of which even mild shortfall undermines the body's ability to defend against damaging oxidizing byproducts. Vitamin C deficiency has been implicated in joint trauma (Padh 1990), heart disease (Cangemi et al. 2007; Heinig and Johnson 2006), cancer (Carroll and Kritchevsky 1994), as well as motor control and degenerative conditions (Sauberlich 1994). Linus Pauling proposed that high doses of vitamin C would prevent the common cold, a claim that has been hotly debated (Hemilä 1997), and some investigators have even suggested that infusion of extremely high doses of vitamin C may have beneficial effects as a prodrug in the treatment of some

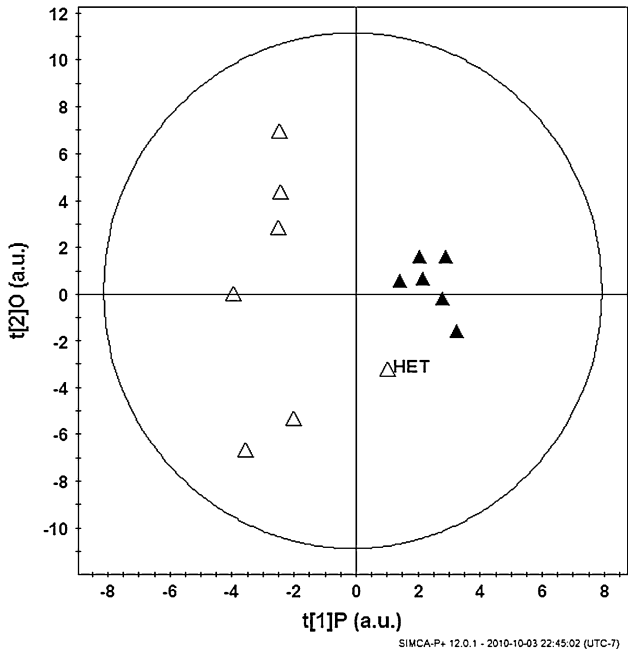

Fig. 3 WT (*black*) vs KO (*white*) OPLS model scores of modeling samples after removal of intrabatch variation using hierarchical OPLS. First component (*horizontal*) score indicates the degree of vitamin C deficiency perceived by the model. The one modeling sample (HET/Triangle) misclassified by the model was the heterozygous mutant (See Fig. 2)

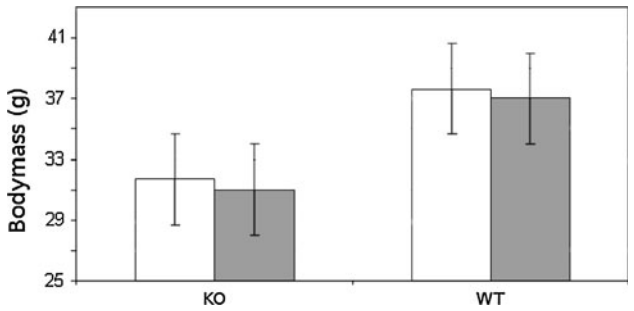

Fig. 4 External validation results: Predicted (*light*) and actual (*dark*) masses for batch 2 samples not used in any model construction. *Standard error* bars show good separation between predictions for samples, notwithstanding the fact that they were harvested pre-symptomatically

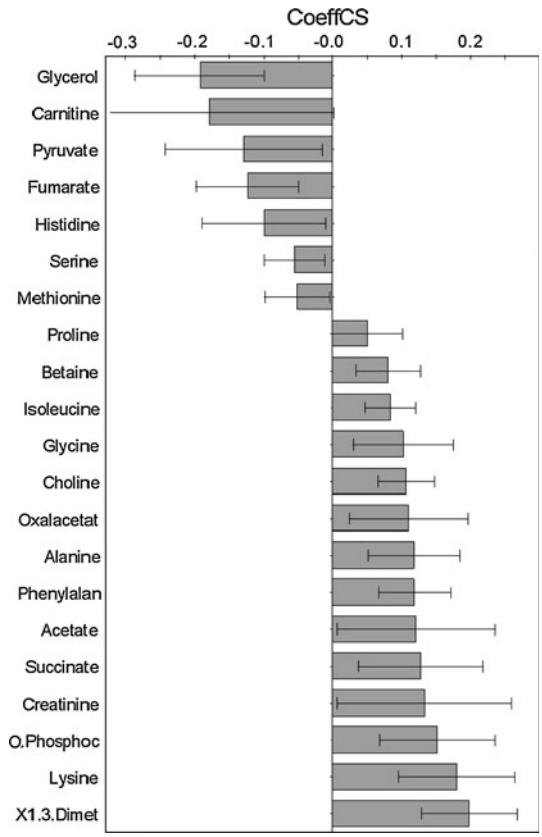

Fig. 5 First component OPLS loadings of WT vs KO mice. Values indicate relative change in compounds in knockout mice after 12 weeks of vitamin C deficiency

cancers (Chen et al. 2005, 2008; Cabanillas 2010). Its popular appeal as a panacea may be grounded in some degree of biochemical fact since ascorbic acid is a known cofactor for over a dozen biological pathways (Padh 1990), and a prominent antioxidant in many others. Vitamin C is one of the nutrients being studied in the European Prospective Investigation into Cancer and Nutrition (EPIC) population study (e.g. Gonzalez and Riboli 2010).

Its significance notwithstanding, the mechanisms by which vitamin C affects various conditions have not been fully characterised (Naidu 2003). Broad-spectrum metabolic state profiling, in conjunction with known networks of metabolic functionality, gives a contextualized view of changes and the *Gulo* knockout mouse provides an ideal platform for the study of vitamin C deficiency (Inai et al. 2003; Harrison et al. 2010). The ability of this mutant to be crossed with other disease models also provides abundant avenues for further investigation. Herein, we have used 1D proton NMR of serum from *Gulo*−/− mice to study the differences in metabolism induced when vitamin C was withheld after weaning.

Because direct analysis of NMR spectra can be difficult, 64,000 spectral intensity points for each serum sample were translated to corresponding concentrations for 50 metabolites. This translation to named compounds prior to analysis was instrumental in model interpretation, refinement, and establishment of significance in differences between knockout and wild-type animals. In this study, the animals' body mass was used as a supervisory measure of signs of vitamin C deficiency (Fig. 1). While the training cohort was small, testing of the models supported their validity as the predicted values were in good agreement with actual values for Batch 2 samples (Fig. 2). Moreover, despite the early harvesting of the external validation

samples, prior to the onset of symptoms, the model showed a significant ($P < 0.05$) ability to separate KO and WT samples from the second batch of unseen animals (Fig. 4). Taken together with the confidence intervals for the metabolite changes, we have evidence that the model accurately describes the changes induced by vitamin C shortfalls and not other phenomena or random variation.

The model loadings (Fig. 5) reflect the significant changes between WT and KO mice. The difficulty of interpretation stems from the complexity of the interactions within the animal systems; no single metabolite can both capture and segregate the multiple responses to various oxidative stresses. Some are ubiquitous indicators of non-specific pathology, while others are involved in numerous pathways. By viewing changes in the context of the total response the loadings can highlight specific pathway segments of interest. In addition to literature sources, the Kyoto Encylopedia of Genes and Genomes (Kanehisa et al. 2010) and the Human Metabolome Database (Wishart et al. 2007) were used to identify functional groups of metabolites with significant changes. Several functional groups were associated with changes in vitamin C availability, which together highlight major shifts in metabolism: an increase in glutathione production to compensate for ascorbic acid's loss, and/or an increase in glycerophospholipid metabolism for either energetic or precursor-provision reasons.

Energy regulation (Pyruvate, Acetate, Oxaloacetate, Succinate, Fumarate)

The TCA cycle's central role in energy metabolism features prominently in many metabolomic profiles, and central TCA players can be affected by so many responses (e.g. injury, diet, uptake, repair) that direct attribution is often difficult. The known role of ascorbate in activating prolyl 4-hydroxylase to increase succinate creation from 2-oxoglutarate raises some questions about succinate's perceived increase despite the decreasing levels of fumarate, pyruvate, and oxaloacetate. This may imply an ascorbate-shortage induced shunt from either pyruvate or succinate (or both) via alanine (which demonstrated a significant increase) and aspartate. While aspartic acid exhibited no consistent increase in concentration there was a large increase in variance indicating possible increases in flux; the changes in both lysine and phenylalanine levels could be attributed to disruption of aspartic acid production from alanine and oxaloacetate.

Carnitine biosynthesis

Two of the most significant and coherent changes seen are the decrease in carnitine levels and an increase in lysine. Carnitine can act as a transporter of fatty acids into the mitochondria, and the twofold role of ascorbate in its synthesis is well established (Padh 1990). Ascorbic acid acts as a cofactor for hydroxylation of trimethyl-lysine and butyrobetaine, both precursors for the production of carnitine. The direct implications of this can be seen in the significant drop in carnitine and O-acetylcarnitine in KO mice, concurrent with a significant increase in lysine and betaine. Whether it is a cause or effect requires further investigation: the resulting shift in aspartate-to-lysine conversion would result in increased flux towards cysteine and glutathione production, or an increase in lipid metabolism could result from other oxidative stress responses (Hopps et al. 2010) (see below).

Glutathione synthesis

As part of the GSH/ascorbate redox chain the significant change in glutathione levels is of immediate interest. In the absence of dehydroascorbic acid, GSSG conversion to GSH is impeded and the normal cycle of reductive protection is cut off from the terminal outlet of vitamin E quinones (Winkler et al. 1994). While increased production in GSH would eventually be overwhelmed it could serve as a short-term compensatory measure, and a number of metabolic shifts support the possibility of increased glutathione production in these knockout mice. Cystathionine-β-synthase (CBS) is the committed enzyme step in production of cysteine from methionine, and is regulated by the oxidative state of its heme group. The decrease in methionine levels supports the notion that an absence of ascorbate results in oxidation of CBS and upregulation of cysteine production, one of two required precursors for the increased production of glutathione. The other precursor for glutathione production, glycine, is also seen to increase while serine (its precursor) decreased significantly (Fig. 6). Significant depletion of serine is also supported by a potential increase in glycerophospholipid metabolism (via conversion to O-phosphatidylserine, see below).

Glycerophospholipid metabolism

The additional role of ascorbate as a primary regulator of glycerophosphocholine-cholinephosphodiesterase enzymes (Sok 2002) may explain the differential levels of choline, phosphocholine, and glycerol in our results. If the loss of ascorbate's stabilizing action on GPC-cholinediesterase gave rise to an increase in free choline, the corresponding increase in betaine would feed the glutathione production above. It seems reasonable that the concomitant release of diacylglycerols into the cell may be an evolutionary response. DAG is a known secondary messenger, activating protein kinase C. Moreover, release of those free lipids

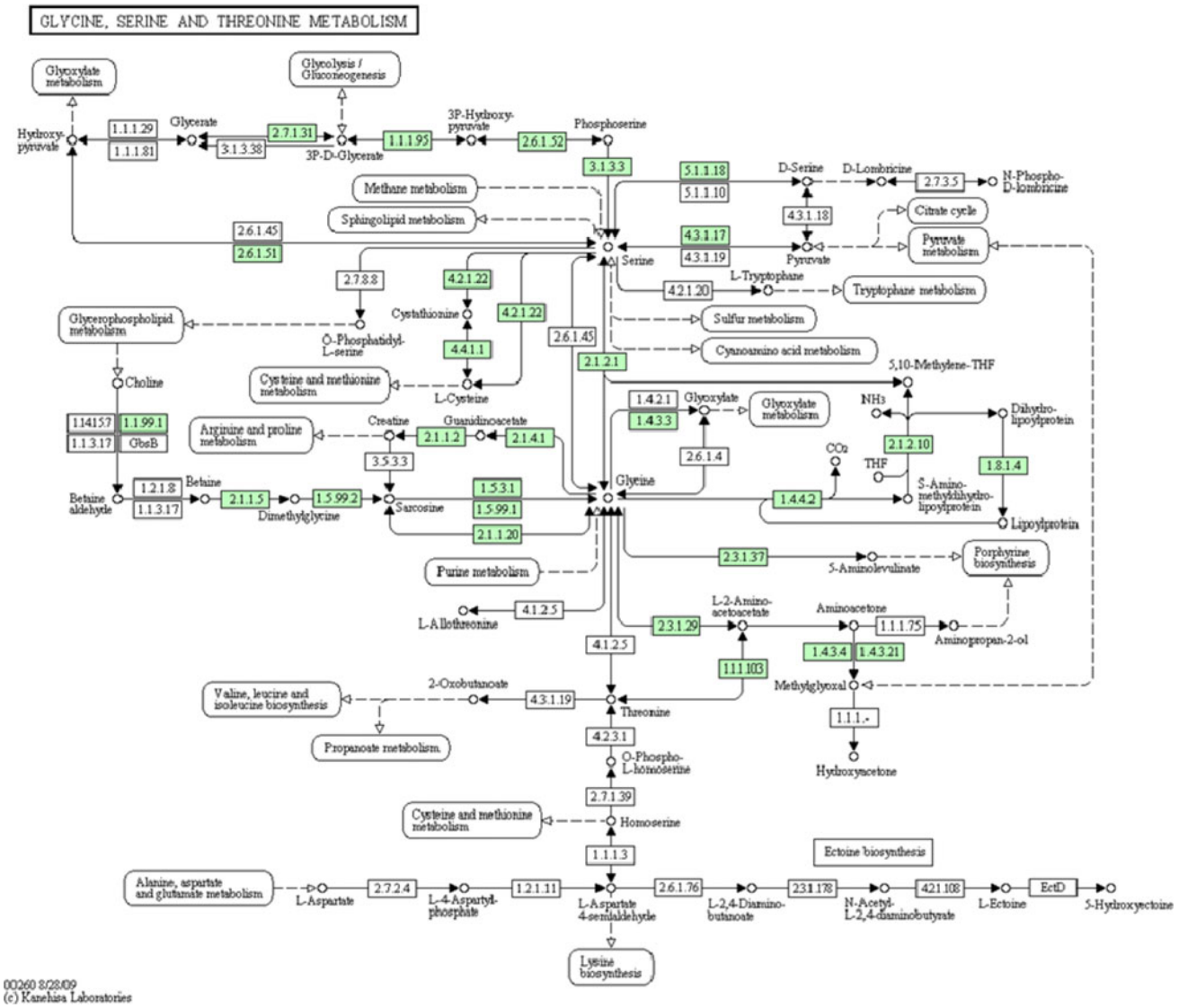

Fig. 6 KEGG pathway diagram displaying serine and glycine associated metabolism, which was significantly modified by removal of vitamin C from the animals' diet

may be related to the reports of insulin resistance in some cases of long term, mild, ascorbate deficiency. Further investigation of intermediate energy metabolism using other techniques (e.g.: lipidomics, hyperinsulinaemic clamp), might be used to distinguish between these possibilities. Regardless, it suffices to say that the implications of low ascorbate levels on lipid metabolism seem both profound and far-reaching.

The one metabolite of high significance that cannot be directly ascribed to any particular ascorbate-related reaction within the scope of this study is 1,3-dimethyluric acid. The importance of uric acid as a secondary antioxidant (Ames et al. 1981), particularly in primates that lack the *GULO* gene (Sevanian et al. 1991), suggests a number of possible functions however currently no literature linking the two phenomena is available. A number of dimethylxanthine-related interactions (Lee 2000) have been described in the literature as having ascorbic acid associations but in light of the numerous possibilities there is insufficient evidence in these results to extrapolate. The lack of significant change in some related metabolites, such as allantoin, further complicates the issue.

Conclusions

Our results highlight the ability of the *Gulo* knockout mouse to present a clear picture of the metabolic changes induced by vitamin C deficiency, and of 1D proton NMR to capture those changes. Quantitative profiling simplified the

NMR results to a manageable set of identified metabolites, and PLS was used to identify coherent patterns (statistical models) of change between wild-type and KO mice. Orthogonalization of those PLS loadings clarified the results and allowed interpretation of the models with respect to vitamin C deficiency induced changes. They also allowed comparison of model loadings between two sample batches, and validation of the resulting models by virtue of accurately predicting the condition of unknown animals. Finally, our results are in good agreement with several metabolites known to require ascorbate for enzymatic activation and proper flux. Almost all of the metabolic changes seen in the animal can be attributed to a few pathways of known importance, which provides a coherent picture of how the absence of ascorbate's reducing action upregulates production of other antioxidants, primarily glutathione, and possibly uric acid. The links between those pathways and the perceived changes underscores the ability of NMR-based metabolomics to capture the impact of complex diseases in a quantitative way.

Acknowledgments The authors gratefully acknowledge funding assistance in the form of research grants from the Arthritis Society of Canada (FRJ) and the Alberta Cancer Foundation (HJV). FRJ is supported by a Canada Research Chair. HJV is a Scientist of the Alberta Heritage Foundation for Medical Research.

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