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Metabolic adaptation of short-living growth hormone transgenic mice to methionine restriction and supplementation

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Abstract

Extension of mammalian health and life span has been achieved using various dietary interventions. We previously reported that restricting dietary methionine (MET) content extends lifespan only when growth hormone signaling is intact (no lifespan increase in GH deficiency or GH resistance). To understand the metabolic responses of altered dietary MET in the context of accelerated aging (high GH), the current study evaluated MET and related pathways in short-living GH transgenic (GH Tg) and wild type mice following eight weeks of restricted (0.16%), low (0.43%) or enriched (1.3%) MET consumption. Liver MET metabolic enzymes were suppressed in GH Tg compared to diet-matched wild type mice. Methionine metabolite levels were differentially affected by GH status and diet. SAM:SAH ratios were markedly higher in GH Tg mice. Glutathione levels were lower in both genotypes consuming 0.16% MET but reduced in GH Tg mice when compared to wild type. Tissue thioredoxin and glutaredoxin were impacted by diet and GH status. The responsiveness to the different MET diets is reflected across many metabolic pathways indicating the importance of GH signaling in the ability to discriminate dietary amino acid levels and alter metabolism and lifespan.

Keywords

aging; amino acid; metabolomics; hormones; mice; glutathione

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H.M.B.B. conceived and designed the project, and drafted the paper. S.G.R., J.A.W., and L.R. cared for the animals, collected tissues and plasma, recorded body weights, and performed the gene and protein expression assays. J.A.W., K.E.B. and H.M.B.B. performed statistical analyses. All authors have read and approved the final manuscript.

Competing Interests

The authors declare that they have no competing interests.

Introduction

Altering the diet of an organism represents a relatively non-invasive means to intervene and improve the health of that individual or population. Successful efforts to enhance health and lifespan have been realized utilizing dietary restriction (DR) protocols as well as amino acid restriction and small molecule interventions.¹ More specifically, it has been shown that decreasing the amount of dietary methionine (MET) exerts many beneficial effects on metabolism and extends the lifespan of normal rodents.^{2–5}

The endocrine system has profound effects on health and longevity across mammalian species and others.^{6,7} Growth hormone, in particular, impacts multiple aspects of metabolism including glucose regulation, protein synthesis, inflammatory status, mitochondrial function, energy, detoxification and oxidative defense.^{8,9} GH deficiency in rodents leads to elevated antioxidative defenses and stress resistance, insulin sensitivity and reduced incidence of age-related diseases concomitant with 50% longer lifespans. In contrast, mice that overexpress GH (GH Tg) exhibit many aspects of accelerated aging, living half as long as their wild type siblings following lifetime ad libitum consumption of normal rodent chow.^{10,11} The lifespan benefit produced by altered methionine in the diet requires an intact growth hormone signaling system thus, longevity is not improved with MET restriction in mutant GH-deficient or -resistant mice.¹² In contrast, a 50% increase in lifespan occurs in GH Tg mice when fed diets with reduced levels of this amino acid.

Many but not all of the health benefits of MET restriction and GH deficiency are shared and include improvements to insulin sensitivity, reduced tumor incidence and delays in aging of the immune system.¹³ To better understand the metabolic responses imposed by different levels of dietary MET in the context of accelerated aging and age-related disease, we conducted studies feeding GH Tg and WT animals different levels of methionine for eight weeks. We examined the MET metabolic pathway as well as components of detoxification and antioxidant defense. We found that in general, GH Tg mice expressed lower levels of many transcripts and metabolites when compared to wild type mice and that dietary MET levels, whether deficient or enriched, were influenced by GH status.

Material and Methods

Animals

The mice utilized in this study were bred and maintained at the University of North Dakota Center for Biomedical Research under controlled conditions of photoperiod (12:12 h light/dark cycle), temperature ($22 \pm 1^\circ\text{C}$) and ad libitum access to food (birth to eight weeks of age; Teklad #8640) and water. The growth hormone transgenic mice (GH Tg) were originally provided by Dr. Andrzej Bartke and were developed from a single male founder (strain B6SJL) produced by microinjection of the phosphoenolpyruvate carboxykinase promoter region/bGH hybrid gene into the male pronucleus of single-cell embryos. The initial characterization and production of these animals (transgenic males crossed to C57Bl/6J x C3H/J F1 females) was previously described.¹⁴ Male animals were started on the MET diets at eight weeks of age and fed for eight weeks (until four months of age). Female mice were not available at the time of this study. Animal procedures were reviewed

and approved by the UND IACUC following the Guide for the Care and Use of Laboratory Animals (NRC, 2011). Thirteen to 18 wild type (WT) and GH Tg male mice were utilized in each group. Body weights were determined before the MET diets were started and immediately prior to tissue collection. Liver tissue weights were also recorded.

Diets

Amino acid defined diets were manufactured by Harlan Teklad based on the AIN-93 diet (TD.10230, TD.10231, TD.10232). Three levels of methionine (0.16%-restricted, 0.43%-low, 1.3%-enriched MET) were incorporated into the diets while glutamic acid was adjusted to keep each of the diets isonitrogenous (Harlan Laboratories, Madison, WI). The only source of sulfur amino acids in these diets was L-methionine. The 0.16% MET level was chosen based on the literature reporting extended longevity in rodents and to avoid issues of rectal prolapse and early mortality that have been published previously in mice.⁴ Diet containing 0.43% MET represented half of the level found in many rodent chows as well as a level of MET that might be more achievable in human diets. The 1.3% MET level represented a 50% increase above normal levels in chow enriching yet supplementing this amino acid below the reported level of toxicity.^{15,16} Supplementation of sulfathiazole was not provided in these diets.

Methionine and Glutathione Metabolism

Samples of liver and kidney tissues were collected from GH Tg and WT mice following eight weeks on the diets (n=13–18/genotype/diet). Tissues were rapidly frozen and stored at –80°C until analysis (food was not removed the night before collection). For enzyme assays, samples were homogenized on ice in buffer (20 mM MOPS, 300 mM sucrose, and 0.1 mM EDTA at pH 7.2; Bullet Blender, Next Advance, Averill Park, NY). Homogenates were centrifuged (30 minutes@13000g) and the supernatant fractions used for analysis following determination of protein concentrations. The enzyme activities of glutathione S-transferase (GST), γ -glutamyl transpeptidase (GGT) and glutaredoxin (Grx) were determined using supernatants and the spectrophotometric methods of Holmgren.^{17–20} Thioredoxin (Trx) activity was measured by its ability to reduce insulin disulfide in the presence of NADPH and thioredoxin reductase (TrxR).^{20,21} For each assay, activity was calculated following absorbance readings at 340 nm with appropriate blanks subtracted from total absorbance. The ratio of the specific reduction/oxidation pair, GSH/glutathione disulfide (GSSG) was measured to determine the cellular oxidation state using procedures described by Griffith.^{22,23}

Standard RT-PCR protocols were used to evaluate tissue gene expression.^{22,24} Total RNA was extracted and equal amounts of RNA for the gene of interest (for primer sequences see Ref 24) and the reference gene, β 2 microglobulin were utilized to perform one-step real-time semi-quantitative PCR. The amount of target DNA was normalized to the endogenous reference gene and compared relative to the control group (diet-matched wild type mice) following calculation of gene expression using the comparative CT method.

Protein levels of the GSH and MET pathways were determined using standard immunoblotting procedures.^{25,26} Antibodies to mouse proteins were obtained from: Cbs,

MatI/II/III (Proteintech, Chicago, IL), mTOR, phosphorylated mTOR (Cell Signaling Technology, Danvers, MA), Bhmt, Cth, Gnm1 (Santa Cruz Biotechnology, Santa Cruz, CA) and CDO (Abcam, Cambridge, MA). Chemiluminescence (BioRad; Hercules, CA) and densitometry (Omega-Lum, Aplegen) were used for detection and analysis of protein levels.²⁷ Membrane staining with Ponceau-S was used to evaluate equal loading of protein.

Metabolomics

A non-targeted metabolomic protocol was used to gain more specific information regarding the response of the liver tissue to the effects of GH status and dietary MET. Liver tissues from GH Tg and WT animals consuming the 0.16% and 1.3% MET diets (n=8/genotype/diet) were evaluated by Metabolon, Inc. (Research Triangle Park, NC). The tissues were processed utilizing standard procedures for protein precipitation, aliquoted for evaluation using three different mass spectrometry platforms, and analyzed as detailed previously.²⁴ Liquid chromatography/mass spectrometry (LC/MS) was performed using a Waters Acquity UPLC system and a Thermo-Finnigan LTQ mass spectrometer, including an electrospray ionization source and linear ion-trap (LIT) mass analyzer. Aliquots of vacuum-dried samples were reconstituted, one each in acidic or basic LC-compatible solvents containing 8 or more injection standards at fixed concentrations (to both ensure injection and chromatographic consistency). Extracts were loaded onto columns (Waters UPLC BEH C18–2.1 × 100mm, 1.7 μm) and gradient-eluted with water and 95% methanol containing 0.1% formic acid (acidic extracts) or 6.5mM ammonium bicarbonate (basic extracts). Samples for gas chromatography /MS analysis were dried under vacuum desiccation overnight prior to being derivatized under nitrogen using bistrimethyl-silyl-trifluoroacetamide (BSTFA). Derivatized samples were separated on a 5% diphenyl/95% dimethyl polysiloxane fused silica column (20m x 0.18mm ID; 0.18μm film thickness) with helium as carrier gas and a temperature ramp from 60° to 340°C in a 17.5 min period, and then analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. Mass accuracy and calibration of the instrument were checked daily. Quality control measures included using columns and reagents from a single lot to complete all related experiments; randomization of experimental samples and controls throughout each day's run; the use of pooled experimental samples serving as technical replicates (for precision calculations) throughout the analyses; extracted water samples serving as process blanks; and a cocktail of standards spiked into every sample to monitor instrument performance.

Metabolites identified in the experimental samples were compared to a reference library of purified chemical standards using chromatographic properties and mass spectra matching to the specific compound or an isobaric entity using proprietary visualization and interpretation software.²⁸ Library matches for each compound were verified for each sample. The liver metabolome consisted of 348 named biochemicals. Specific to this analysis, the median relative standard deviation for the internal standards (instrument variability) was 4% and for the endogenous biochemicals (total process variability) was 12%.

Statistics

For the activity assays, gene and protein expression, and metabolomic data comparisons, a two-way analysis of variance and when appropriate, Bonferroni post-hoc testing as well Tukey's were used to determine significant differences among means (Prism GraphPad, San Diego, CA). Students t-tests were utilized for protein expression data assayed by diet. Data are reported as means $\pm$ SEM.

Results

The actions of elevated plasma GH can be observed both phenotypically and metabolically. Growth hormone Tg mice are much larger than their WT counterparts averaging 16–18 grams more in body weight at eight and 16 weeks of age, regardless of diet. Wild type and GH Tg mice consuming 0.16% MET lost 0.7–3.0 g, respectively (Figure 2A; Table S1). Liver weights were lowest in mice fed 0.16% MET and similar within WT mice fed higher levels of MET (Figure 2B).

Gene and protein expression of MET pathway enzymes and associated metabolites were evaluated to determine the role that high plasma GH may play in response to changes in dietary amino acids levels. Metabolomic analysis was conducted only in liver tissue from mice fed 0.16% and 1.3% diets while the transcriptional, translational and enzyme activity response to all three diets was determined in liver and when relevant, kidney tissue. Related data (metabolites, gene expression) are presented together. We have previously observed that transcription levels of MET components are indicative and reflect enzyme activity levels in liver tissue.²⁹

Dietary MET levels impact components of the MET pathway in liver tissue. In animals consuming 0.16% MET, liver MET was 21 and 26% higher compared to levels from mice fed 0.43 and 1.3% MET (Figure 3). GH Tg mice fed high MET had 79% higher levels of N-acetylMET (nutrient equivalent of MET), compared to diet-matched WT mice (Figure S1). Gene expression of Mat1a, the enzyme that converts MET to S-adenosylmethionine (SAM), was significantly lower in GH Tg compared to WT regardless of diet ($P < 0.001$; Figures 3, S2). Antibodies specific to Mat1a were not available, however protein detected by an antibody that recognizes MATI, MATII and MATIII (translated products of Mat) indicated diet ($p < 0.0001$) and genotype effects ($p < 0.0292$; Figure S3). Liver SAM levels were reflective of MET with higher values observed in animals consuming low MET. GH Tg mice maintained higher (95%) SAM than WT on the 1.3% MET diets.

Glycine N-methyltransferase (Gnmt) removes a methyl group from SAM resulting in S-adenosylhomocysteine (SAH). Liver Gnmt mRNA levels were suppressed 66 and 77% in GH Tg mice on the restricted and enriched MET diets, respectively, when compared to diet-matched WT mice (Figure 3, S2, S3). The metabolite, SAH, was similarly affected with transgenic mice exhibiting 54 and 75% less on 0.16% and 1.3% MET, respectively, compared to WT mice. The resulting SAM:SAH ratios were higher in both genotypes (GH Tg 5.6 vs WT 2.6) on 0.16% MET. Compared to WT, GH Tg mice maintained much higher SAM:SAH ratios on both diets (1.3% 3.27 vs 0.40, GH Tg and WT, respectively).

Homocysteine is the resulting metabolite following the actions of adenosylhomocysteinase (Ahcy) on SAH. Liver homocysteine levels were not different between genotypes and diets. Transcript levels of Ahcy were 68 and 74% lower in GH Tg mice compared to WT regardless of dietary MET content (0.16% and 1.3% MET, respectively; Figure 3, S2). Ahcy action also produces adenosine, the levels of which mirrored those of SAH in GH Tg mice. MET restriction (0.16%) resulted in lower levels of adenosine compared to supplemented MET (1.3%) levels in both genotypes.

Homocysteine can be metabolized via one of two pathways. It can be recycled back to MET or flow through the transsulfuration pathway (Figures 3, 4). Homocysteine recycling involves metabolism of betaine to dimethylglycine and conversion of 5-methyltetrahydrofolate (5-meTHF) to THF by betaine homocysteine methyltransferase (Bhmt) and methionine synthase (Mtr), respectively. Liver betaine was higher in mice fed 0.16% MET. Bhmt and Mtr transcript levels were lower in GH Tg mice compared to WT mice (Figures 3, S2, S3). Liver 5-MeTHF was markedly decreased when GH Tg (55%) and WT (90%) mice were fed 1.3% MET compared to 0.16% diets. GH Tg mice also maintained higher levels compared to WT on both diets (54 and >600%, 0.16% and 1.3% MET, respectively). GH Tg mice also exhibited low sarcosine levels compared to WT mice.

If homocysteine is not recycled to MET, it flows irreversibly through the transsulfuration pathway. Cystathionine beta-synthase (Cbs) combines serine and homocysteine to form cystathionine (Figure 4). While the Cbs liver transcripts tended to be lower in GH Tg versus WT mice, cystathionine γ -lyase (Cth) was dramatically suppressed in these mice compared to diet-matched WT mice (Figures 4, S2, S3). Protein levels of Cth were similarly reduced (38, 66 and 49%) in GH Tg versus WT mice on 0.16, 0.43 and 1.3% MET, respectively, (Figure S3). Serine values were significantly increased in both genotypes consuming restricted MET compared to the MET supplemented mice. Cysteine levels were 41 and 260% higher in GH Tg vs WT mice, respectively, consuming restricted compared to supplemented MET (Figure 4).

In GSH biosynthesis, cysteine is conjugated to glutamate by the rate-limiting enzyme γ -glutamyl cysteine ligase (GCL). GH Tg mice exhibited higher glutamate and glutamine compared to WT mice on the 0.16% MET diet (Figure 5). GCL has two subunits (Gclc, Gclm) that combine to create the active enzyme both of which were reduced 40–76%, respectively, in GH Tg compared to WT mice on each of the three diets (Figures 4, S2). The product of GCL, in turn, is conjugated to glycine to form GSH. Metabolomic analysis indicated that the resulting levels of reduced GSH were significantly lower in GH Tg mice on MET restriction compared to the levels in diet-matched WT mice and to both genotypes fed 1.3% MET (Figure 4, S4; confirmed in an additional assay). Similarly, GH Tg mice had lower levels of oxidized GSH compared to WT and animals fed 1.3% MET. The GSH:GSSG ratios generated in the metabolomic analysis were unaffected by genotype but significantly lower in both genotypes consuming restricted MET.

Cysteine is also derived from the degradation of GSH by GGT. Liver GGT activity was unchanged in GH Tg mice across diets while increased GGT activity was observed with increasing MET in WT mice (Figure 6). As a functional correlate of GSH utilization, we

determined that detoxification activity of GST decreased with increasing MET content of the diets. Mice with high GH levels exhibited higher GST activity compared to WT mice ($p<0.0001$; Figure 6). Kidney GST activity was also elevated in GH Tg mice on each diet (Figure 6). Related kidney GSH values also indicated significant effects of diet (Figure S5).

Some of the cysteine generated is utilized in the formation of the amino acid, taurine. Cysteine dioxygenase, which metabolizes cysteine to cysteinesulfinate, was similar in both genotypes on restricted MET while the 1.3% MET diets increased CDO proteins levels 200 and 258% in GH Tg and WT mice, respectively (Figures 4, S3). Hypotaurine levels mirrored those of CDO protein. Taurine levels were significantly increased in both genotypes on 1.3% MET compared to animals on restricted MET but with no genotype effects on this amino acid (Figure 4).

Liver transcript expression of the redoxins, Trx1 and Trx2 and the Trx reductases, decreased with increasing MET diet content in GH Tg mice. In WT mice, Trx1 and TrxR2 followed a similar pattern (Figure S7). However, when activity of Trx was examined, higher dietary MET content increased activity. Kidney redoxin transcripts (Trx1, Trx2, TrxR1, TrxR2) were not dramatically reduced (by half on average) in GH Tg mice regardless of diet (Figure S8). Liver Grx transcript expression was lower in transgenic mice however Grx activity was markedly higher (Figure S7; $p<0.0001$).

Liver mTOR protein was elevated in transgenic mice regardless of diet while phosphorylated mTOR was higher only in the GH Tg mice fed 0.16% MET (Figure 7). The resulting pmTOR:mTOR ratios were significantly reduced in these animals on each MET diet.

Liver metabolite analysis also revealed significant genotype-specific responses in components of glycolysis and Krebs cycle intermediates (Figures 8, 9). The presence of elevated plasma GH reduced the levels of glucose, glucose 6-phosphate, fructose 6-phosphate, the hexose diphosphates, glycerol 3-phosphate and glycerate significantly. However, different dietary MET levels had no effect on the measured glycolytic components in either genotype. Nicotinamide, NAD⁺, and coenzyme A were decreased in GH Tg mice regardless of diet while MET restriction increased citrate, fumarate and malate intermediates in mice with elevated GH.

We also examined components of the MET pathway in kidney tissue and observed that transcript levels of every gene examined indicated significant genotype effects (Mat1a, Mat2a, Gnm1, Ahcy, Mtr, Mthfr, Bhmt, Cbs, Cth, Gcl, Gclm). In general, GH Tg mice expressed lower values in each case (Figure S6).

Discussion

Limiting consumption of the essential amino acid methionine extends health span and lifespan in rodents, similar to interventions reducing caloric intake. We previously showed that an intact GH signaling system was necessary for MET restriction to promote longevity via altered MET and GSH metabolism.^{12,24} Long-living GH-deficient (Ames dwarf) and GH-resistant (GH receptor knock out) mice did not experience further lifespan extension when fed reductions of 50% and 80% MET as well as 150% supplementation. In contrast,

short-living animals that exhibit very high levels of circulating GH lived 50% longer (from 12 months to 18 months) when fed 80% less MET throughout their life.¹² These GH transgenic mice exhibit significant disease burden including a high incidence of hepatic and mammary cancers and succumb to glomerulosclerosis and glomerulonephritis.³⁰ We observed significant reductions in tumor incidence and enlarged bladders, two findings identified by necropsy, in the accelerated aging GH Tg mice consuming 0.16% MET from eight weeks of age to death.¹² In addition, although female animals were not utilized in this study, others have shown that several signaling mediators in liver are significantly elevated (expression, activation) in GH transgenic mice without sexually dimorphic differences detected as found in mice with normal levels of GH exhibiting sexually dimorphic GH secretion patterns.^{78–80}

In the current study, GH Tg mice were fed different levels of MET to examine the metabolic responses of methionine, glutathione and corresponding systems to further understand the relationship between hormones, accelerated aging and metabolism. An 80% reduction of this essential amino acid extends lifespan in rodents. The 0.43% MET level represented a 50% reduction in typical rodent chow and more importantly, a level that could be easily applied in human interventional studies. The metabolic response of supplementary MET (50% above typical levels of rodent diet; 1.3%) was of interest but incorporating a level well below that shown to be toxic (>2.5%).^{31,32} We focused on liver as it is the primary organ contributing to MET metabolism, GSH biosynthesis, detoxification and stress resistance and is dramatically impacted by GH status. The kidney plays an integral role with the liver in detoxification and degradation of GSH.

GH has somatic and metabolic actions that are exaggerated when the hormone is expressed outside of the physiological range. Plasma IGF-1 levels are significantly elevated in GH transgenic mice in comparison to WT mice regardless of diet.¹² Body weights of GH Tg and WT mice subjected to different levels of dietary MET were similar to our previous report. Animals on MET restriction maintained the same or slightly lower weight over the eight-week period while mice on higher levels of MET grew at similar rates within genotype.^{2–4,33} The IGF-1 levels along with body and liver weights are indicative of the somatic effects of GH. As dietary MET decreases, IGF-1 levels also decrease.¹²

In general, metabolite levels were more often affected by diet in WT mice compared to GH Tg mice. This may reflect the ability of high GH to mask some of the effects of nutrient sensitive pathways. The responsiveness of these pathways in GH Tg mice likely contributed to lifespan extension as similar components were affected by diet in WT mice that also respond to MET restriction with lifespan extension.

Genotype MET levels were comparable within diet with higher levels when MET was restricted compared to supplementation. This pattern held true for several other metabolites including glycine, SAM, betaine, 5-MeTHF, serine, carnitine, glutamate, opthalmate, norophthalmate, 5-oxoproline and all of the γ -glutamyl metabolites detected. This response to restricted MET suggests that: 1) there is conservation of important factors during times of nutrient loss, and 2) high GH does not interfere with ability of this tissue to respond appropriately. This responsiveness is maintained despite the GH suppression of genes that

are translated to key enzymes contributing to these metabolic pathways such as Mat1a, Gnmt, Ahcy, Gclc, Gclm and those that impact the γ -glutamyl cycle. There is a report demonstrating that downregulation of Cbs occurs when animals are fed diets lacking MET indicating that a possible sensing system determines when MET intake is low and signals the liver to activate Cbs to maintain MET levels.³⁴ GH Tg mice were unresponsive to altered dietary MET thus maintaining Cbs protein levels while WT mice decreased Cbs protein with increasing MET levels. Wild type mice generated higher levels of MET and cysteine on MET restriction suggesting conservation of sulfur AA possibly through recycling as reflected in the folate pathway (5-methyl THF). In contrast, others reported that MET restriction decreased sulfur amino acids of rats inducing hyperhomocysteinemia.^{35,36} The only sulfur AA that differed between GH Tg and WT mice on either diet was cysteine even though metabolic enzyme levels differed significantly for each. Thus, these systems are able to respond to a wide range of biochemical alterations and maintain levels of key metabolites.

Three important biochemical pathways: methylation, transsulfuration and polyamine synthesis, use SAM as a precursor. The protein products of Mat transcripts (MATI, II, III) have been shown to be regulated, in part, by GH thus, the low levels of the Mat transcripts and protein were not unexpected.³⁷ However, the 0.16% MET diet elevated SAM in both genotypes regardless of the level of the Mat enzymes suggesting the physiological importance of preserving of hepatic SAM. SAH is a potent inhibitor of most methyltransferases. Wild type mice fed high MET had higher SAH, Gnmt and Bhmt mRNA levels but lower corresponding protein levels when compared to those fed restricted MET suggesting posttranscriptional regulation. Protein and message expression of the methyltransferases, Gnmt and Bhmt, were very low in GH Tg mice despite low SAH indicating dysregulation of feedback inhibition in the presence of elevated GH. GH has been shown to downregulate Gnmt activity thus agreeing with our observations.^{25,38} Elevated GH suppressed Ahcy resulting in reduced adenosine and SAH in GH Tg mice. Although flux analyses were not performed in the current study, we have previously shown that the flux of the carbon backbone through transsulfuration is highly increased in GH deficiency with concomitant high Cth activities.²⁹ It is possible that homocysteine was not as rapidly utilized via transsulfuration as evidenced by the decreased Cth in the presence of elevated GH. It is more likely that the homocysteine was used for remethylation to form MET as 5-MeTHF levels were upregulated in GH Tg mice and MET levels were maintained. The metabolic pathway of MET appears to be suppressed at many junctions in the presence of high GH but overall maintenance of the sulfur AA, MET, cysteine, homocysteine and taurine as well as serine are preserved compared to WT mice.

The MET conserving enzymes (Mat, Ahcy, Bhmt, Mtr, Mthfr) tend to be inhibited by their products and other metabolites. For example, rats fed high MET exhibit reduced levels of these liver enzymes. In GH Tg mice, we observe low levels of all of the conserving enzymes except Mthfr when consuming any level of MET. Gnmt, Cbs and Cth represent MET catabolizing enzymes that are activated by the cycle metabolites and increase with additional MET intake. In our study, these enzymes were lower in transgenic mice regardless of dietary MET content when compared to their WT counterparts. Ahcy can be considered as an MET conserving and MET catabolizing enzyme as it has intermediate properties. The thermodynamics of this reaction ensures the conversion of excess homocysteine to SAH

unless adenosine is limiting.³⁹ Adenosine and SAH were both very low in GH Tg mice. Together, the levels of conserving and catabolizing enzymes suggest a marked dysregulation of methionine metabolism in the face of elevated circulating GH. Additional support for this assertion stems from studies showing that GH administration reduces many of the MET enzyme transcripts and proteins.²⁵

Further variation of this pathway is observed in GH Tg mice. High levels of SAM are known to inhibit Mthfr and activate Cbs activity.⁴⁰ MET excess results in higher SAM from more homocysteine degradation via transsulfuration. However, GH Tg mice maintain the same level of Mthfr transcript and low levels of Cbs transcript regardless of MET content. Wild type mice responded appropriately decreasing Mthfr in the presence of high MET. Mthfr is critical in the regulation of 5-MeTHF for homocysteine remethylation and the availability of methyl groups for methylation reactions. The preservation of methyl donors (betaine, folate, SAM) in GH Tg mice is interesting in the face of significantly suppressed methyltransferase enzymes.

GH and MET appear to modulate Cth and downstream cysteine and GSH levels.²⁴ GH Tg mice exhibit >50% reductions in Cth (mRNA, protein) while in GH deficient mice, Cth is 3–5 fold higher than in animals with normal GH levels. Cysteine is used for de novo GSH, hypotaurine and taurine biosynthesis, and it is incorporated into a variety of proteins, as well as hydrogen sulfide. Elevated GCL subunits in MET restriction did not raise GSH compared to mice receiving supplemented MET. In the absence of GH signaling, GSH is elevated and contributes to enhanced stress resistance likely via Nrf2 activation.^{9, 24, 42} MET restriction in normal mice does not alter cellular stress resistance suggesting that GH may be the key factor in managing resistance.^{13, 41} Related GSH metabolites within the γ -glutamyl cycle (glutamate, glutamine) support this evidence and indicate that restricting MET increases metabolite levels in the presence of GH whereas in its absence, the system does not detect dietary AA differences. While there appears to be adequate (actually high) levels of GSH components (glutamate, glycine, cysteine) present following MET restriction, and corresponding elevated biosynthetic transcript levels, the resulting GSH concentrations are lower suggesting possible utilization in the formation of opthalmate and related compounds as occurs under oxidative stress conditions (Figure 4; although flux analysis was not performed).⁴³ The tripeptide, opthalmate, is a structural analog of GSH that utilizes the same biosynthetic and degradative pathways (GCL, GS, GGT) as GSH. In our study, high levels of opthalmate, noropthalmate (20–30 times) and γ -glutamate cycle metabolites were observed with MET restriction suggesting that this dietary condition mimics an oxidative stress without concomitant upregulation of antioxidative enzymes.⁴⁴ The three amino acids comprising GSH intersect at metabolic ‘centers’ that mirror sulfur and nitrogen metabolism, one-carbon metabolism and overall energy levels in a tissue.⁴⁵ Therefore, the availability of these precursors is indicative of the metabolic status of tissues.⁴⁶ During metabolic stress, cysteine is diverted from transmethylation into transsulfuration. Our previous MET flux work demonstrated that the rates of transmethylation and transsulfuration are two and three times greater, respectively, in the absence of GH signaling.²⁹ In the current study, when normal or high GH is present, it appears that the allosteric and regulatory mechanisms present within the MET metabolic network are mostly intact.

GSH serves as a continuous source of cysteine via the γ -glutamyl cycle.⁴⁷ The γ -glutamylamino acids and 5-oxoproline were potentially influenced by MET intake in mice conserving cysteine during restricted MET intake and utilizing cysteine for GSH and hypotaurine synthesis (via CDO) when MET levels were high. These findings would be consistent with increased recycling of GSH via the γ -glutamyl cycle. GH Tg mice are unable to maintain GSH at WT levels under restricted MET possibly due to the high demand for amino acids for growth versus defense and cellular protection. In addition, cysteine availability strongly regulates CDO levels such that in animals fed restricted MET, CDO is low (conserving cysteine) and is increased in hepatic tissue of animals fed excess sulfur AA, to remove high cysteine and prevent cytotoxicity.^{59,60} Our study indicates that less cysteine is being routed through these pathways as oxidative (low CDO) and desulfurative (low Cth) mechanisms of cysteine metabolism were downregulated in GH Tg mice.

The SAM:SAH ratio is a marker of methylation capacity and a primary regulator of most methyltransferase activities in the cell.⁴⁸ Our study revealed that high SAM:SAH ratios in GH Tg mice likely suppressed methyltransferases. SAH levels are tightly linked to DNA methylation with liver and kidney tissues most sensitive to methylation changes as both tissues maintain complete transsulfuration pathways.^{49, 50} Age associated methylation changes are markedly suppressed in long-living, GH-deficient Ames dwarf mice.⁵¹ Thus, GH Tg mice are predicted to exhibit exaggerated age-associated changes to their DNA methylation pattern indicative of accelerated aging, increased cancer incidence and shortened lifespan based on the MET pathway components observed and our previous work examining GH deficiency.^{51,52}

The activities of liver Mtr and Mat1a are reduced under conditions of oxidative stress.⁵³ GH Tg mice exhibit suppressed levels of antioxidative enzymes and increased superoxide radicals and oxidative damage suggesting a high oxidative stress environment. Perhaps the pro-oxidant situation in GH Tg mice provides an additional explanation for reduced levels of these transcripts in our study as well as the increased ophthalmate levels.⁹ In addition, the redoxin systems function in redox signaling and antioxidative defense.^{61–65} Hepatic and renal redoxin expression was mostly suppressed in a high GH environment representing another example of systems that are sensitive to circulating GH levels. The high levels of methionine sulfoxide in GH Tg mice may reflect the altered Trx and Grx (Figure S1). These results support the hypothesis that elevated GH levels suppress multiple oxidant defense mechanisms.

Further regulation of MET metabolism occurs via the MET salvage (5-methylthioadenosine; MTA) pathway that involves polyamine synthesis. GH Tg mice had high MTA and markedly higher putrescine and spermidine levels compared to WT mice suggesting a significant upregulation in polyamine synthesis (Figure S1). These high levels of polyamines reflect known stimulatory effects of GH on ornithine decarboxylase activity and represent systemic indicators of malignancy, especially hepatic.^{54–57} Overall, MTA is a key player in the balance between cell death and survival in hepatocytes and the high levels in GH Tg mice may contribute to the increased incidence of neoplastic disease observed and ultimately, shortened lifespans.^{30,58}

The liver metabolite analysis also revealed changes in other key pathways providing a more global picture of genotype and diet effects. Several components of glycolysis reveal significant genotype-specific responses differing from those reported in calorie restriction where the glycolytic pathway was downregulated when compared to ad libitum-fed animals and by reports in WT mice where MET restriction improved glucose homeostasis and insulin sensitivity.^{66–68} Major components within the Krebs cycle appeared to be differentially impacted by genotype and diet, too. Thus, Krebs cycle metabolites are sensitive to the combination of high GH:low MET and likely impinge on downstream activities such as mitochondrial function, oxidative phosphorylation and energy production.^{69,70}

Coenzyme A is a requisite thiol cofactor in all organisms functioning as an acyl carrier and carbonyl activating group in key metabolic pathways including the Krebs cycle and fatty acid metabolism.⁷¹ Coenzyme A is one of several thiol-containing proteins that are reduced in GH Tg mice including GSH, GSH peroxidase, and selenoprotein R (Figure S3).^{9,24} This data contrasts that observed in long-living GH deficient mice. Long term MET restriction increases energy expenditure limiting fat deposition despite increased food intake suggesting that these diets lead to increased metabolic flexibility.³³ However, metabolic flexibility is in question in mice with elevated GH. GH Tg mice lose body weight despite exhibiting hyperphagia when fed restricted MET (each mouse consumed 70–80 g of food per week compared to 30 g in diet-matched WT mice in our previous study).¹² GH overexpressing mice on normal chow or high fat diets are also hyperphagic yet maintain leanness as energy expenditure is increased or similar to wild type mice.^{72,73} In addition, they appear to be protected against diet-induced obesity in part due to the elevated lipolysis stimulated by GH.^{74,75}

As a sensor of nutrient availability, mTOR regulates cell metabolism, protein synthesis, growth and proliferation by integrating intra- and extracellular signals.⁷⁶ Growth factors increase mTOR and TORC1 expression. In GH Tg mice, mTOR was less elevated in animals fed restricted MET compared to higher levels of MET positively correlating with IGF1 levels and indicative of reduced mTOR signaling with low MET.¹² GH signals via the GH receptor through a variety of systems including JAK2, MAPK, PI3K and HNF4 α , thus exerting pleiotropic effects. GH also stimulates IGF1 that can act via PI3K on C/EBP β and Nrf2 amongst other transcription factors. Both GH and IGF1 impact Phase I and Phase II enzyme activation via CCAAT/XRE and AREs and affect many of the cytochrome P450 enzymes and antioxidative defense systems. GH modulates levels of enzymes within the MET metabolic pathway (possibly via mTOR and JAK-STAT) but the signaling between GH/IGF1 and this pathway remain unclear. It has been suggested that the selective sensitivity of the GH/IGF1 system to MET may ensure that the drive to grow is modulated not by general amino acid availability but by the amino acid least available in the nutrient supply.⁷⁷ In an elevated GH environment, MET metabolism appears to be dysregulated.

Conclusions

GH Tg mice live half as long as wild type mice and exhibit many signs of accelerated aging. The high levels of GH impact many aspects of metabolism. In the current study, we showed

that despite a major suppression of many enzymes in the MET pathway, certain key metabolite levels were maintained in liver tissue of animals with elevated circulating GH and include many amino acids in addition to methionine and cysteine (glutamine, glutamate, serine, tryptophan, phenylalanine). Methyl donors were also elevated in GH Tg mice (betaine, SAM) despite low methyltransferase expression. Dysregulation of MET metabolism was indicated by significant downregulation of nearly every enzyme measured, some of which were previously shown to be affected by GH and/or oxidative stress. mTOR and polyamine synthesis were increased regardless of diet in these mice reflecting the strength of the elevated GH signal. In a previous study, we showed that MET restriction extended the lifespan of these mice dramatically and dampened gross aspects of age-related disease pathology. Reduced dietary MET may have tempered the extreme GH levels by altering GH effects on MET and GSH metabolism and resulting in extended lifespan (Figure 10). Our study suggests that the level of GH signaling may modulate the responsiveness of an organism to nutrient content.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

MET	methionine
GH	growth hormone
GSH	glutathione
DR	dietary restriction
Mat	methionine adenosyltransferase
Gnmt	glycine N-methyltransferase
Ahcy	adenosylhomocysteine hydrolase
Cbs	cystathionine β -synthase
Cth	cystathionase
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
5-MeTHF	5-methyltetrahydrofolate
THF	tetramethylhydrofolate

Mtr	methionine synthase
Mthfr	5,10 methylenetetrahydrofolate reductase
Bhmt	betaine homocysteine methyltransferase
GCL	γ -glutamyl cysteine ligase
Gclc	γ -glutamyl cysteine ligase catalytic subunit
Gclm	γ -glutamyl cysteine ligase modifier subunit
GSSG	glutathione disulfide
GGT	γ -glutamyltranspeptidase
GST	glutathione S-transferase
Trx	thioredoxin
TrxR	thioredoxin reductase
Grx	glutaredoxin
CDO	cysteine dioxygenase
IGF1	insulin-like growth factor 1
MTA	5-methylthioadenosine
CoA	coenzyme A

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Figure 1.

The methionine metabolic pathway. The pathway begins with methionine and includes remethylation of homocysteine to methionine and transsulfuration of homocysteine to cysteine. Enzymes are shown in red with green arrows indicating their location of activity. Cysteine can be incorporated to cysteine sulfinic acid (via CDO) through to taurine or to hydrogen sulfide via CBS. The contribution of cysteine from glutathione degradation pathways (GGT) as well as the use of cysteine in glutathione biosynthesis is shown. MAT - methionine adenosyltransferase; SAM - S-adenosylmethionine; GNMT - glycine N-methyltransferase; SAH - S-adenosylhomocysteine; AHCY - adenosylhomocysteine hydrolase; CBS - cystathionine beta-synthase; CTH - cystathionase (aka cystathionine gamma-lyase); CDO - cysteine dioxygenase; GS - glutathione synthase; GCL - glutamylcysteine ligase; GGT - gamma-glutamyl transpeptidase; GPX - glutathione peroxidase; GR - glutathione reductase; GSH - glutathione; GSSG - glutathione disulfide; H₂S - hydrogen sulfide; MTR - methionine synthase; BHMT - betaine homocysteine S-methyltransferase; THF - tetrahydrofolate.

Figure 2.

Body weight change (A) and liver weights (B) in GH Tg and wild type mice following eight weeks of 0.16, 0.43 or 1.3% dietary methionine consumption. N=11–17 mice/genotype/diet. Values in bar graphs represent means $\pm$ SEM. Asterisks represent significant genotype differences within diet. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ in GH Tg versus wild type mice.

Figure 3.

Liver metabolite (solid bars; scaled intensity) and enzyme gene expression (shaded bars; relative expression) of methionine pathway components in GH Tg and wild type mice following eight weeks of 0.16% (red/yellow bars) or 1.3% (blue/green bars) dietary methionine consumption. *Asterisks represent significant differences between diets within a genotype. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. †Crosses represent significant genotype differences within a diet. †p<0.05; ††p<0.01; †††p<0.001; ††††p<0.0001. Values represent means ± SEM (n=8/genotype/diet). Orange arrows indicate location of enzyme action. Blue arrows indicate metabolite progression through metabolic pathway.

Figure 4.

Liver metabolite (solid bars; scaled intensity) and enzyme gene expression (shaded bars; relative expression) of transsulfuration, glutathione and taurine pathway components in GH Tg and wild type mice following eight weeks of 0.16% (red/yellow) or 1.3% (blue/green) dietary methionine consumption. *Asterisks represent significant differences between diets within a genotype. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. †Crosses represent significant genotype differences within a diet. †p<0.05; ††p<0.01; †††p<0.001; ††††p<0.0001. Values represent means ± SEM (n=8/genotype/diet). Orange arrows indicate location of enzyme action. Blue arrows indicate metabolite progression through metabolic pathway.

Figure 5.

Liver glutathione metabolites (scaled intensity) in GH Tg and wild type mice following eight weeks of 0.16% (red/yellow) or 1.3% (blue/green) dietary methionine consumption.

*Asterisks represent significant differences between diets within a genotype. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. †Crosses represent significant genotype differences within a diet. †p<0.05; ††p<0.01; †††p<0.001; ††††p<0.0001. Values represent means ± SEM (n=8/genotype/diet).

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Figure 6.

Liver γ -glutamyltranspeptidase (GGT; nmol/mg protein) and glutathione S-transferase (GST; nmol/min*mg protein or μ mole/min*mg protein) activities in GH Tg and wild type mice following consumption of 0.16, 0.43 or 1.3% methionine for eight weeks. Values represent means $\pm$ SEM (n=10–18/genotype/diet). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 GH Tg versus wild type mice.

Figure 7.

Liver phosphorylated mTOR and mTOR proteins (relative optical density units) and the phospho-mTOR/mTOR ratios in GH Tg and wild type mice following eight weeks of dietary methionine consumption. Values represent means $\pm$ SEM (n=11–12/genotype/diet). Students t-test p values shown on each graph. P<0.05 considered significant.

Figure 8.

Liver glycolysis and pentose phosphate metabolites (scaled intensity) in GH Tg and wild type mice following eight weeks of 0.16% (red/yellow bars) or 1.3% (blue/green bars) dietary methionine consumption. *Asterisks represent significant differences between diets within a genotype. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. †Crosses represent significant genotype differences within a diet. †p<0.05; ††p<0.01; †††p<0.001; ††††p<0.0001. Values represent means ± SEM (n=8/genotype/diet).

Figure 9.

Liver Krebs cycle metabolites (scaled intensity) in GH Tg and wild type mice following eight weeks of 0.16% (red/yellow bars) or 1.3% (blue/green bars) dietary methionine consumption. *Asterisks represent significant differences between diets within a genotype. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. †Crosses represent significant genotype differences within a diet. †p<0.05; ††p<0.01; †††p<0.001; ††††p<0.0001. Values represent means $\pm$ SEM (n=8/genotype/diet). Blue arrows indicate metabolite progression through metabolic pathway.