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Original Article

Metabolic reprogramming of hydrogenosomal amino acids in *Trichomonas vaginalis* under glucose restriction

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Abstract *Background:* Glucose is the major energy source that is converted to pyruvate for ATP generation in the trichomonad hydrogenosome. Under glucose restriction (GR), the regulation of amino acids metabolism is crucial for trichomonad growth and survival. RNA-sequencing (RNA-seq) analysis has been used to identify differentially expressed genes in *Trichomonas vaginalis* under GR, leading to significant advances in understanding adaptive responses of amino acid metabolism to GR. However, the levels of amino acid metabolites modulated by GR are unknown in *T. vaginalis*.
Methods: Herein, we describe a comprehensive metabolomic analysis of amino acid metabolites in the hydrogenosome using liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry (LC-FT MS). The relative abundance of 17 hydrogenosomal amino acids was analyzed under GR and high-glucose (HG) conditions.

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Results: Levels of most amino acids were higher in GR culture. Arginine was not detectable in either HG or GR cultures; however, its metabolic end-product proline was slightly increased under GR, suggesting that the arginine dihydrolase pathway was more activated by GR. Additionally, methionine catabolism was less stimulated under GR because of greater methionine accumulation. Furthermore, branched chain amino acids (BCAA), including leucine, isoleucine and valine, as well as phenylalanine and alanine, markedly accumulated under GR, indicating that glutamate-related metabolic pathways were remarkably enhanced in this setting. Our metabolomic analysis combined with previous RNA-seq data confirm the existence of several amino acid metabolic pathways in the hydrogenosome and highlight their potentially important roles in *T. vaginalis* under glucose deprivation.

Introduction

Human trichomoniasis, which is caused by *Trichomonas vaginalis*, is the most widespread non-viral sexually transmitted infection (STI), with approximately 276 million cases reported annually worldwide.[1] *T. vaginalis* colonizes the human of urogenital tract and leads to serious health outcomes for women, including vaginitis, preterm delivery, infertility, low birth weight infants, and susceptibility to cervical cancer.[2] Trichomoniasis has also been implicated as a risk factor for HIV transmission[3] and lethal prostate cancer.[4] *T. vaginalis* lacks conventional mitochondria and instead contains specialized double-membrane organelles termed hydrogenosomes.[5] Pyruvate that forms in the cytosol by glycolysis is metabolized to acetate and CO_2 and is imported into the hydrogenosome to generate molecular hydrogen (H_2) and ATP.[6–8] Several genes that encode enzymes involved in amino acid metabolic pathways in the hydrogenosome have been identified in *T. vaginalis*.[9] However, the levels of metabolites derived from these pathways upon different nutritional cues, which could potentially shed light on their importance for metabolic adaptions, have not yet been investigated in this parasite.

Metabolomics, which aims to catalog metabolites within a given organelle, cell, tissue, or organism, has been applied to systems biology analyses of several protozoan parasites. For example, a metabolomics pipeline was developed to characterize the global metabolic differences between antimonial-sensitive and -resistant *Leishmania donovani* isolates.[10] Another study used ^{13}C NMR to monitor the metabolism of proline in procyclic *Trypanosoma brucei*,[11] demonstrating that ^{13}C proline is converted into succinate in the presence of glucose, whereas ^{13}C is principally incorporated into alanine upon glucose withdrawal. The metabolic profiles of red blood cells infected with different *Plasmodium* strains were analyzed by ^{1}H NMR spectroscopy, which revealed strain-specific differences in a range of metabolites.[12] Moreover, those metabolites related to *Cryptosporidium parvum* infection have been evaluated by GC–MS to understand the effects of infection on the host environment.[13] Metabolomic analyses have been used to investigate the global metabolic responses to encystation, oxidative stresses, and L-cysteine deprivation in *Entamoeba*, which revealed previously unidentified metabolic signatures and their potential significance in response to specific environmental cues.[14]

Transcriptomic and proteomic approaches have been employed to elucidate the metabolic roles of genes or proteins that are differentially expressed in *T. vaginalis* under certain nutritional conditions, such as glucose restriction (GR) and iron deficiency.[15,16] Nevertheless, careful studies of the levels of metabolites are essential to confirm the existence of metabolic pathways and changes induced by diverse nutrient stimuli. The *T. vaginalis* genome shows an absence of several essential enzyme-encoding genes in the synthesis and degradation pathways of nearly all lipids.[17] In our previous transcriptomic analysis of *T. vaginalis* upon GR, we did not find any remarkable changes in the expression levels of lipid-related genes, suggesting that lipid metabolism may not play an essential role in *T. vaginalis* upon glucose limitation. As we have previously demonstrated that many aminotransferases are significantly up-regulated in *T. vaginalis* upon GR, we performed for the first time a metabolomics survey of changes in amino acid metabolism in the hydrogenosome of *T. vaginalis* under GR compared with high-glucose (HG) conditions, which led to the discovery of metabolic reprogramming and hydrogenosomal amino acid adaptions in *T. vaginalis* under conditions of glucose deficiency.

Methods

Parasite and culture conditions

T. vaginalis (ATCC30236) trophozoites were cultivated in YIS medium,[18] pH 5.8, containing 10% heat-inactivated horse serum and 1% glucose at 37 °C, which was served as a high-glucose (HG) condition. Trophozoites were grown in the same medium without glucose supplementation for the GR condition.[15]

Isolation of hydrogenosomes

T. vaginalis trophozoites were harvested from stationary-phase cultures (24 h of HG and GR cultivation), after which cells were resuspended in SM buffer [250 mM sucrose, 10 mM morpholinepropanesulfonic acid (MOPS, pH 7.2)].

Cytosolic fraction (supernatant) and large granules of *T. vaginalis* homogenates were separated, as previously described,[19] with some modifications. To isolate hydrogenosomes, the large granule fraction that contained lysosomes and hydrogenosomes was added to 45% Percoll in SM buffer and centrifuged at 30,000 rpm for 30 min at 4 °C.[20] The upper (white) and lower (brown) layers were collected, representing the lysosomes and hydrogenosomes, respectively. They were washed with 10 volumes of SM buffer and centrifuged at 13,500 rpm for 30 min at 4 °C. Finally, cytosolic fraction and hydrogenosomes were stored in small volume of SM buffer at −80 °C.[21]

Western blot analysis

Hydrogenosomal and cytosolic proteins (10 μg) from HG and GR cultures (24 h) were separated on 15% SDS-PAGE and gels were transferred to nitrocellulose membrane (GE Healthcare, Amersham) using a semi-dry transfer unit (GE Healthcare, Amersham) under 15 V for 1 h. Membranes were blocked with 5% skim milk in TTBS buffer (Tris-buffered saline containing 0.1% Tween 20) for 1 h. The membranes were then incubated with the indicated primary antibodies in blocking buffer at 4 °C overnight. The following primary antibodies were used: rubrerythrin (Rbr; 1:2000 dilution), Pyruvate:ferredoxin oxidoreductase (PFO; 1:2000), and GAPDH (1:2000 dilution). Bound antibody was detected using anti-rabbit IgG secondary antibodies (GeneTex) (1:5000 dilution) in blocking buffer for 40 min. Stable peroxide solution and enhanced solution (1:1; Millipore, Billerica, MA, USA) were added on membranes and the intensity of protein bands was visualized and quantified using Biospectrum Imaging System (UVP) and ImageJ, respectively.

Enzyme assays

The malic enzyme activity was assayed by observing the reduction of nicotinamide adenine dinucleotide (NAD^+), replacing nicotinamide adenine dinucleotide phosphate ($NADP^+$) as described previously.[22] The hydrogenosomal malic enzyme preferentially utilizes NAD^+ to $NADP^+$ to catalyze oxidative decarboxylation of L-malate to pyruvate.[23] Lactic dehydrogenase activity was assayed by monitoring the oxidation of NADH.[24]

Metabolomic analysis of hydrogenosomal amino acids in HG and GR cultivation by LC-FT MS

Dansylation reaction

Hydrogenosomal proteins from HG and GR cultivation (24 h) were extracted in 3× volume of iced methanol and incubated for 2 h at −20 °C. After centrifugation, supernatant was dried by SpeedVac and reconstituted in 50 μL water. The dansylation reaction protocol was adapted from previous study.[25] Briefly, samples were mixed with 25 μL sodium carbonate/sodium bicarbonate buffer (500 mM, pH9.5), 25 μL acetonitrile (ACN), and 50 μL freshly prepared ^{12}C- or ^{13}C-dansyl chloride (DnsCl; 18 mg/mL in ACN). After incubation for 45 min at 40 °C, 10 μL NaOH (250 mM) was added and incubated for 10 min to quench the labeling reaction. To neutralize the solution, 50 μL formic acid (FA) in ACN/H_2O (425 mM) was added.

LC—UV analysis

To measure the amounts of labeled metabolites, each sample was individually subjected to LC—UV analysis. Solvent A was 5% ACN in 0.1% FA, and solvent B was ACN in 0.1% FA. The flowrate was 450 μL/min with a 6 min gradient (0 min, 0% B; 1.00 min, 0% B; 1.01 min, 95% B; 2.50 min, 95% B; 3.00 min, 5% B; 6.00 min, 5% B) through a reverse-phase column (ACQUITY UPLC BEH C18, 1.7 μm, 2.1 × 50 mm, Waters).

LC-FT MS analysis

The light (^{12}C)- and heavy (^{13}C)-labeled samples were equally mixed based on UV absorbance and subjected to a 9.4 T Fourier Transform Ion Cyclotron Resonance Mass Spectrometry (FTICR-MS; apex-Qe, Bruker Daltonics, Billerica, MA, USA) coupled with reverse-phase HPLC. Two injections of LC-FT MS were performed. Solvent A was 5% ACN in 0.1% FA, and solvent B was ACN in 0.1% FA. The flowrate was 60 μL/min with a 34-min gradient (0 min, 5% B; 2.0 min, 5% B; 3.0 min, 15% B; 13.0 min, 35% B; 25.0 min, 70% B; 28.0 min, 99% B; 32.0 min, 99% B; and 32.1 min, 5% B) through a reverse-phase column (ACQUITY UPLC BEH C18, 1.7 μm, 1.0 × 100 mm, Waters). All acquired MS spectra were processed by DataAnalysis (v.4.1, Bruker Daltonics) and IsoMS[26] to identify and quantify metabolite pairs.

Results and discussion

Isolation of hydrogenosomes from *T. vaginalis* cultured under HG and GR

The flow chart of this study was shown (Fig. 1), including isolation of hydrogenosomes and sample preparation for LC-FT MS analysis. To investigate the regulation of amino acid metabolism in the hydrogenosome under GR, hydrogenosomes of GR- and HG-cultured trophozoites were purified using gradient density centrifugation. Immunoblot analysis was performed to confirm the purity of the hydrogenosomal and cytosol fractions using the corresponding markers PFO/Rbr and GAPDH, respectively (Fig. 2A). This analysis revealed that PFO/Rbr and GAPDH were distinctly expressed in hydrogenosomal and cytosolic fractions, indicating the isolation of hydrogenosomes to high purity. In addition, enzyme assays were performed to support that cross contamination between cytosol and hydrogenosomal fractions was very minute (Fig. 2B and C).

Metabolomic analysis of hydrogenosomal amino acids in *T. vaginalis* under GR

A LC-FT MS approach was developed to examine the levels of amino acids in hydrogenosomes of *T. vaginalis* cultured

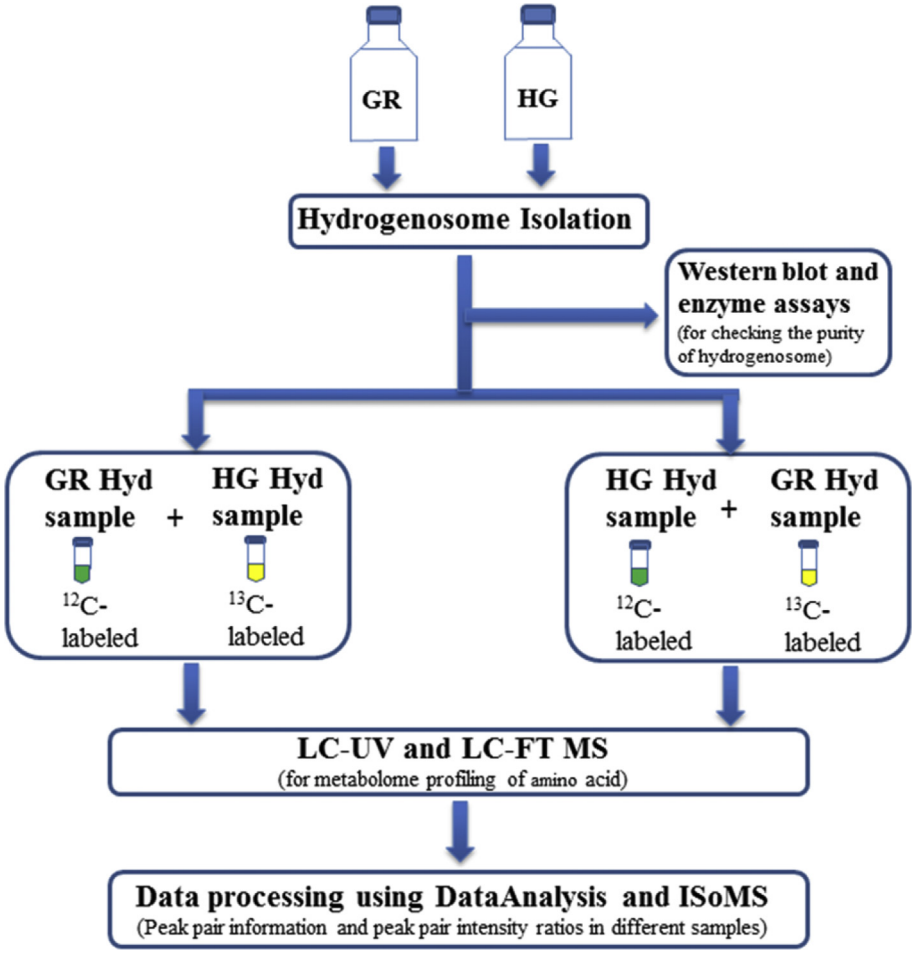

Figure 1. Experimental flow chart of this study. Hydrogenosomal fractions isolated from GR and HG cultures were subjected to western blot and enzyme assays to check the purity of hydrogenosome. Hydrogenosomal proteins from GR culture with ^{12}C-labeled was mixed with hydrogenosomal proteins from HG culture with ^{13}C-labeled. LC—UV analysis and LC-FT MS were performed. Data including peak pair information and intensity ratios was analyzed using DataAnalysis and ISoMS.

under GR versus HG. Twenty-five μL of hydrogenosomal samples purified from GR and HG cultures were ^{12}C-dansylation and ^{13}C-dansylation labeled, respectively. After that, they were proceded for LC—UV analysis, and were equally mixed and subjected to LC-FT MS analysis. The label-swap experiment was performed in which HG and GR samples were labeled with ^{12}C and ^{13}C, respectively (Fig. 1). The relative abundance of 17 amino acids in the hydrogenosomes isolated from HG- and GR-cultured cells was determined (Table 1). Interestingly, the levels of most hydrogenosomal amino acids were higher in hydrogenosomes cultured under GR compared with HG, with the exception of glycine and serine. Among amino acid metabolites that we tested, leucine, isoleucine, methionine, phenylalanine, valine, threonine, and alanine markedly accumulated under GR compared with HG (greater than 2-fold accumulation in GR-cultured cells). A previous report showed that alanine, valine and leucine were more abundant in intracellular amino acid pools of *T. vaginalis* [27]cultured under glucose-rich (30 mM) conditions, suggesting that the parasite further enhanced synthesis of these abundant amino acids under glucose limitation. Together, these data implied that most amino acid metabolism was activated in the hydrogenosome of *T. vaginalis* in response to glucose deprivation.

Arginine, asparagine, cysteine, lysine, and tyrosine were not detectable in the metabolomic analysis of both GR- and HG-cultured trophozoites, suggesting that they were rapidly consumed by *T. vaginalis*. Previous studies indicated that greater amounts of amino acids, such as arginine, were consumed by this parasite in the absence of maltose in culture medium.[28] Similarly, it has been proposed that *Giardia intestinalis* and *Entamoeba* species prefer to use arginine as an energy substrate to generate ATP after glucose is withdrawn.[29,30] These findings suggest that energy generation via amino acid catabolism may be a feature of anaerobic protists under conditions of carbohydrate deficiency.

L-cysteine is the major low molecular weight thiol that exerts various biological functions in *Entamoeba histolytica*, including cell survival, growth, attachment, elongation, motility, gene regulation, and antioxidant ability.[31—33] *In vitro* growth of amebic trophozoites requires high concentrations of L-cysteine, which suggests that extracellular cysteine or thiols have a crucial role in parasite growth.[34] L-cysteine is supplied in culture medium

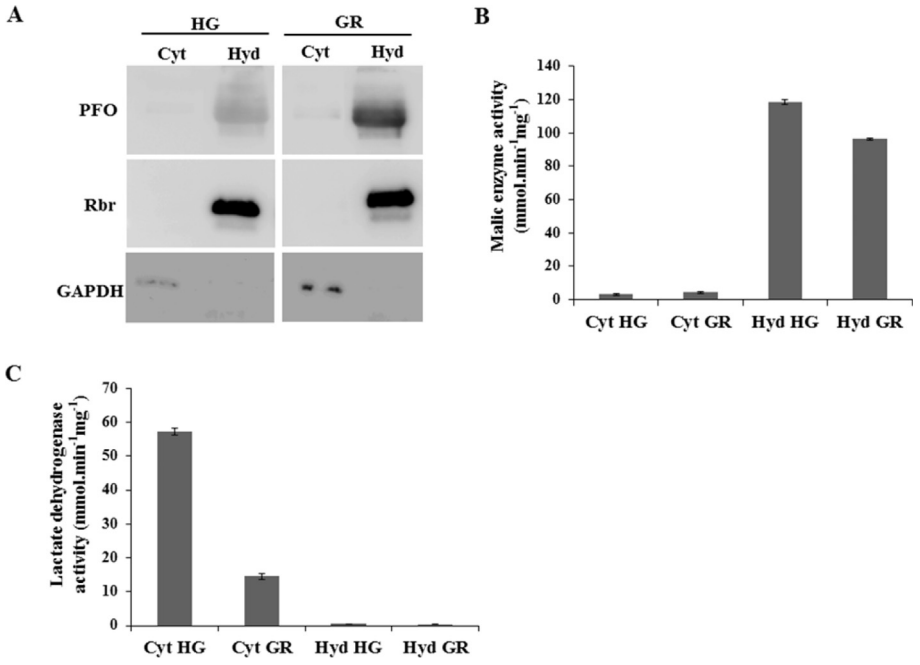

Figure 2. Isolation of hydrogenosomes from *T. vaginalis* cultured in GR and HG. Hydrogenosomal fractions were purified from GR and HG cultures (24 h of cultivation) using 45% Percoll. (A)Western blot analysis was performed to determine hydrogenosomal and cytosolic fractions. PFO and Rbr served as hydrogenosomal markers; GAPDH was used as a cytosolic marker. (B) Enzymatic activities of malic enzyme (hydrogenosomal marker) and (C) lactate dehydrogenase (cytosolic marker) were detected in the cytosolic and hydrogenosomal fractions. Cyt: Cytosol; Hyd: Hydrogenosome.

Table 1 Relative abundance of hydrogenosomal amino acids in GR and HG cultures.

Compound	Ratio of GR-to-HG		[a]Mean of two inj.	Ratio of HG-to-GR		Mean of two inj.
	Inj. 1	Inj. 2		Inj. 1	Inj. 2	
Dns—Ala	2.34	2.66	**2.50**	0.34	0.28	**0.31**
Dns—Arg	—	—	—	—	—	—
Dns—Asp	—	—	—	—	—	—
Dns—Cystine	—	—	—	—	—	—
Dns—Glu	1.45	1.46	**1.46**	0.49	0.55	**0.52**
Dns—Gly	0.73	0.67	**0.70**	1.1	1.10	**1.10**
Dns—His	1.82	1.79	**1.81**	0.59	0.57	**0.58**
Dns—Leu/Ile	5.32	4.74	**5.03**	0.18	0.17	**0.18**
	4.74	4.88	**4.81**	0.16	0.15	**0.16**
Dns—Lys	—	—	—	—	—	—
Dns—Met	4.6	4.64	**4.62**	0.14	0.14	**0.14**
Dns—Phe	3.55	3.67	**3.61**	0.22	0.22	**0.22**
Dns—Pro	1.87	1.85	**1.86**	0.39	0.41	**0.40**
Dns—Ser	1.13	1.13	**1.13**	0.69	0.84	**0.77**
Dns—Thr	2.46	2.72	**2.59**	0.4	0.38	**0.39**
Dns—Tyr	—	—	—	—	—	—
Dns—Val	3.29	2.79	**3.04**	0.23	0.21	**0.22**

[a] Two injections of LC—FTMS analysis were performed and mean of these two injections was calculated.
'—' indicates non-detected.
Bold indicates the mean of two injections.

to support the growth of trichomonads; however, it was not detectable in the metabolomic analysis of hydrogenosomes that were isolated from both GR and HG cultures, which suggests that it was further catabolized to carry out its cellular functions. Hence, more efforts will be needed to clarify whether cysteine has multiple critical functions in *T. vaginalis*, as it does in *Entamoeba histolystica*.

Integration of transcriptomic and metabolomic analyses of amino acid metabolic pathways in hydrogenosomes under GR and HG conditions

The arginine dihydrolase pathway

The main energy source of *T. vaginalis* is carbohydrates, which contribute to fermentative metabolism under both aerobic and anaerobic conditions.[28,35] The arginine dihydrolase pathway has been reported to contribute to energy metabolism in *T. vaginalis*.[36] We previously showed that the genes encoding ornithine carbamoyltransferase (TVAG_041310) and carbamate kinase (TVAG_261970), which mediate energy production in this pathway, were up-regulated under GR.[15] Metabolomic analysis in our present study revealed that arginine was not detectable under both GR and HG conditions, suggesting that it was consumed rapidly because of the elevated enzyme levels to generate ATP or downstream metabolites such as proline. Proline, the end metabolite of this pathway, could be detected in both GR and HG conditions, confirming that the arginine dihydrolase pathway is present in *T. vaginalis* hydrogenosome. A slight increase in proline levels under GR conditions implies that the arginine dihydrolase pathway becomes more activated in this parasite in response to glucose deprivation (Table 1).

The methionine metabolic pathway

Methionine can be catabolized into either α-ketobutyrate or homocysteine. Our previous transcriptomic analysis reported that two genes encoding enzymes that catalyze the degradation of methionine, S-adenosylmethionine synthetase (TVAG_252200) and adenosylhomocysteinase (TVAG_405240), were significantly down-regulated under GR.[15] Down-regulation of adenosylhomocysteinase, which was accompanied by ATP consumption, suggested that the energy-consuming reaction could be suppressed under glucose limitation. Consistent with our RNA-seq data, levels of methionine largely accumulated in the hydrogenosome in response to GR (the ratio of GR-to-HG was 4.62; Fig. 3). These observations confirm that methionine catabolism was less stimulated, resulting in the prevention of ATP depletion in *T. vaginalis* under GR conditions.

Glutamate-related metabolic pathways

The genes that encode enzymes involved in glutamate-related metabolic pathways have been shown to be up-regulated in *T. vaginalis* under GR.[15] Intriguingly, we noted that several amino acids involved in glutamate metabolism markedly accumulated in the hydrogenosome under glucose deprivation (Fig. 4). Specifically, levels of branched-chain amino acids (BCAAs), including leucine, isoleucine and valine, were significantly elevated in the absence of glucose. Leucine and isoleucine represent the most up-regulated amino acids in the hydrogenosome under GR, which show 5.03- and 4.81-fold increases under GR compared with HG cultivation, respectively. Additionally, levels of valine increased by 3.04-fold under GR compared with HG. Moreover, genes encoding BCAA aminotransferase (TVAG_026740 and TVAG_139240), which catabolize BCAAs into glutamate, were up-regulated.[37] Together, these observations suggest that the BCAA metabolic pathways are activated and may have an indispensable role in *T. vaginalis* in response to GR. Phenylalanine and alanine, which are also linked to glutamate metabolism, accumulated under GR (ratios of GR-to-HG for phenylalanine and alanine were 3.61 and 2.50, respectively; Table 1). Those enzymes that catalyze the reversible conversion of these amino acids and glutamate were also up-regulated. In summary, these findings established that glutamate-related metabolic pathways were highly activated in *T. vaginalis* under GR.

We previously reported that glutamate dehydrogenase (GDH) is the most up-regulated gene in *T. vaginalis* in response to GR.[15] It has been demonstrated that GDH is positively regulated by a low energy status (GDP and ADP); therefore, it is likely that GDH is up-regulated to produce α-ketoglutarate, which serves as an intermediate that enters the TCA cycle for energy production under low energy conditions.[38] GDH has been identified in the hydrogenosome of *T. vaginalis*, which is thought to catalyze the conversion of glutamate to α-ketoglutarate. Our integration of transcriptomic and metabolomics analysis revealed that BCAAs and BCAAs aminotransferase were markedly up-regulated under GR, suggesting that more glutamate was generated from BCAA-dependent catabolism. The slight increase in glutamate levels under GR partially supports this hypothesis. This observation points to the possibility

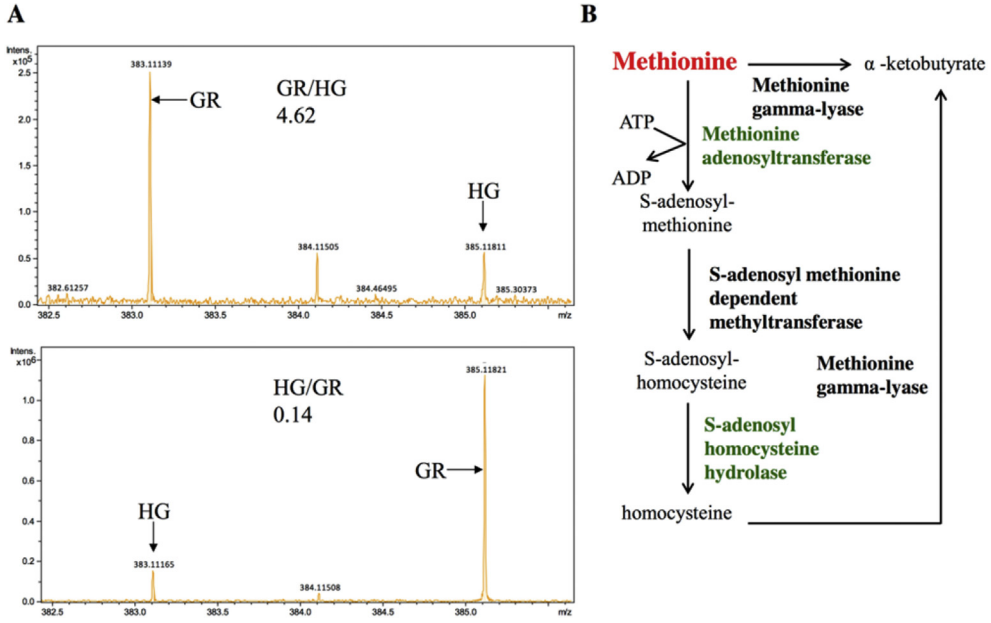

Figure 3. Reduced Methionine catabolism in *T. vaginalis* under GR. (A) A representative mass spectrum showing the Dns-Met ion pair of ^{12}C-dansylated. Hydrogenosome from a GR culture combined with a ^{13}C-dansylated hydrogenosome from a HG culture (upper; a peak pair of 383.1114 and 385.1181). The ratio of GR-to-HG intensity was 4.62. The mass spectrum showing the Dns-Met ion pair of ^{12}C-dansylated hydrogenosome from a HG culture combined with a ^{13}C-dansylated hydrogenosome from a GR culture (bottom; a peak pair of 383.1116 and 385.1182). The ratio of HG-to-GR intensity was 0.14. (B) Methionine significantly accumulated in the hydrogenosome of *T. vaginalis* under GR. Differentially expressed genes were mapped to known putative methionine metabolic pathways based on RNA-seq data. Metabolites or genes shown in red and green represent up- and down-regulation in *T. vaginalis* under GR, respectively.

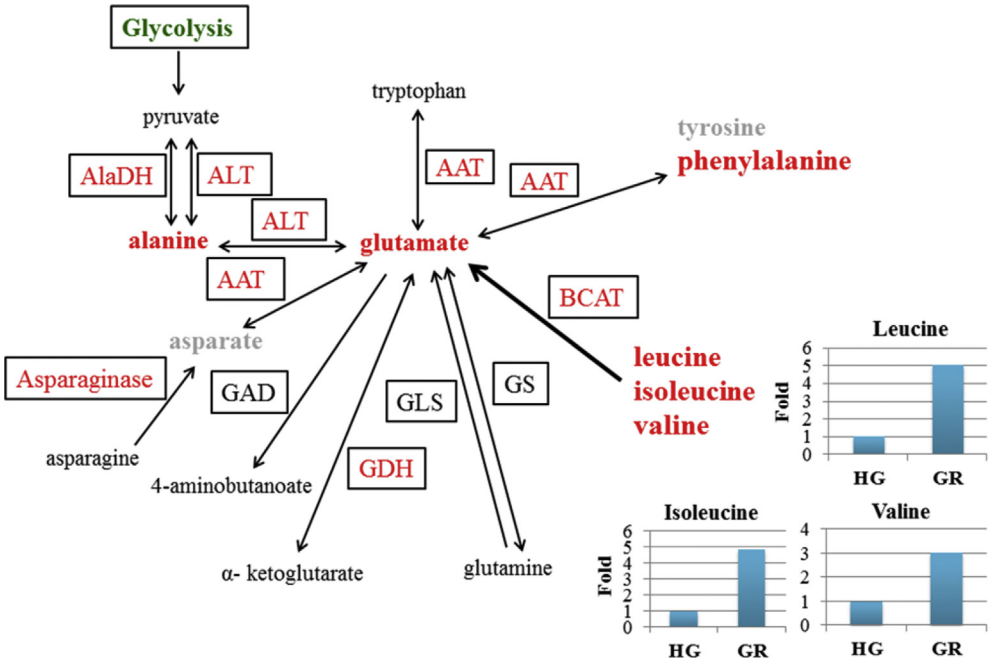

Figure 4. Marked activation of branched chain amino acid metabolism in *T. vaginalis* under GR. Differentially expressed genes encoding enzymes related to glutamate metabolism were mapped to known putative metabolic pathways based on RNA-seq data. Metabolites or genes shown in red represent up-regulation in *T. vaginalis* under GR. Right panels showed the fold changes of BCAAs levels under GR compared with HG. AlaDH, Alanine dehydrogenase; ALT, Alanine aminotransferase; AAL, Aspartate aminotransferase; GAD, Glutamate decarboxylase; GDH, Glutamate dehydrogenase; GLS, Glutaminase; GS, Glutamine synthetase; BCAT, Branched-chain amino acid aminotransferase.

that glutamate is further converted to other amino acids, such as alanine and α-ketoglutarate, in a manner mediated by the up-regulation of aminotransferases under GR. BCAAs have also been shown to be closely associated with energy generation. For example, in a malnourished condition with liver cirrhosis, increased amount of BCAAs is consumed by skeletal muscles for energy generation.[39] Hence, we propose that massive accumulation of BCAAs serves as energy sources for *T. vaginalis* in response to glucose-limited environment.

Collectively, this is the first report to assess the levels of amino acid metabolites in the hydrogenosome of *T. vaginalis* using metabolomic analysis. We identified several amino metabolic pathways by direct detection of their intermediates or end-products, which helps to support previous transcriptomic or proteomic studies. Activation of the arginine dihydrolase pathway and suppression of the methionine metabolic pathway represent more energy production and less energy consumption, respectively, in *T. vaginalis* upon GR. Significant up-regulation of BCAAs also implies that more energy resources are generated in *T. vaginalis* upon glucose shortage. Alterations of the levels of amino acids in *T. vaginalis* under GR not only represent a critical adaptation of this parasite, but also represent a possible new drug target for the treatment of trichomoniasis.

Conflicts of interest

All authors declare no conflict of interest.

Acknowledgments

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