ORIGINAL PAPERS

Metformin modifies glutamine metabolism in an *in vitro* and *in vivo* model of hepatic encephalopathy

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ABSTRACT

Aim: to analyze the effect of metformin on ammonia production derived from glutamine metabolism *in vitro* and *in vivo*.

Methods: twenty male Wistar rats were studied for 28 days after a porto-caval anastomosis (n = 16) or a sham operation (n = 4). Porto-caval shunted animals were randomized into two groups (n = 8) and either received 30 mg/kg/day of metformin for two weeks or were control animals. Plasma ammonia concentration, Gls gene expression and K-type glutaminase activity were measured in the small intestine, muscle and kidney. Furthermore, Caco2 were grown in different culture media containing glucose/glutamine as the main carbon source and exposed to different concentrations of the drug. The expression of genes implicated in glutamine metabolism were analyzed.

Results: metformin was associated with a significant inhibition of glutaminase activity levels in the small intestine of porto-caval shunted rats (0.277 ± 0.07 IU/mg *vs* 0.142 ± 0.04 IU/mg) and a significant decrease in plasma ammonia (204.3 ± 24.4 µg/dl *vs* 129.6 ± 16.1 µg/dl). Glucose withdrawal induced the expression of the glutamine transporter SLC1A5 (2.54 ± 0.33 fold change; $p < 0.05$). Metformin use reduced MYC levels in Caco2 and consequently, SLC1A5 and GLS expression, with a greater effect in cells dependent on glutaminolytic metabolism.

Conclusion: metformin regulates ammonia homeostasis by modulating glutamine metabolism in the enterocyte, exerting an indirect control of both the uptake and degradation of glutamine. This entails a reduction in the production of metabolites and energy through this pathway and indirectly causes a decrease in ammonia production that could be related to a decreased risk of HE development.

Key words: Hepatic encephalopathy. Metformin. Ammonia. Glutaminase. Glutamine. Cirrhosis. ASCT2. mTOR. Porto-caval anastomosis.

INTRODUCTION

Hepatic encephalopathy (HE) is a common entity caused by liver failure that results from acute and chronic disorders such as hepatitis and cirrhosis and is one of the main complications of cirrhosis (1). This syndrome involves neuropsychiatric dysfunction ranging from subtle psychological abnormalities to profound coma due to a metabolic disturbance (2). Clinical studies strongly suggest a major role of ammonia in the development of brain disturbances in liver disease and systemic hyperammonemia is commonly found in patients with cirrhosis and HE (3). The pathophysiological mechanisms include alteration of blood-brain-barrier permeability, cytokine production, changes in neurotransmitter synthesis and release, neuronal oxidative stress, impaired mitochondrial function and osmotic disturbances resulting from astrocytic metabolism (4).

Ammonia is mainly produced in the small intestine during protein digestion and nitrogen metabolism carried out by epithelial cells and bacterial flora (5). The majority of the gut ammonia is a by-product derived from glutamine metabolism into glutamate and ammonia by the enzyme glutaminase (EC 3.5.1.2) (4). In a damaged liver, especially a cirrhotic one which can have up to four times more ammonia producing activity, hepatocytes cannot handle the overload and urea cycle is saturated (6). Neural amino acid transporter B(0) (ATB[0] or ASCT2 encoded by SLC1A5) is a glutamine transporter that also plays an important role in metabolism and amino acid homeostasis of cells in most tissues and it may be relevant in glutamine-derived ammonia production (7). Both proteins (ASCT2 and glutaminase) are critical for glutaminolytic

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metabolism, which in turn is regulated by the MYC proto-oncogene, a master regulator of metabolism (8). One of the most important signaling functions of ASCT2 is the link with the mammalian target of the rapamycin (mTOR) pathway. This integrates signals from five major routes, including growth factors, cellular stress, energy status, oxygen and amino acids (9). In fact, it is thought that glutamine metabolism could sustain cell energy requirements and be essential for growth and viability when regulated rapid cell division is required. This is important in processes such as wound healing (i.e., liver regeneration), immune responses to specific antigens or even cancer (10). Furthermore, stress and hyperammonemia could rapidly increase the ratio a-ketoglutarate/citrate, triggering enhanced glutaminolysis and sensitizing the cells to the action of several agents (11).

Almost 95% of cirrhotic patients may be glucose intolerant and type 2 diabetes mellitus was found in a third of cases (12). Furthermore, the association of diabetes and insulin resistance in HE risk and cirrhosis progression has been previously demonstrated. These diseases have been related to enhanced cytokine production and an inflammatory state, an increased ammonia production via a glutaminase activity increase and constipation (13-15). Metformin is a widely used antihyperglycemic drug that has demonstrated pleiotropic effects and has also been reported to reduce the chronic inflammatory response by inhibiting TNFα production (16-17). It also blocks endogenous reactive oxygen species production by interfering with mitochondrial complex I activity (18). Ampuero et al. (2012) demonstrated in a retrospective study that metformin-exposed cirrhotic patients had an eight-fold lower HE risk (19). Furthermore, they also demonstrated an *in vitro* glutaminase activity reduction followed by an ammonia production decrease after metformin treatment.

The aim of this study was to analyze the effect of metformin on glutaminase (GLS) gene expression and K-type glutaminase (KGA) enzyme activity in a rat model of HE and to determine the impact of the drug on glutamine metabolism.

MATERIAL AND METHODS

Experimental design

In vivo *study*

All animals used in the study were maintained before, during and after experiment according to the directive 2010/63/EU regarding the use of animals for experimentation and other scientific purposes (Royal Decree-Law 53/2013, of February 8, laying down basic rules for the protection of animals used for experimental and other scientific purposes, including teaching). All animal procedures were performed with the approval of the University of Seville Animal Ethics Committee. All experiments were conducted on the premises of Service Production and Animal Experimentation of the Institute of Biomedicine of Seville/HUVR (registered and authorized center no. ES410910008015). All guidelines and regulations concerning the housing conditions of animal experimentation were fulfilled. Appropriate measures were taken to minimize the pain or discomfort of animals.

Twenty male Wistar rats with a body weight of 400 ± 24 g were individually housed in standard conditions (12 h/12 h light/dark cycle at 23 ± 2 °C, 50% humidity) with *ad libitum* access to food and water. These animals underwent a sham operation (n = 4) or porto-caval shunt (PCS) (n = 16) following the method proposed by Numata (20). Two weeks after surgery, operated rats were randomized into two groups; PCS as a control group (n = 8) and PCS + Met (n = 8). The latter group received 30 mg/kg/day of metformin (Acofarma, Madrid, Spain) with the food during the experimental period of 14 days. Animals were sacrificed by exsanguination and blood was withdrawn from the descending aorta and immediately put into ice cold EDTA tubes, centrifuged at 3,000 rpm at 4 °C, and plasma was collected. Muscle, kidneys and small intestine (scrapings of intestinal villi) were collected and washed in cold phosphate saline buffer. Approximately 100 mg of tissue were processed for enzymatic activity as described below. The remaining tissue was stored at -80 °C until RNA extraction.

In vitro *study*

The human colonic epithelial cell line Caco2 was maintained in DMEM Low Glucose (w/glucose, w/o glutamine and w/ sodium pyruvate) or DMEM (w/o glucose, w/glutamine and w/o sodium pyruvate), supplemented with 100 ml/l fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, non-essential amino acids and 2 mmol/l L-Glutamine (ThermoFisher, MA, USA) in 50 ml/l CO_2 at 37 °C. Cell concentration was determined with a Neubauer chamber. Cell assays were initiated 24 hours after seeding. Caco2 (10,000 cells/cm^2) were cultured in the presence of 2, 5 and 10 mM of metformin, based on the work of Javeshghani et al. (21). An aliquot of supernatant was collected for every group 24 hours after initiation of the experiment. After 48 hours, cells were collected and stored at -80 °C until assayed.

Gene expression

Total RNA was extracted from cell culture or tissue homogenization using TRIzol (22). Reverse transcription reactions were performed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Gene expression was analyzed by quantitative polymerase chain reaction (qPCR) using the SensiFAST™ SYBR® Lo-ROX Kit (Bioline, MA, USA) and the Illumina Eco Real-Time PCR System (Illumina, CA, USA). Nucleic acid concentration was measured in a Qubit 2.0 Fluorometer using kits for the specific measurement of RNA and DNA (Qubit® dsDNA HS Assay Kit, Qubit® RNA HS Assay Kit) (ThermoFisher, MA, USA). Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as a housekeeping gene in rat and ribosomal RNA 18S (RNA18S5) in Caco2. Gene expression levels of GLS, MYC proto-oncogene (MYC), mTOR, SLC1A5 and Gls were determined by the Delta Ct method, assuming a high efficiency of the reaction. Fold change was calculated as the sample/control ratio in three independent experiments and values were expressed as the mean ± SEM.

Biochemical measurements

Ammonia was measured in cell culture supernatant and in rat plasma immediately after collection following the gluta-

mate-dehydrogenase enzymatic assay in a COBAS Integra® 700 (ROCHE, Switzerland).

Enzyme activity

Tissue samples from the small intestine (ileum and duodenum), muscle and kidney were homogenized within two hours after sacrifice using homogenization buffer (sucrose 320 mM, Trizma-HCl 10 mM, PMSF 0.2 µM, EDTA 1 mM, pH 7.4) (Sigma-Aldrich®, MO, USA) before centrifugation at 12,000 rpm for 10 min at 4 °C. The mitochondrial-enriched pellet was then suspended in incubation buffer (sucrose 70 mM, EGTA 1 mM, mannitol 210 mM, K_2HPO_4 150 mM, pH 8) (Sigma-Aldrich®, MO, USA) and exposed to an ultrasonic cell disruptor to ensure the rupture of all cell and mitochondrial membranes and the release of free glutaminase from the mitochondria. Protein concentration was measured using the Bradford method (23).

Glutaminase activity assays were performed following the colorimetric protocol described by Heini et al. (24). Specific activity of the enzyme was expressed in international units (IU) per milligram of total homogenate of protein. One IU was defined as a micromole of substrate transformed per minute.

Statistical analysis

Continuous variables are described as the mean and the error of a minimum of three independent experiments. Data were compared using the ANOVA test with the Tukey's test as post-hoc multiple comparison analyses. The Student's t-test for unpaired samples was used for comparisons between two groups. p values $p < 0.05$ (*) and $p < 0.01$ (**) were considered as statistically significant. The SPSS (version 21.0; SPSS, Inc., IL, USA) statistical package was used in all analyses and graphs were generated using GraphPad PRISM (version 6.0, GraphPad Software, Inc, CA, USA).

RESULTS

Metformin and ammonia production

In PCS rats, metformin treatment for two weeks significantly reduced ammonia levels in plasma. Sham-operated rats had a mean of 110.8 ± 16.1 µg/dl of plasma ammonia. These levels were increased in the PCS-animal model ($p = 0.025$) and significantly decreased after metformin treatment (204.3 ± 24.4 µg/dl *vs* 129.6 ± 16.1 µg/dl; $p = 0.038$), reaching levels similar to sham animals (Fig. 1A).

In the *in vitro* model after 24 hours of incubation, a dose-dependent decrease in the production of ammonia was found in the groups treated with 2, 5 and 10 mM of metformin in comparison to the control (Fig. 1B).

Glutaminase expression and activity

As shown in figure 2, no changes in Gls gene expression levels were associated with PCS or metformin use in rats (Fig. 2A). However, PCS induced an increase in KGA activity in peripheral tissues such as kidney (0.153 ± 0.05 IU/mg *vs* 0.337 ± 0.08 IU/mg) and muscle (0.015 ± 0.005 IU/mg *vs* 0.086 ± 0.026 IU/mg; $p < 0.05$) compared to sham operation (Fig. 2B). In addition, a reduction in KGA activity was observed after metformin treatment in the small intestine (0.277 ± 0.07 IU/mg *vs* 0.142 ± 0.04 IU/mg) and muscle (0.086 ± 0.026 IU/mg *vs* 0.054 ± 0.002 IU/mg), but not in kidney (0.337 ± 0.08 IU/mg *vs* 0.286 ± 0.08 IU/mg) (Fig. 2B). However, these data did not reach statistical significance.

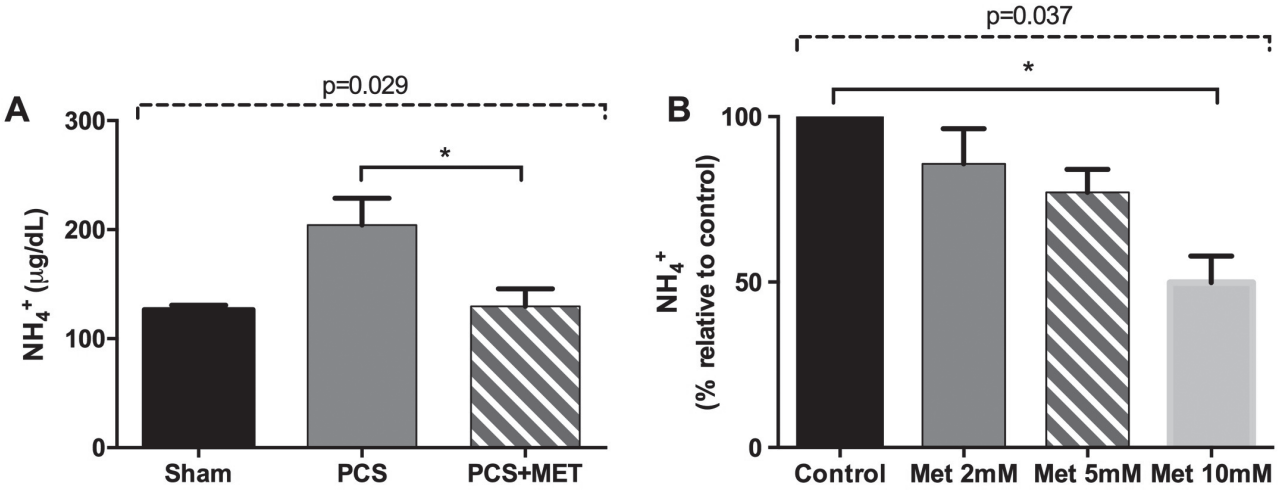

Fig. 1. *Ammonia concentrations in plasma of PCS rats and cell culture. A. Plasma ammonia levels in sham rats (n = 4), PCS (n = 8) or PCS + metformin (30 mg/kg/day) (n = 8) expressed as µg/dl; *p < 0.05. Outlier trimming (exclusion) at the 5th and 95th percentile was performed for every group in the analysis. B. The percentage of ammonia concentration in the culture media of Caco2 exposed to 2, 5 and 10 mM of metformin compared with non-treated cells in three independent replicates. Differences between groups were assessed by ANOVA (dashed line) with Tukey's post-hoc test (continuous line); *p < 0.05. Data are expressed as means ± SEM.*

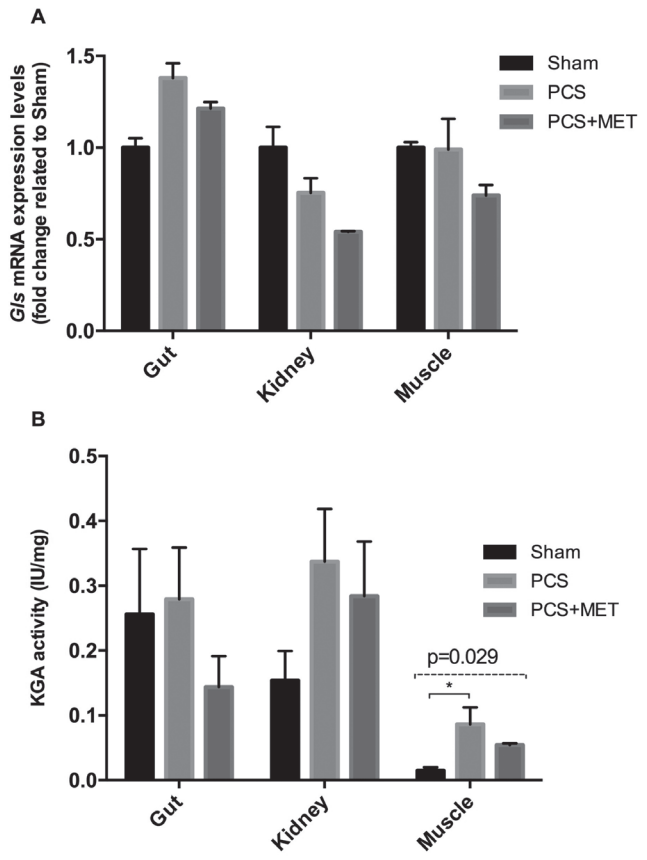

Fig. 2. *Changes in gene expression and activity of K-type glutaminase in different tissues. A. Gls mRNA expression levels in PCS rats (n = 8) and PCS + metformin (30 mg/kg/day) (n = 8) expressed as fold change compared with the sham group (n = 4) in the small intestine, kidney and muscle. B. KGA activity levels expressed as IU/mg. Data are the mean value ± SEM and the differences between groups were assessed by ANOVA with Tukey's* post-hoc *test; *p < 0.05.*

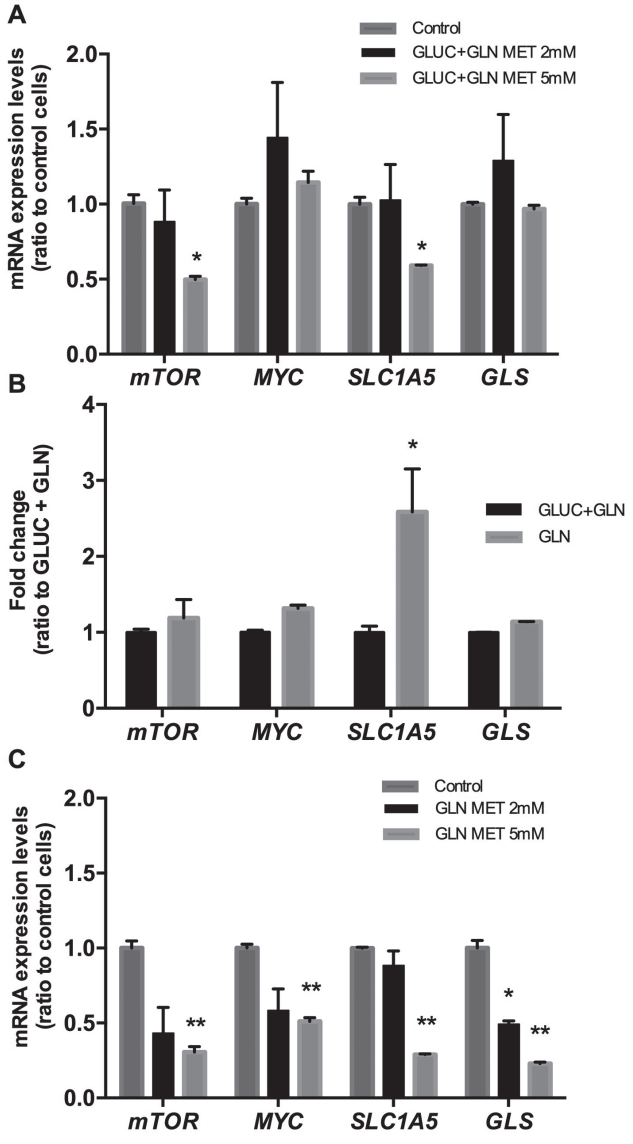

Fig. 3. *Changes in gene expression in Caco2. A. Gene expression levels of candidate genes involved in glutamine metabolism in cells exposed to glucose-containing medium and treated with 2 and 5 mM of metformin. B. Relative mRNA expression levels in Caco2 cells seeded in the presence or absence of glucose (GLN/GLN+GLU). C. Gene expression levels of Caco2 grown in the absence of glucose treated with 2 and 5 mM of metformin. Data are expressed as means ± SEM and differences between groups were assessed by the unpaired t-test or ANOVA; *p < 0.05, **p < 0.01.*

Metformin and glutamine metabolism *in vitro*

Despite the fact that ammonia was measured at 2, 5 and 10 mM, we chose 2 and 5 mM for gene expression level analysis based on the results obtained in the proliferation MTT assay (data not shown). Treatment with 5 mM metformin for 48 hours produced a slight downregulation both in mTOR and SLC1A5 expression in Caco2 with access to glucose and glutamine (0.49 ± 0.03 and 0.59 ± 0.01 fold change respectively; p < 0.05). However, no significant changes were found for the other genes analyzed, neither at 5 mM nor 2 mM doses (Fig. 3A).

As shown in figure 3B, the absence of glucose in the medium induced the expression of SLC1A5 in comparison to cells with free access to both glucose and glutamine (2.54 ± 0.33 fold change; p < 0.05) (Fig. 3B). In the context of glucose deprivation, metformin treatment induced changes in the expression levels of mTOR and MYC in a dose-dependent manner (0.43 ± 0.24 and 0.58 ± 0.20 fold change, at 2 mM [p = ns] and 0.30 ± 0.05 and 0.51 ± 0.03 at 5 mM [p < 0.01], respectively). In the same way, GLS and SLC1A5 expression were downregulated after treatment (0.88 ± 0.13 [p = ns] and 0.49 ± 0.03 [p < 0.05] fold change respectively at 2 mM; 0.28 ± 0.01 and 0.22 ± 0.01 at 5 mM [p < 0.01]) (Fig. 3C).

DISCUSSION

In this proof-of-concept study, we investigated the modulation of ammonia production in the small intestine by analyzing glutaminase expression and activity in an animal model of hepatic encephalopathy. The effect of metformin

in regulating glutamine metabolism was also assessed. PCS animals show hyperammonemia, alterations of circadian rhythms, different aromatic/branched chain amino acid ratios, decreased brain glucose utilization, oxidative/nitrosative stress and an interrupted function of multiple neurotransmitters similar to HE in human cirrhotic patients (25).

Although circulating levels of ammonia depend on the action of various organs, this study was focused on KGA. The activity of this enzyme in the duodenum has been proposed as the main source of the neurotoxin related to the presence of HE and the degree of hepatic dysfunction in patients with cirrhosis (6,26). Besides, higher concentrations of metformin were found in the small intestine compared to other tissues or in plasma (up to about 500 mg/g of tissue). This raises the possibility that the small intestine might be a significant site of action of the drug (27). We previously showed that the increase in ammonia was related to an increased activity of the glutaminase enzyme in the gut of PCS rats. This is consistent with the concept that gut ammonia production is increased in cirrhotic patients after oral glutamine challenge (6,19). In this study, our results indicate that metformin treatment resulted in a reduction of ammonia production reflected by a decrease in glutaminase activity, which was directly measured in plasma and cell culture supernatant. However, this study has some limitations, largely related to its proof-of-concept design. The absence of statistical significance in some points might be due to the low sample size in the *in vivo* study. Besides, the *in vitro* study may be performed in more than one cell line. Nevertheless, our data seem to corroborate the influence of this drug on ammonia homeostasis, postulating it as an alternative therapy against HE. The fact that no changes were found in glutaminase gene expression suggests a posttranscriptional effect, as proposed previously (28). In accordance with our data, Jover-Cobos et al. showed a reduction in glutaminase activity in the gut after treatment with ornithine phenylacetate in bile-duct ligated rats (29). However, since this study was conceived as a proof of concept, further studies with a larger sample size are needed.

Several compounds have recently been tested that inhibit KGA such as DON (6-diazo-5-oxy-i-norleucine), an unspecific glutamine-competitive irreversible KGA inhibitor, which is the most commonly used (30). Other molecules such as 968, BPTES or THDP17 have also been reported to inhibit glutaminase via different mechanisms with *in vitro* efficacy. However, despite all efforts, its high *in vivo* toxicity means it cannot be used for treatment (31,32). Nevertheless, these studies suggest the possibility of many other scaffolds that inhibit glutaminase enzyme activity, enabling further investigations in the field.

Recently, it has become clear that overexpression of MYC causes glutamine addiction via a coordinated transcriptional program by the stimulation of glutamine uptake and metabolism, both directly and indirectly. MYC directly binds to the promoters and stimulates the expression of genes involved in glutaminolysis, such as the transporter ASCT2 and several enzymes utilizing glutamine (8). MYC also influences post-transcriptional regulation, as its overexpression correlates with a significant upregulation of glutaminase protein levels. This occurs indirectly by repression of the expression of miR-23a/b, which target GLS in the 3' untranslated region (UTR) (33).

Here, we confirmed that glucose deprivation induces an overexpression of SLC1A5 leading to increased glutamine uptake, in order to sustain ATP generation through the TCA cycle. In fact, glutamine could sustain the oxidative TCA cycle in MYC-driven cells, even in a glucose deprivation environment, and cells would undergo apoptosis in the absence of this amino acid (34).

A further role for glutamine in cell protein translation stems from observations that a master regulator of protein translation, the mammalian target of rapamycin complex 1 (mTORC1), is responsive to glutamine levels (35). MYC and mTOR are involved in a positive feedback loop. mTORC1/S6K1 controls MYC expression by modulating mRNA translation initiation efficiency via phosphorylation of the initiation factor eIF4B. On the other hand, MYC promotes mTORC1 activity by facilitating leucine uptake (36). The study of Fuchs et al. in 2007 showed that silencing ASCT2 in hepatoma cells caused a reduction of mTOR activity leading to apoptosis. Glutamine taken up by ASCT2 stimulates leucine uptake by a parallel leucine/glutamine antiport catalyzed by the large neutral amino acid transporter 1 (LAT1), this promotes mTORC1 assembly and lysosomal localization (37,38). Furthermore, rapamycin decreases ASCT2 expression, thus indicating a reciprocal effect between this transporter and mTOR activity (37).

Our results show a feedback between MYC and mTOR under glucose withdrawal and ratify its role in the regulation of glutamine metabolism via ASCT2 and KGA. Metformin treatment caused a downregulation of both MYC and mTOR, as previously demonstrated (39,40). In addition, a study by Akinyeke et al. in 2013 showed that the drug stimulates cell cycle arrest and apoptosis with a reduction of MYC protein levels by at least 50% *in vitro* and *in vivo* (41). They addressed the effects on the AMP-activated kinase (AMPK) pathway. However, a recent report suggested that MYC deregulation was a direct consequence of metformin-mediated upregulation of miR-33a/b, an intronic miRNA located within the sterol regulatory binding protein (SREBPs) genes (42).

Along these lines, we suggest that an increased use of glutamine as a carbon source for the cells, the greater the impact that metformin would have on them. Increased glutaminolysis was reported to be induced by a high α-ketoglutarate/citrate ratio. Stress and hyperammonemia have been described to inhibit α-ketoglutarate dehydrogenase, which catalyzes decarboxylation of α-ketoglutarate in the TCA cycle. This increases the ratio and triggers enhanced glutaminolysis (43). The MYC oncogene controls the glutaminolytic pathway and induces glutamine addiction. Metformin use would reduce MYC levels in these cells and therefore, ASCT2 and KGA. This would result in a reduction in the production of metabolites and energy through this pathway and as an indirect consequence, a decrease in ammonia production that could be related to a decreased risk of HE development.

In conclusion, our findings lead us to hypothesize that the reduction of hyperammonemia in PCS rats caused by metformin treatment could be explained by a slight modulation of glutaminase activity. Besides, *in vitro* glucose withdrawal enhances the expression of genes involved in glutamine metabolism, which confers sensitivity to metformin by

decreasing both absorption and degradation of this amino acid in the enterocyte. This could explain, at least in part, the lower prevalence of HE in patients taking metformin.

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