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Prolonging the survival of *Tsc2* conditional knockout mice by glutamine supplementation

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ABSTRACT

The genetic disease tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by loss of function mutations in either *TSC1* (*hamartin*) or *TSC2* (*tuberin*), which serve as negative regulators of mechanistic target of rapamycin complex 1 (mTORC1) activity. TSC patients exhibit developmental brain abnormalities and tuber formations that are associated with neuropsychological and neurocognitive impairments, seizures and premature death. Mechanistically, *TSC1* and *TSC2* loss of function mutations result in abnormally high mTORC1 activity. Thus, the development of a strategy to inhibit abnormally high mTORC1 activity may have therapeutic value in the treatment of TSC. mTORC1 is a master regulator of growth processes, and its activity can be reduced by withdrawal of growth factors, decreased energy availability, and by the immunosuppressant rapamycin. Recently, glutamine has been shown to alter mTORC1 activity in a TSC1-TSC2 independent manner in cells cultured under amino acid- and serum-deprived conditions. Since starvation culture conditions are not physiologically relevant, we examined if glutamine can regulate mTORC1 in non-deprived cells and in a murine model of TSC. Our results show that glutamine can reduce phosphorylation of S6 and S6 kinase, surrogate indicators of mTORC1 activity, in both deprived and non-deprived cells, although higher concentrations were required for non-deprived cultures. When administered orally to TSC2 knockout mice, glutamine reduced S6 phosphorylation in the brain and significantly prolonged their lifespan. Taken together, these results suggest that glutamine supplementation can be used as a potential treatment for TSC.

1. Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant neurodevelopmental disease that causes significant morbidity and mortality. The brains of TSC patients are characterized by focal areas of cortical disorganization called tubers, subependymal nodules, and other developmental lesions. These anatomical defects are often associated with epilepsy, intellectual disability, and autism spectrum disorders. In addition, subependymal nodules can degenerate into low grade subependymal giant cell astrocytomas (SEGAs), which are associated with a wide variety of clinical presentations, in up to 20% of TSC patients [1,5,18]. More than 80% of patients with TSC have loss of function mutations within the genes *TSC1* and *TSC2*, which encode the proteins hamartin and tuberin, respectively [11,23].

Hamartin and tuberin form a heterodimer (TSC1/2) that is a central regulator of the mechanistic Target of Rapamycin Complex 1 (mTORC1). mTORC1 is a multiprotein complex that functions as a cellular kinase, and is composed of a core set of at least 5 key proteins (mTOR, Raptor, PRAS40, Deptor, and mLst8). mTORC1 integrates a diverse array of intra- and extracellular signals, including energy status, growth factors, amino acid and nutrient availability to regulate protein, lipid and nucleotide synthesis, cell growth and autophagy [4,21,29,30,35]. Binding of the small GTPase protein Rheb (Ras-homolog enriched in brain) is required for mTORC1 activity [31]. The TSC1/2 heterodimer controls mTORC1 activity though the GTPase-activating domain of TSC2. TSC1/2 interacts with GTP-bound Rheb, converting it into its inactive GDP-bound

Abbreviations: mTORC, mechanistic Target of Rapamycin Complex; Rheb, Ras-homolog enriched in brain; S6, ribosomal protein S6; S6K, ribosomal protein S6 kinase; TSC, Tuberous Sclerosis Complex.

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form, thus resulting in decreased mTORC1 activity [16,20,22,36]. To a large extent, the molecular pathogenesis of TSC is caused by the cellular consequences of dysregulated, overactive mTORC1 arising from the loss of TSC1/2 inhibitory regulation [14,25,42]. Pharmacological inhibitors of mTORC1, such as rapamycin and other rapalogues, have successfully treated various mouse models of TSC [9,17,24]. These successful preclinical studies have paved the way for many clinical trials for brain, kidney and lung manifestations of TSC [15,41]. Recently, the FDA approved an additional indication for the rapalogue everolimus to treat refractory SEGAs associated with TSC [10,17,28]. However, in some cases the SEGAs are found to re-emerge after discontinuation of everolimus treatment, and the consequences of long-term treatment with rapalogues remains unclear at this time. Therefore, alternative approaches to the inhibition of mTORC1 may have substantial benefit for the pharmacologic treatment of TSC.

Recent *in vitro* studies have shown that amino acids are able to regulate mTORC1 signaling through a pathway independent of TSC1/2 [3,32,45]. These studies have suggested that glutamine, the most abundant amino acid found in the circulation, can modulate mTORC1 activity, with both inhibition [12,33] and stimulation [6,34,40] of mTORC1 activity being reported. However, these studies examined the influence of glutamine on mTORC1 activity using cultured cells exposed to serum- and amino acid-free conditions, making it difficult to determine if glutamine can alter mTORC1 activity under normal physiological conditions. In the present study, we examined if glutamine can alter mTORC1 activity in cultured cells maintained in non-deprived conditions. Our *in vitro* results showed that high concentrations of glutamine inhibited mTORC1 activity, as assessed by decreased phosphorylation of its downstream targets ribosomal protein S6 and S6 kinase. Based on this finding, we then tested if oral glutamine supplementation could be used to decrease mTORC1 activity in the brains of mice with a conditional knockout of Tsc2 in neurons, and prolong their life-span.

2. Materials and methods

2.1. Reagents

L-glutamine was purchased from Sigma–Aldrich (St. Louis, MO). Antibodies for phosphorylated (Serine 240/244) and total S6, and phosphorylated (Threonine 389) and total S6 kinase were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against the neuronal marker NeuN were obtained from Millipore (Billerica, MA).

2.2. Cell culture and western blotting

Mouse brain endothelial cells (bEnd.3; ATCC, stock #CRL-2299) were grown in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum and penicillin/streptomycin (5000 U/5000 μg/ml). Forty-eight hours after reaching confluence, cells were incubated for 2 h with either serum-free DMEM lacking glutamine or DMEM with serum (Life Technologies, Grand Island, NY). Glutamine at different concentrations was added for 30 min, after which cells were lysed in boiling Laemmli sample buffer, and lysates stored at −20 °C until needed for western blot experiments. Lysate protein concentrations were determined by NanoOrange Protein Quantification Assay (Invitrogen, Carlsbad, CA) using bovine serum albumin (BSA) as the standard. Samples were resolved on Tris-glycine SDS-PAGE gels and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked for 30 min in 5% BSA, and then incubated in primary antibodies overnight at 4 °C. Immunoreactivity was detected using species-specific secondary antibodies conjugated to alkaline phosphatase and a chemiluminescence system.

2.3. Tsc2 conditional knock-out mice and treatment

All protocols involving the use of animals were in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by The University of Texas Health Science Center Animal Care and Use Committee. The *Tsc2*$^{flox/flox}$;*CamKIIα-Cre* mouse line was generated by crossing *Tsc2*$^{flox/flox}$ mice [19] to JAX® mice *B6.Cg-Tg*(*CamKIIα-Cre*)*T29-1Stl/J*, stock #005359. The progeny were backcrossed to *Tsc2*$^{flox/flox}$ mice to get *Tsc2*$^{flox/flox}$; *CamKIIα-Cre* (Tsc2CKO). Control littermates constituted animals lacking *CamKIIα-Cre*. Glutamine treatment was initiated when Tsc2CKO mice were 1 month of age. A suspension of 3 g/kg glutamine in saline was given twice a day, 5 days a week via gavage. Animals were monitored daily and the date of death noted.

2.4. Immunohistochemistry

One month old Tsc2CKO mice were given 2 daily doses of either 3 g/kg glutamine or saline, 5 days a week for 2 weeks via gavage. One hour after the last dose, animals were decapitated, their brains quickly removed and dropped-fixed in 4% paraformaldehyde and 15% picric acid. Brains from age-matched control littermates were also collected. Coronal brain sections (40 μm) were incubated with primary antibodies overnight at 4 °C which were then detected using species-specific secondary antibodies conjugated to Alexafluors. Epifluorescent images of the sections were taken using a Zeiss Axiovert S100 microscope and a MicroFire (Optronics, Goleta, CA) camera. The parameters used for image capture were determined using control littermate samples to minimize background and optimize the signal. These parameters were kept constant across all groups. Three non-overlapping regions in the dentate gyrus of the hippocampus and two sections from each animal (n = 5/group) were used for quantification. The fluorescence intensities were measured using Photoshop software (Adobe, San Jose, CA), averaged for each section, then averaged for each animal.

2.5. Statistical analysis

Western blot and immunohistochemistry data were compared using either two-tailed Student's *t*-test for unpaired variables or by one-way ANOVA. The analysis of survival was performed using the Kaplan–Meier method. Comparisons between survival curves were carried out using the log-rank test. Data were considered significant at $p < 0.05$.

3. Results

3.1. Glutamine inhibits mTORC1 activity in vitro

Previous studies have reported inhibition of mTORC1 activity by glutamine using cultured cells deprived of serum and/or amino acids, which does not reflect physiological situations. This inhibition was observed at concentrations ranging from a low of 4 mM to a high of 80 mM glutamine [12,33]. We therefore examined if these concentrations of glutamine can inhibit mTORC1 in cells grown under normal culture conditions. Consistent with previous studies, the addition of 5 mM glutamine to serum-starved bEnd.3 cells significantly decreased the phosphorylation of S6 (Fig. 1A, $p = 0.042$) and S6 kinase (Fig. 1B, $p = 0.018$), both downstream targets of mTORC1. The total levels of S6 and S6 kinase were unaffected. In contrast, when 5 mM glutamine was added to cells growing under normal serum conditions, it failed to significantly

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