Glutamine Directly Downregulates Glutamine Synthetase Protein Levels in Mouse C2C12 Skeletal Muscle Myotubes\textsuperscript{1,2}

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Abstract

This study examined the regulation of glutamine synthetase protein levels, in response to changes in external glutamine concentration, in mouse C2C12 skeletal muscle cells. Glutamine, at concentrations as low as 0.25 mmol/L, downregulated endogenous and exogenous (plasmid encoded) glutamine synthetase with maximal effect at 2 mmol/L. Glutamine appears to act by changing the stability of the glutamine synthetase protein, and the effect was partially blocked by the proteasome inhibitor MG132. The addition of the glutamine structural analog and glutaminase inhibitor, 6-diazo-5-oxo-L-norleucine, in the presence or absence of glutamine, also resulted in low glutamine synthetase protein levels. Otherwise, the effect was specific for glutamine, and the only compounds able to mimic the effect of glutamine were amino acids, glutamate, alanine, and ornithine, which can be converted to glutamine. Other amino acids, analogs, and products of glutamine metabolism were without effect. Methionine sulfoximine, an inhibitor of glutamine synthetase, stabilized the protein and prevented the glutamine effect. Thus, in mouse C2C12 skeletal muscle cells, glutamine synthetase protein expression is regulated by glutamine through changes in the rate of degradation of the protein. The effect is specific to glutamine, which acts directly without requiring prior metabolism.  


Introduction

Glutamine plays an important role in the interorgan transport of nitrogen, carbon, and energy, and the turnover of plasma glutamine is much higher than that of other amino acids (1,2). In addition, skeletal muscle concentrates free glutamine to high levels, and the maintenance of this intracellular pool has been proposed to be important in skeletal muscle protein homeostasis (3,4). Although it is present in the diet, most ingested glutamine (and glutamate) is metabolized by the small intestine, and thus, under normal dietary conditions, there is no net absorption into the circulation (5). Therefore, all glutamine within the body is synthesized de novo, through the action of glutamine synthetase [L-glutamate ammonia ligase (ADP) EC 6.3.1.2], with skeletal muscle, liver, lungs, and adipose tissue recognized as the major sites of glutamine net production (1,2,6,7). During times of catabolic stress, there is an increased need for glutamine by tissues such as the kidneys, liver, and cells of the immune system, and an early response to such stress is an increase in the release of glutamine from skeletal muscle with a resultant drop in intramuscular glutamine concentrations. Usually, the glutamine concentration in the muscle cell stabilizes, but the release of glutamine continues at elevated rates, indicating increased rates of glutamine synthesis (1,2,8,9). If the stress continues, there is an accompanying upregulation of muscle glutamine synthetase expression in keeping with the continued synthesis and release of glutamine by this tissue.

Glutamine synthetase activity is not known to be subject to short-term regulation by allosteric or covalent modification mechanisms, but it is regulated through changes in the amount of protein at both the pre- and post-translational levels (1,2,9–15). In skeletal muscle, the activity is increased by starvation, insulin-dependent diabetes, sepsis, and denervation (9–14). Glucocorticoids are responsible, at least in part, for such changes, with the primary mechanism involving increased rates of transcription of the glutamine synthetase gene. In addition, it has been well known since the 1950s that glutamine synthetase activity in a variety of cell lines in culture is subject to downregulation in the presence of glutamine. This was first demonstrated by DeMars (16) in HeLa cells and has subsequently been confirmed in chick embryo retinal cells, V79 lung cells, hepatomas, L cells and 3T3 L1 adipocytes (17–27). In most cells, the mechanism involves an acceleration of glutamine synthetase protein degradation in the presence of glutamine with no change in gene transcription. Some earlier studies (28–30) infer that glutamine may also regulate glutamine synthetase mRNA abundance in some cell types, but this has only been confirmed in FTO-B hepatoma cells (31), and we did not detect such changes in Hep G2 hepatoma cells, 3T3 L1 adipocytes, or C2C12 myotubes (11). Despite the importance of skeletal muscle in providing glutamine to the body, few other cell types are known to use glutamine as a metabolic precursor. Therefore, it is surprising that the mechanism of glutamine synthetase regulation has been so elusive, and we believe that the mechanism by which glutamine regulates glutamine synthetase expression is essential to understanding the role of this enzyme in the metabolic regulation of the cell.
studies have examined the regulation of glutamine synthetase in this tissue. Smith et al. (26) demonstrated that glutamine could regulate glutamine synthetase in L6 muscle cells, and this was confirmed by Feng et al. (24), who additionally demonstrated that changes in the abundance of glutamine synthetase mRNA were not involved.

Glutamine is also known to play a signaling role in many processes, including the regulation of metabolism, expression of specific genes, suppression of inflammatory cytokine production, maintenance of cell-to-cell interactions, regulation of blood flow, stimulation of insulin secretion, and general promotion of cell proliferation and protein synthesis, while decreasing proteolysis (32). Many of these effects are due to a direct action of glutamine, but others require metabolism of glutamine to a secondary metabolite, for example, glucosamine, which is then responsible for the specific effects (32-34). But few studies have investigated the mechanisms by which glutamine brings about accelerated degradation of glutamine synthetase. Some work has been done with inhibitors of the enzyme activity in hepatomas (17,22), adipocytes (27), and lung cells (9), and Freikof and Kulka (23) carried out an extensive characterization of glutamine analogs in HTC hepatoma cells. Although such studies indicate that some analogs can act as glutamine mimetics, the question of whether glutamine metabolism is required has not been addressed directly.

The apparent feedback downregulation of glutamine synthetase by glutamine may play a role in vivo, insofar as it is well documented that glutamine levels drop dramatically during stress, such as in response to sepsis, burns, and trauma (1,2,8). In addition, the extensive use of supplemental glutamine in such conditions aims to increase both circulating and intramuscular glutamine concentrations and thus could influence expression of the enzyme. To date, however, it has not been possible to demonstrate the effect of glutamine levels on glutamine synthetase expression in vivo, in part because the conditions that result in lowered glutamine levels are also accompanied by elevated levels of glucocorticoids.

In C2C12 myotubes, we established that glucocorticoids increase glutamine synthetase expression through changes in the abundance of the mRNA (11). In contrast and in confirmation of work done with other muscle cell lines, we found that culture in the absence of glutamine also resulted in higher levels of glutamine synthetase protein without changes in the level of the mRNA. Our study was designed to determine both the mechanism by which glutamine brings about changes in glutamine synthetase in muscle cells and whether the effect required glutamine metabolism. The results provide strong evidence that glutamine acts to accelerate the degradation of the glutamine synthetase protein, and that this effect is not dependent on prior metabolism of the glutamine.

Materials and Methods

Materials. C2C12 cells were obtained from the ATCC. DMEM, with or without glutamine, fetal bovine serum (FBS), trypsin-EDTA, penicillin/streptomycin, PBS, and 4-12% NuPage-Bis-Tris gels were obtained from Invitrogen. MG132, and Protease Inhibitor Cocktail III were from Calbiochem. Protein determination kits were from BioRad Laboratories, albizzi from Fisher Scientific, mouse anti-sheet glutamine synthetase and goat anti-mouse IgG were from BD Biosciences Parningen, and ECL Western Detection Reagents were from Amersham Biosciences. All other chemicals were from Sigma Aldrich.

Cell culture. C2C12 cells were cultured in DMEM containing penicillin (100 KU/L) streptomycin (100 mg/L), 10% fetal bovine serum (FBS) and 4 mmol/L l-glutamine with medium replacement every 2 d. Before the cells reached 80–90% confluence they were trypsinized and split into 100 mm × 20 mm dishes (9.8 × 10⁶ cells/dish) and cultured until confluent. For differentiation, the FBS content of the medium was decreased to 1% and the cells cultured for an additional 2–3 d until complete development of myotubes. Cells were then cultured in medium containing no FBS for 24 h and then subjected to experimental treatments. With the exception of time course studies, all experiments were of 48 h duration. At the end of the experiments, cells were harvested directly into 0.5 mL homogenization solution (0.33 mol/L sucrose, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, 3 mmol/L Hepes pH 7.4, 5 mL/L protease inhibitor cocktail) and homogenized using a model 985-370 Tissue Tearor (Biospec Products). Extracts were centrifuged at 8000 × g for 10 min at 4°C and the supernatants stored at −80°C. In selected experiments, cell viability was assessed after 1 h incubation with thiazolyl blue tetrazolium bromide (0.5 g/L) and determination of the absorbance at 570 nm.

Glutamine synthetase gene transfection. C2C12 cells were cultured in 6-well plates until differentiation into myotubes. Plasmds (4 μg DNA/well), the mouse glutamine synthetase cDNA sequence linked to the cytomegalovirus (CMV) promoter, or the control CMV plasmid, were transfected into the cells using the GenePorter 2 system according to the manufacturer’s instructions. Cotransfection with β-gal showed similar transfection efficiency in all wells. Cultures, in the presence or absence of glutamine, were continued for a further 48 h.

Western blot analysis. The protein content of the extracts was determined by the Bradford method using a Bio-Rad kit and bovine serum albumin as the standard. Equal amounts (10 μg protein) of the extracts were solubilized and subjected to electrophoresis in 4–12% Bis-Tris SDS gels. Proteins were transferred to nitrocellulose membranes and evenness of loading and transfer was checked by Ponceau dye. Membranes were blocked with fat-free dried milk followed by incubation with the primary antibody (antiglutamine synthetase diluted 1:5000). Glutamine synthetase protein bands were detected by incubation with the second antibody (Goat anti-mouse IgG diluted 1:5000) followed by visualization using the enhanced chemiluminescent detection system and exposure to Kodak MR film. Images were digitized and imported into Adobe Photoshop and bands quantitated using the National Institutes of Health Image Software (SCION Image). Glutamine synthetase protein abundance is expressed as arbitrary densitometry units.

Statistical analyses. Experimental limitations, such as the number of electrophoresis systems and wells per gel, did not allow for comparisons of all conditions in the same experiment. Thus, various conditions were tested in many individual experiments to identify potential agents for further investigation. Cells cultured with and without glutamine (0 and 2 mmol/L) were included in every experiment. Results were expressed as means ± SEM and analyzed by 1- or 2-way ANOVA using GraphPad Prism Software, version 4.03. Dunnnett’s multiple comparison tests (1-way ANOVA) or Bonferroni’s post-tests (2-way ANOVA) were used to compare individual means. Significance was determined at P < 0.05.

Results

The abundance of glutamine synthetase protein was highest in cells cultured in the absence of added glutamine and was gradually lower as the glutamine concentration increased (Fig. 1). The effect was clearly seen at external glutamine levels as low as 0.25 mmol/L and was maximal at 2 mmol/L; increasing glutamine levels to 4 mmol/L had no further effect (results not shown). We previously showed that glutamine had no effect on the abundance of glutamine synthetase mRNA in C2C12 cells (11). To confirm that the effects were independent of transcription of the glutamine synthetase gene, we used the CMV promoter to drive the expression of exogenous glutamine synthetase in C2C12 cells. In cells cultured without glutamine this promoter...
resulted in high levels of glutamine synthetase protein but, in the presence of glutamine, the abundance of the enzyme protein was markedly suppressed (Fig. 2).

The culturing of C2C12 cells in the presence of glutamine (2 mmol/L) resulted in minimal levels of glutamine synthetase protein, and a subsequent culture in the absence of added glutamine caused a gradual increase in the abundance of the protein reaching maximal levels by 48 h (Fig. 3A). Changes did not occur when the culture was extended for an additional 48 h (results not shown). In contrast, the addition of glutamine to cells cultured in the absence of glutamine did not change the abundance of the protein over the 48 h of culture. The results obtained from the time course following the addition of glutamine were used to calculate an apparent half-life for glutamine synthetase protein of 7.7 h.

Previous studies in a variety of cell types demonstrated that glutamine exerts its action on glutamine synthetase protein abundance by changing the rate of degradation of the enzyme, with no effect on the rate of enzyme synthesis. To demonstrate this mechanism in C2C12 cells, we attempted to immunoprecipitate radiolabeled (35S-methionine and cysteine) glutamine synthetase protein. Despite many attempts, including the use of protein A sepharose beads, we were unable to obtain clean immunoprecipitates using the commercially available antibodies. To obtain indirect evidence that proteolysis was the major mechanism involved, cells were cultured without glutamine and then incubated in the presence of glutamine with or without MG132, an inhibitor of the ubiquitin-multicatalytic proteasome system. In the absence of inhibitor, the amount of glutamine synthetase protein decreased by 40 ± 6% over 12 h of culture with glutamine but by only 25 ± 6% (P < 0.05) in cultures containing the inhibitor MG 132 (0.01 mmol/L). Increasing the concentration of MG132 to 0.05 mmol/L to further block proteolytic degradation was unsuccessful because it resulted in a marked decrease in cell viability.

The addition of glutamate, alanine, ornithine, or arginine (all at 2 mmol/L) resulted in lower levels of glutamine synthetase protein, but the effects were not as large as those seen with glutamine, and leucine and proline were without effect (Fig. 4).
Similarly, ammonium chloride (both a substrate for glutamine synthesis and a product of glutamine metabolism), and glucosamine (a product of glutamine metabolism) were without effect. The addition of aminooxyacetate (0.5 mmol/L), an inhibitor of pyridoxal phosphate–dependent enzymes such as aminotransferases, prevented most of the effects of alanine and ornithine.

Amino acids that were able to lower glutamine synthetase levels, glutamate, alanine, and ornithine, are all potential substrates for glutamine synthetase and thus could be acting indirectly after being converted to glutamine. To test this possibility, the cells were cultured with methionine sulfoximine (MSO, 0.1 mmol/L), a reasonably specific inhibitor of glutamine synthetase. The presence of MSO blocked the effects of all treatments and resulted in levels of glutamine synthetase protein higher than those for the control (no glutamine) incubations (Fig. 4). The exception was when MSO was included together with glutamine, where the abundance of the protein was similar to that seen with glutamine alone.

The effect of glutamine on glutamine synthetase protein levels could be a direct effect of glutamine or it may require glutamine metabolism to some secondary signaling molecule. As indicated above, a number of other amino acids were not able to mimic the action of glutamine, and glucosamine, a metabolite of glutamine known to be involved in the signaling effects of glutamine in some other systems, was without effect. Quantitatively, the most important enzyme of glutamine metabolism is hydrolysis to glutamate and ammonia via glutaminase; otherwise, glutamine is utilized by a family of amidotransferases and a few minor transaminases. Diazonorleucine (DON, 6-diazo-5-oxo-L-norleucine) is a strong inhibitor of glutaminase, although it does block the amidotransferases to a limited degree (35). Incubation of C2C12 cells with glutamine and DON (0.5–2 mmol/L) for 48 h resulted in low levels of glutamine synthetase protein, which is similar to results of glutamine alone (results not shown). The addition of DON (2 mmol/L) in the absence of glutamine also mimicked the effect of glutamine, but effects were not seen until 12–24 h of culture, in contrast to the 6–12 h that occurred with glutamine (Fig. 5). Two other glutamine analogs (both at 1–4 mmol/L), acivicin (L-[αS,5S]-α amino-3-chloro-4,5, dihydro-5-isoxazolacetic acid) and albizziin (L-2-amino-3-ureidopropionic acid), were without effect in the presence or absence of glutamine (2 mmol/L) (results not shown).

**Discussion**

The effect of glutamine on glutamine synthetase in C2C12 cells, and other cells in culture, occurs at extracellular glutamine concentrations similar to levels seen in the plasma of healthy subjects.
even under such conditions, muscle glutamine levels remain relatively high (>2 mmol/L) and it is difficult to envision an effect on glutamine synthetase expression. However, compared with the proposed role of glutamine in the regulation of muscle protein turnover, it may be that the change in intracellular glutamine concentration is part of the signaling mechanism, rather than the absolute glutamine concentration (4). Another problem in determining whether glutamine has a regulatory role in vivo is that most conditions that result in changes in muscle glutamine synthetase activity are accompanied, not only by a drop in glutamine levels, but also by rises in glucocorticoids that are known to increase the rate of transcription of the glutamine synthetase gene. Labow et al. (28,29) noted that the abundance of glutamine synthetase mRNA increased by 400–800% in lung and skeletal muscle of dexamethasone-treated rats, whereas the amount of protein and enzymatic activity increased by only 40–100% and concluded that post-translational regulation was involved. Recently, Meynial-Denis et al. (36) attempted to use the glutamine chelating agent, phenylbutyrate, to lower glutamine levels but obtained only minor changes in circulating (not intramuscular) glutamine levels, and skeletal muscle glutamine synthetase expression was unchanged. Some evidence for a role of glutamine, at least in breast cancer-derived tumor cells, was seen in the work of Collins et al. (37) where there was an inverse correlation of glutamine synthetase expression and tumor glutamine concentration.

In conclusion, the work presented here provides strong evidence that glutamine regulates the level of glutamine synthetase protein in C2C12 by changing the rate of degradation of the enzyme. The effect is specific for glutamine, which acts directly, without requiring prior metabolism. Although intramuscular glutamine levels appear to be sufficiently high to rule out a physiological role for glutamine in glutamine synthetase regulation in vivo, the fact that such levels can drop dramatically suggests that the effect may function in catabolic states and may be important when circulating glutamine levels are elevated in response to intravenous glutamine delivery or very large oral glutamine supplementation.

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