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Glutamine, insulin and glucocorticoids regulate glutamine synthetase expression in C2C12 myotubes, Hep G2 hepatoma cells and 3T3 L1 adipocytes

Yanxin Wang and Malcolm Watford

Department of Nutritional Sciences, Thompson Hall, Cook College, Rutgers, The State University of New Jersey, New Brunswick, NJ 08901

Summary

The cell-specific regulation of glutamine synthetase expression was studied in three cell lines. In C2C12 myotubes, glucocorticoids increased the abundance of both glutamine synthetase protein and mRNA. Culture in the absence of glutamine also resulted in very high glutamine synthetase protein abundance but mRNA levels were unchanged. Glucocorticoids also increased the abundance of glutamine synthetase mRNA in Hep G2 hepatoma cells but this was not reflected in changes in protein abundance. Culture of Hep G2 cells without glutamine resulted in very high levels of protein, again with no change in mRNA abundance. Insulin was without effect in both C2C12 and Hep G2 cells. In 3T3 L1 adipocytes glucocorticoids increased the abundance of both glutamine synthetase mRNA and protein, insulin added alone had no effect but in the presence of glucocorticoids resulted in lower mRNA levels than seen with glucocorticoids alone, although protein levels remained high under such conditions. In contrast to the other cell lines glutamine synthetase protein levels were relatively unchanged by culture in the absence of glutamine. The results support the hypothesis that in myocytes, and hepatomas, but not in adipocytes, glutamine acts to moderate glutamine synthetase induction by glucocorticoids.

Keywords

glutamine; glutamine synthetase; skeletal muscle; liver; adipocyte; insulin; glucocorticoids

Glutamine is the most abundant free α amino acid in most mammalian species. The plasma glutamine pool is turning over very rapidly since it plays important roles in the interorgan transport of carbon, nitrogen and energy [1]. Although present in the diet, most ingested glutamine is metabolized by the small intestinal mucosa and thus the large body pool of glutamine is synthesized de novo [2]. The only enzyme capable of glutamine synthesis is glutamine synthetase (L-glutamate:ammonia ligase (ADP) EC 6.3.1.2) which is found in relatively high activity in skeletal muscle, adipose tissue, liver, lungs, brain and small intestine, but its regulation is poorly understood [3–9]. The enzyme is not known to be subject to regulation by allosteric or covalent modification mechanisms and those agents that show stimulatory or inhibitory actions in vitro are unlikely to play any physiological role [5,7]. The enzyme is however, subject to long-term regulation via changes in the amount of enzyme protein. Diabetes increases expression of the enzyme in skeletal muscle and adipose tissue and

Address for correspondence: Malcolm Watford, D.Phil., Watford@aesop.rutgers.edu, Tel. 732 932 7418, Fax. 732 932 6837.

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some models, including exogenous glucocorticoids, of hypercatabolic stress increase the activity in muscle, adipose tissue and lung [4,5,8–22]. Extensive study has shown that the hormonal regulation of glutamine synthetase expression is mainly at the level of gene transcription [3,6,9,23].

In addition to hormonal regulation, it has been known since the 1950s that glutamine synthetase activity in a variety of cell lines in culture is subject to down-regulation in the presence of glutamine [24–39]. The principal mechanism involved has been established as glutamine causing a rapid acceleration of the degradation of the glutamine synthetase protein with little change in transcription or translation in most cells. Some earlier studies [8,18] have indicated that glutamine also regulates the level of glutamine synthetase mRNA through a post-transcriptional mechanism and changes in mRNA stability in the presence of exogenous glutamine were recently reported in FTO-2B hepatoma cells [9]. A limited number of studies have attempted to dissect the effects of glucocorticoids from those of glutamine in the regulation of glutamine synthetase in lung and muscle in vivo but these have yielded equivocal results [8,20,40]. The work reported here was designed to determine if glutamine synthetase was subject to common regulatory patterns and mechanisms in response to glutamine and hormones in two cell lines representing major tissues (skeletal muscle and adipose tissue) of glutamine synthesis in the body and in the Hep G2 hepatoma line.

Materials and Methods

Materials

HepG2, C2C12, and 3T3 L1 cells were obtained from the ATCC (Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM) with or without glutamine, fetal bovine serum (FBS), trypsin-EDTA solution, penicillin/streptomycin, phosphate buffered saline (PBS), Trizol, and 4%–12% NuPAGE-Bis-Tris gels were from Invitrogen Corp. (Grand Island, NY). Protease inhibitor cocktail III was from Calbiochem (San Diego, CA). Protein determination kits were from BioRad (Hercules, CA) and dCTP ^{32}P from PerkinElmer (Boston, MA). Mouse anti-sheep glutamine synthetase and goat anti-mouse IgG were from BD Biosciences, Pharmingen (San Jose, CA). ECL Western Blotting Detection Reagents were from Amersham Biosciences (Piscataway, NJ) and MR film from Kodak (Rochester, NY). All other chemicals were from Sigma Aldrich Inc (Atlanta, GA).

Cell culture

C2C12, 3T3 L1 and Hep G2 cells were seeded in 75cm² flasks with DMEM containing penicillin (100U/ml)/streptomycin (100µg/ml), 10% FBS and 2mM glutamine. When C2C12 or 3T3 L1 cells reached 90–95% confluence, and Hep G2 about 75% confluence, cells were trypsinized and split into 100 x 20 mm Corning dishes (9.8×10^5 cells per dish). Two days after achieving confluence C2C12 cells were switched to 1% FBS medium for differentiation into myotubes (2 to 3 days). 3T3 L1 cells were held at confluence for two days and were then stimulated to differentiate into adipocyte-like cells by culture in differentiation medium (0.5mM 3-isobutyl-1-methylxanthine, 10µg/ml insulin, 10µM dexamethasone and 8µg/ml biotin in 10% FBS DMEM) for three days followed by cultured in regular 10% FBS DMEM for a further three days, medium was changed daily. C2C12 myotubes, mature 3T3 L1 adipocytes, and Hep G2 cells that had been cultured for two to three days at around 75% confluence, were pretreated by culture in serum free medium in the presence or absence of 2mM glutamine. After 24h pre-treatment, cells were treated with hormones (7nM insulin and/or 25nM dexamethasone) in the presence or absence of 2mM glutamine and cultured for a further 48h.

RNA extraction and Northern Blotting

Cells from one dish for each treatment were washed three times with PBS and RNA extracted using Trizol according to the manufacturers instructions. Total RNA (20µg per lane) was subjected to denaturing electrophoresis, northern blotting and hybridization to ³²P dCTP labelled mouse glutamine synthetase cDNA [10] as previously described [41]. The intensity of the bands was quantified using Un-scan it software (Silk Scientific, Orem UT) and data are expressed as the ratios of absolute pixel intensity of GS mRNA and ethidium bromide stained 18S rRNA.

Western blotting

Cells from one dish per treatment were washed three times with PBS and then homogenized (Tissue Tearor, Biospec Products, Bartlesville OK) in 0.4ml 0.33M Sucrose, 1mM DTT, 1mM EDTA, 5mM HEPES pH 8.0, containing 5µl/ml Protease inhibitor cocktail Set III. The total protein content of the homogenate was determined by the Bradford method using bovine serum albumin as the standard. Equal amounts (20µg) of protein were separated by electrophoresis in a 4%–12% gradient gel, followed by eletrotransfer to nitrocellulose with evenness of transfer checked by Ponceau staining. Membranes were blocked with fat-free dry milk followed by incubation with mouse anti-glutamine synthetase antibody (diluted 1:10,000). Glutamine synthetase protein bands were detected by incubation with anti-mouse immunoglobulin conjugated with horseradish peroxidase (diluted 1:10,000) and visualization by the ECL detection system and exposure to X-ray film. Fluorographs were analyzed using the Un-scan it software. Results are expressed as relative pixel intensities (arbitrary units). Samples from groups to be compared were always run on the same gel. Values from different gels were normalized to an arbitrary value for a pooled sample included on every gel.

Statistical analysis

Results are expressed as mean ± SEM and were analyzed by two-way ANOVA (hormone and glutamine) using GraphPad Prism Software (San Diego, CA) version 4.03. Bonferroni Post-tests were used to compare individual means between the absence and presence of glutamine within each hormone treatment. The same method was also used to compare individual means amongst hormone treatments (with and without glutamine) and in the absence or presence of glutamine (with and without hormones). P values less 0.05 were considered significantly different.

Results

C2C12 skeletal muscle cells

The abundance of glutamine synthetase mRNA (Figure 1.A) in C2C12 cells was highest when the cells were cultured in the presence of the synthetic glucocorticoid, dexamethasone ($p<0.05$). Insulin had no effect, either alone or when added in combination with dexamethasone. The abundance of glutamine synthetase mRNA was slightly higher ($p<0.05$) in cells cultured with dexamethasone and insulin in the absence of glutamine than when glutamine was present. In other conditions the presence or absence of glutamine had no effect on glutamine synthetase mRNA levels. In contrast, the abundance of glutamine synthetase protein (Figure 1.B) was much higher in all cells cultured in the absence of glutamine when compared to those cultured with glutamine ($p<0.05$). Dexamethasone resulted in elevated levels of glutamine synthetase protein in all cases regardless of the presence or absence of glutamine ($p<0.05$). Similar to the results for mRNA levels, glutamine synthetase protein levels were not affected by insulin.

HepG2 hepatoma cells

In Hep G2 cells the abundance of glutamine synthetase mRNA (Figure 2.A) was higher ($p<0.05$) in the presence of dexamethasone. Insulin alone was without effect on glutamine synthetase mRNA levels. Although the level was lower with insulin and dexamethasone than with dexamethasone alone in the absence of glutamine ($p<0.05$), no such differences were seen in the presence of glutamine. In contrast to the differences seen in mRNA levels, in cells cultured with glutamine, the amount of glutamine synthetase protein (Figure 2.B) was unchanged by insulin or dexamethasone. In all cultures in the absence of glutamine however, the abundance of glutamine synthetase protein was very high ($p<0.05$) with no differences seen between the hormonal treatments.

3T3 L1 adipose cells

Culture of 3T3 L1 cells with dexamethasone resulted ($p<0.05$) in very high glutamine synthetase mRNA levels (figure 3.A). Insulin alone had no effect but insulin together with dexamethasone resulted in lower, compared to dexamethasone alone ($p<0.05$), levels of the mRNA. The presence or absence of glutamine had no effect on glutamine synthetase mRNA levels in 3T3 L1 cells. Culture with dexamethasone resulted in higher ($p<0.05$) levels of glutamine synthetase protein (Figure 3.B) regardless of the presence or absence of insulin. In contrast to the other cell lines, culture with glutamine had little effect on the levels of the protein. Only culture with insulin in the absence of glutamine resulted in higher levels of glutamine synthetase protein when compared to similar culture conditions in the presence of glutamine. In this regard it is worth noting that culture with insulin alone, in the presence of glutamine, appeared to result in a slightly lower level of glutamine synthetase protein when compared to the control cultures with glutamine, but the result was not statistically significant.

Discussion

Glutamine plays important roles in the inter-organ transport of nitrogen, carbon and energy. Since most dietary glutamine is metabolized by the small intestinal mucosa, the large pool of glutamine in the body is synthesized de novo. The primary sites of net glutamine synthesis are skeletal muscle, liver, lungs and adipose tissue and in catabolic states the increased glutamine needs of the kidney, liver and immune system are met by increased glutamine synthesis. Although such conditions are usually accompanied by hormonal changes, including a large increase in glucocorticoids, an early event is a large drop in both circulating and intra-muscular glutamine levels [1,4,8,20]. Glutamine is known to down-regulate glutamine synthetase levels in a variety of cell types. Thus it is possible that the changes seen in glutamine synthetase activity in catabolic states are a result of both hormonal increases in gene transcription and a stabilization of glutamine synthetase protein due to the lower glutamine levels.

Glucocorticoid induction of glutamine synthetase has been described for many cell types with the mechanism involving increased protein synthesis, higher mRNA abundance, and, in L6 skeletal muscle and 3T3 L1 cells at least, an increase in gene transcription [42,43]. In addition, there is evidence of post-translational regulation in fao hepatoma cells [23]. Working with rats in vivo Abcouwer et al [16] showed that glucocorticoid treatment increased glutamine synthetase mRNA abundance in skeletal muscle, lung, heart and possibly liver, but not in thymus. In the same study the abundance of the protein was only significantly increased in lung, with small increases in skeletal muscle and thymus. The short time (4h) course of the experiments may have precluded the detection of changes in the amount of protein in some tissues given that glutamine synthetase protein has a reported apparent half-life from hours to many days [2,27,35,36,44].

In C2C12 and 3T3 L1 cells we confirmed increased expression of glutamine synthetase mRNA in response to dexamethasone that was mirrored by changes in protein levels. In contrast although glucocorticoids increased the level of glutamine synthetase mRNA in Hep G2 cells there was no detectable changes in the level of the protein. In this case the duration of the experiment (48h) should have been sufficient to see changes in protein abundance and therefore the presence of 2mM glutamine was probably suppressing the effect of the elevated mRNA levels. In liver, expression of glutamine synthetase is limited to a very small layer of parenchymal cells surrounding the venous outlet [1,3,5,6]. This activity is very stable and only changes in response to somewhat severe perturbations such as feeding low protein diets and after partial hepatectomy, portal-caval shunts, or hypophysectomy [3,5,6,9,45,46]. Expression in primary hepatocytes [47] is not subject to regulation by glucocorticoids but our findings of higher levels of glutamine synthetase mRNA in response to glucocorticoids in Hep G2 cells is in agreement with work with other hepatoma cell lines [28,31,33–36]. However, in those studies there was an accompanying increase in glutamine synthetase protein synthesis and enzyme activity not seen in our work. This may be due to the sensitivity of glutamine synthetase in Hep G2 cells to medium glutamine levels such that any changes in glutamine synthetase protein synthesis were nullified by the accelerated rate of protein breakdown caused by the presence of 2mM glutamine. In this regard it is interesting that glutamine synthetase protein levels were extremely high in all conditions in the absence of exogenous glutamine that probably masked any changes due to hormonal treatments.

The effect of insulin on glutamine synthetase is not clear, the activity is increased in skeletal muscle and adipose tissue in response to insulin dependent diabetes but direct effects of insulin have been difficult to establish. In 3T3 L1 cells differentiation (in the presence of high insulin and glucocorticoids) results in an 80-fold increase in glutamine synthetase expression but, in contrast, in mature 3T3 L1 adipocytes, where expression is increased by glucocorticoids and cAMP, insulin results in a decrease in expression [43,48–50]. In our work insulin had little effect when added alone but was able to almost totally suppress the increase in glutamine synthetase mRNA abundance brought about by glucocorticoids. This effect was only seen at the level of mRNA but, despite the lower mRNA levels, the protein levels remained high. It may be that effects at the level of protein require longer to be seen but it is also possible that insulin is in some way changing the level of glutamine synthetase protein by either increasing synthesis or decreasing degradation. Whatever the mechanism the effect is in the opposite direction to the decrease seen in mRNA levels with insulin alone and thus does not resolve the role of insulin in vivo.

It has been known since the 1950s that glutamine synthetase is subject to down-regulation by glutamine. DeMars [24] was first to demonstrate the effect in HeLa cells and others have shown it in chick embryo retinal cells, V79 and L2 lung cells, hepatomas, L cells, astrocytes, IEC intestinal cells, and L6 muscle cells [25–39]. Where examined, the effect has usually been found to be independent of changes in mRNA abundance or enzyme protein synthesis, and the primary mechanism involves an acceleration of glutamine synthetase protein degradation in the presence of glutamine. Some earlier reports indicated an effect of glutamine on glutamine synthetase mRNA abundance [8,18] and this has recently been confirmed in FTO-2B cells where the stability of the mRNA is decreased in the presence of exogenous glutamine [9]. In contrast, we found no changes in mRNA abundance in any of the three cell lines used in the present study. We have recently confirmed changes in protein turnover as the major mechanism in C2C12 cells where we, similar to the findings of Labow [4] in L2 lung cells, have evidence for involvement of the ubiquitin-proteasome system (Y.F. Huang and M Watford, unpublished results). In the present work the effect of glutamine was most pronounced in Hep G2 cells where it appears that 2mM glutamine is sufficient to completely block the dexamethasone induced changes (about a 50% increase) in mRNA abundance. Conversely, culture in the absence of glutamine was sufficient to result in a more than doubling in the amount of protein

that was not related to changes in mRNA abundance. In C2C12 cells glucocorticoids, in the presence of glutamine, resulted in an approximately 6-fold higher mRNA abundance that was matched by a difference of similar magnitude in protein level. In the absence of glutamine however, a similar 6-fold higher level of mRNA in the presence of glucocorticoids was accompanied by only a doubling of the amount of protein. This was due, in part, to higher levels of glutamine synthetase protein in the control cells cultured in the absence of glutamine but the results illustrate the strong effect that glutamine has in this cell line. Thus in Hep G2 and C2C12 cells glutamine is clearly moderating the effects of glucocorticoids. In contrast, and as previously reported by Bhandari and Miller [49], glutamine appears to have very little effect on glutamine synthetase in 3T3 L1 adipocytes.

An important question arising from such work, is whether the effects are of physiological and/or pathological significance. Extensive studies [3,9,47] demonstrate that glutamine synthetase in primary hepatocytes is not subject to such regulation but the activity of hepatomas appears to be regulated differently in each cell line. Similarly, the activity in 3T3 L1 adipocytes shows only mild regulation by glutamine levels and is therefore unlikely to be of importance in vivo. In contrast the activity in C2C12 myocytes is clearly very sensitive to exogenous glutamine. Plasma glutamine levels in healthy animals are in the range of 0.5–0.8mM and can fall by 50% in catabolic states [1,2]. The effects of glutamine on glutamine synthetase in a variety of cells are seen at extracellular concentrations in this range (0.2–0.8mM) [31,35] and we have recently confirmed this in C2C12 cells (Y. Wang, Y.-F. Huang, M. Watford, unpublished). Most conditions that result in increased glutamine synthetase expression are accompanied by increased levels of glucocorticoids and thus it has been difficult to determine if glutamine plays a role in vivo. A number of studies have attempted to separate the effects of glucocorticoids from those of glutamine, but the results have been equivocal. Labow and co-workers [8,20] used a variety of treatments, including feeding zero glutamine diets, treatment with methionine sulfoximine (an inhibitor of glutamine synthetase), and acute treatment with endotoxin, and examined the results in lung and skeletal muscle. Unfortunately the only conditions that were successful in lowering glutamine levels were those employing methionine sulfoximine that also stabilized the glutamine synthetase protein. Recently, Meynial-Denis et al [40] attempted to utilize the glutamine chelating agent, phenylbutyrate, to decrease glutamine levels in rats but although circulating glutamine levels were decreased by 20%, muscle glutamine levels and glutamine synthetase expression were unchanged. Also in the rat, Hickson [18,19] showed that infusion of glutamine, or the dipeptide alanyl-glutamine, suppressed the induction of muscle glutamine synthetase activity in response to glucocorticoids. In these studies however, muscle glutamine levels were unchanged and therefore the mechanism is not clear. Some evidence for a glutamine effect can be seen in the work of Collins [51] who studied breast cancer derived tumors implanted in vivo. They reported an inverse correlation with intra-tumor glutamine levels, those tumors with lower glutamine content had higher glutamine synthetase levels. Similarly in recent work with neonatal and adult horses we also found an inverse correlation of muscle glutamine synthetase protein levels with muscle glutamine levels (H. Manso Filho and M. Watford, unpublished).

In summary, the work clearly shows that glutamine synthetase is subject to cell-specific regulation in response to hormones and glutamine. Glucocorticoids up-regulate expression at a pre-translational level in all three cell types but this does not result in changes in glutamine synthetase protein levels in Hep G2 hepatoma cells, possibly because the protein in these cells is very sensitive to glutamine down-regulation. In contrast, insulin only affects glutamine synthetase in 3T3 L1 adipocytes where it suppresses the effect of glucocorticoids on mRNA abundance but not on the level of enzyme protein. Others have seen a down-regulation of glutamine synthetase activity by insulin in such cells although they also indicated that changes in both transcription and protein stability were involved [43,44,48–50]. In a variety of cell types it has been well documented that the magnitude of changes in glutamine synthetase

mRNA abundance do not always correlate with those in protein abundance, with the latter usually being much smaller [4,8,9,20]. In the present work, culture of C2C12 cells in the presence of glutamine and dexamethasone resulted in changes of similar magnitude in both mRNA and protein levels. This was not seen in other conditions, or cells, and the results indicate that, in C2C12 myocytes, medium glutamine is acting to temper the effect of dexamethasone. If such findings are indicative of the situation in skeletal muscle in vivo it suggests that the high levels of glutamine in healthy animals is acting to maintain sufficient glutamine synthetase levels that can rapidly be increased in response to catabolic states by increased stability of the protein at the same time as transcription of the gene is increased in response to glucocorticoids.

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Figure 1. Glutamine synthetase mRNA and protein abundance in C2C12 muscle cells

C2C12 myotubes were cultured for 48h in the presence or absence of glutamine (2mM), with the following additions, B (Basal, no additions), I (7nM insulin), D (25nM dexamethasone), ID (7nM insulin + 25nM dexamethasone). Results are expressed as arbitrary densitometry units and are means $\pm$ SEM of four separate experiments. Values with different letters within a group of with glutamine or without glutamine are statistically different ($p < 0.05$). Differences between similar culture conditions in the absence of glutamine when compared to the presence of glutamine are indicated by * $p < 0.05$, ** $p < 0.01$.

Figure 2. Glutamine synthetase mRNA and protein abundance in Hep G2 hepatoma cells

Cells were cultured for 48h in the presence or absence of glutamine (2mM), with the following additions B (Basal, no additions), I (7nM insulin), D (25nM dexamethasone), ID (7nM insulin + 25nM dexamethasone). Results are expressed as arbitrary densitometry units and are means $\pm$ SEM of four separate experiments. Values with different letters within a group of with glutamine or without glutamine are statistically different ($p < 0.05$). Differences between similar culture conditions in the absence of glutamine when compared to the presence of glutamine are indicated by * $p < 0.05$, ** $p < 0.01$.

Figure 3. Glutamine synthetase mRNA and protein abundance in 3T3 L1 adipocytes

Mature adipocytes were cultured for 48h in the presence or absence of glutamine (2mM), with the following additions, B (Basal, no additions), I (7nM insulin), D (25nM dexamethasone), ID (7nM insulin + 25nM dexamethasone)). Results are expressed as arbitrary densitometry units and are means $\pm$ SEM of four separate experiments. Values with different letters within a group of with glutamine or without glutamine are statistically different ($p < 0.05$). Differences between similar culture conditions in the absence of glutamine when compared to the presence of glutamine are indicated by * $p < 0.05$, ** $p < 0.01$.