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Glutamine, gene expression, and cell function

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1. ABSTRACT

Glutamine is the most abundant free amino acid in the body and is known to play a regulatory role at the gene and protein level in several cell specific processes including metabolism (e.g. oxidative fuel, gluconeogenic precursor and lipogenic precursor), cell integrity (survival, cell proliferation), protein synthesis and degradation, redox potential, respiratory burst, insulin resistance, insulin secretion and extracellular matrix synthesis. Glutamine has been shown to regulate the expression of many genes related to metabolism, signal transduction, cell defense and repair and to activate intracellular signaling pathways. Thus, the function of glutamine goes beyond that of a simple metabolic fuel or protein precursor as previously assumed. In this review, we have attempted to identify some of the common mechanisms underlying glutamine dependent changes in gene and protein expression and cellular function.

2. INTRODUCTION

Glutamine is the most abundant free amino acid in the body, and has its primary source in skeletal muscle, from where it is released into the bloodstream and transported to a variety of tissues (1, 2). In mammals the impact of nutrients, especially amino acids and fatty acids, on gene expression has become an important area of research. Control of gene expression by nutrient availability has been well documented in prokaryotes and lower eukaryotes, which are able to adjust their metabolic activity to variations in the nutrient supply by altering their pattern of gene expression. However, the mechanisms responsible for amino acid control of mammalian cell gene expression have only recently been investigated. Amino acids may exert influence via mTOR dependent stimulation of protein synthesis and indirectly, gene expression (3).

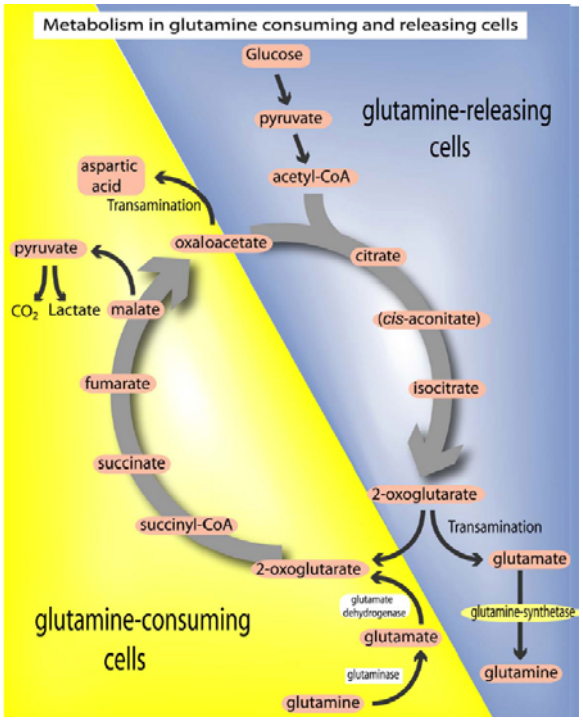

Figure 1. Metabolism in glutamine consuming and releasing cells. Metabolism associated with glutamine-releasing cells is indicated on the right, whereas metabolism associated with glutamine-consuming cells is indicated on the left.

Amino acid starvation can lead to tRNA accumulation, transcriptional factor activation and up regulation of several genes that are involved in amino acid synthesis (4). Interestingly, supra-physiological concentrations of amino acids have been shown to regulate gene expression in hepatocytes via cell swelling dependent changes (5). Supra-physiological concentrations of L-alanine were shown to impact on anti-oxidant and cell defense mechanisms in the pancreatic beta cell (6). In context to the work reported here, glutamine supra-physiological and physiological concentrations can specifically regulate pro-inflammatory cytokine gene and secretory protein expression in several cell types including leukocytes and insulin secreting cells, respectively (3). Deficiency of glutamine (glutamine depletion) can lead to a wide variety of detrimental effects in normally glutamine consuming cells, including cells of the immune system (7) and epithelial cells of the intestine (8, 9). However this area is outside the scope of this review, and will not be discussed further. We have specifically described the effects of an elevation in glutamine concentration on cell function and gene expression. While we appreciate that some of the cell types we have described consume glutamine at much lower rates compared to others, we hope that we have conveyed an integrative effect on gene expression and cell function.

Glutamine may be released by a number of cells and tissues for example skeletal muscle and liver, but glutamine synthesis and release should be considered in the context of glutamine consumption by other cells and tissues (see Figure 1). Indeed blood glutamine concentration is a reflection of the balance between synthesis and release and consumption by the relevant cell types. A sudden change in this balance eg rapid expansion and rapid consumption by cells of the immune system must be balanced by enhanced synthesis and release by for example skeletal muscle.

Intracellular glutamine concentration varies between 2 and 20 mM (depending on cell type) whereas its extracellular concentration averages 0.7 mM (1). Glutamine plays an essential role, promoting and maintaining function of various organs and cells such as kidney (10), intestine (11, 12), liver (13), heart (14), neurons (15), lymphocytes (16), macrophages (17), neutrophils (18, 19, 20), pancreatic β-cells (21) and white adipocytes (22). At the most basic level, glutamine serves as important fuel in these cells and tissues. A high rate of glutamine uptake is characteristic of rapidly dividing cells such as enterocytes, fibroblasts and lymphocytes (23, 24) where glutamine is an important precursor of peptides and proteins, as well as of amino sugars, purines and pyrimidines, thus participating in the synthesis of nucleotides and nucleic acids (25, 26, 27). Glutamine metabolism additionally provides precursors for the synthesis of key molecules, such as glutathione (GSH) (28, 29). Flaring *et al.* (30) showed that glutamine supplementation attenuates glutathione depletion in human skeletal muscle following surgical trauma. Recently, Brennan *et al* (31) have demonstrated that glutamine metabolism in clonal pancreatic beta cells is related to optimal glutathione production via the gamma-glutamyl cycle and hence influences insulin secretion. Recently the same laboratory reported that approximately 1% of 10, 000 genes assessed by micro-array techniques in the pancreatic beta cell were altered on addition of glutamine. As changes in gene expression will impact on cell function then a change in glutamine concentration *in vivo* will alter many clinical parameters. For example, plasma glutamine concentration decreases by up to 50% in patients with HIV, severe burns, sepsis or post-surgery (32, 33), which was correlated with patient outcome. In cultures of neutrophils recovered from burnt and post-operative patients, addition of glutamine augmented the *in vitro* bacterial killing activity (34). In another study glutamine was shown to be important to the production of reactive oxygen species (18, 19).

Thus, the importance of glutamine for cell function, first recognized by Prof. Hans Krebs (reviewed in 35), is now firmly established. However, Kreb's early assumptions that glutamine provided a source of respiratory fuel and nitrogen for biosynthetic reactions has been replaced by a realization that this amino acid plays diverse regulatory roles in the relevant target cells. The mechanisms underlying these diverse actions of glutamine are only now becoming clear and are discussed in the present review.

A summary of reported glutamine-regulated cell functions and the possible transcriptional mechanisms involved are shown in Figure 2.

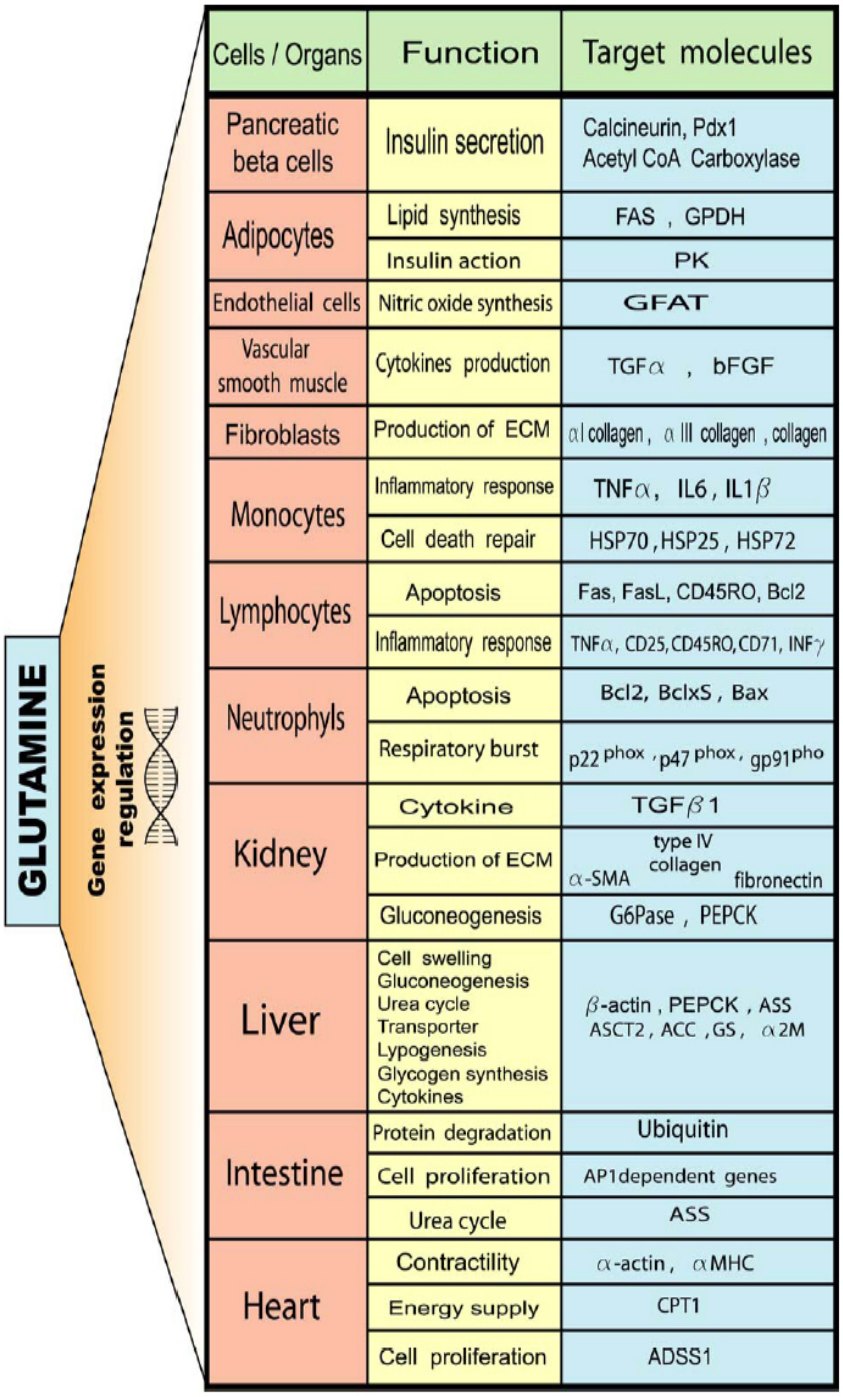

Figure 2. Schematic representation of the glutamine-regulated cell functions and the corresponding changes in gene expression and protein activation (target molecules). PEPCK- phosphoenolpyruvate carboxykinase; ASS- argininosuccinate synthase; JNK- c-Jun-N-terminal kinase; SAPK-stress-activated protein kinase; QRS- glutaminyl-tRNA synthetase; ASK1-apoptosis signal-regulating kinase 1; αSMA-smooth muscle cell α-actin; αMHC- alfa myosin heavy chain; $p70^{S6K}$- p70 ribosomal protein S6 kinase; CPT-1-carnitine palmitoyl transferase I; ADSS-1- adenylosuccinate synthase; HSP25- heat shock protein 25 kDa; HSP70- heat shock protein 70 kDa; HSP72- heat shock protein 72 kDa; ERK-extracellular signal-regulated kinases; AP-1- activating protein 1; IL-6-interleukin-6; IL-1β- interleukin-1 beta; Fas-cell death receptor 95kDa; FasL-cell death receptor 95 kDa ligand; CD45RO- lymphocyte cell surface marker; Bcl-2- b-cell lymphoma-2; TNF-α- tumor necrosis factor-α; IFN-γ - interferon gama; ASCT2 – transporter sodium-dependent systems isoform 2; bcl-xL - b-cell lymphoma-2-associated x protein long; bax– b-cell lymphoma-2-associated x protein membrane; bcl-xS - b-cell lymphoma-2-associated x protein short.

3. CELL METABOLISM

3.1. Respiratory Fuel

Glutamine is quantitatively the most important fuel for a number of rapidly dividing cells and tissues including intestinal tissue (1, 36, 37, 38, 39). It is metabolized to L-alanine in intestinal epithelial cells by a route involving conversion to glutamate, then 2-oxoglutarate via glutaminase and glutamate dehydrogenase, respectively, then TCA cycle conversion to malate followed by the action of $NADP^+$-dependent malic enzyme to create pyruvate, which undergoes amination to produce L-alanine via the action of alanine aminotransferase. In other cells, pyruvate may be converted to lactate via lactate dehydrogenase and released, or it may enter the TCA cycle via pyruvate dehydrogenase, resulting in complete oxidation of glutamine-derived carbon.

The NADH and $FADH_2$ generated via these pathways are used for electron donation to the electron transport chain in the mitochondria and thus they promote ATP synthesis. The L-alanine produced in the intestinal epithelial cell pathway is exported to the hepatic portal vein for transport to the liver (1, 35, 40).

3.2. Gluconeogenesis

Glutamine plays an important role in gluconeogenesis in the liver and kidney. The carbon skeleton of glutamine can provide substrate for the pathway and also may influence the expression and activity of phosphoenolpyruvate carboxykinase (PEPCK), a key regulatory enzyme of gluconeogenesis (41). It has been reported that at supraphysiologic concentrations glutamine induces an increase in PEPCK transcript level (41), in support of earlier work (39) in perfused rat liver.

Several studies in humans have shown that in the post-absorptive state, glutamine is an important glucose precursor and makes a significant contribution in the addition of new carbon to the glucose carbon pool (for review, see 42). The contribution of renal gluconeogenesis to whole-body glucose production is in the order of 20 to 25%, but this rate increased in humans with type II diabetes (42). Alanine can additionally contribute to the supply of new carbon for gluconeogenesis. It appears that glutamine is a major gluconeogenic substrate predominantly in the kidney, whereas alanine dependent gluconeogenesis is essentially confined to the liver (13). In support of this observation, Ikeda & Iwata (43) confirmed gluconeogenesis from glutamine in the kidney had a significant role in whole body glucose homeostasis.

3.3. Urea cycle

Glutamine is an important substrate of urea cycle. This amino acid, via glutaminase and glutamate dehydrogenase provides the first 'N' atom for urea synthesis. Quillard *et al* (44) showed that glutamine increases argininosuccinate synthase (ASS) activity and expression in cultured hepatocytes from fetal and adult rats. ASS converts citrulline to arginosuccinate. Husson *et al.* (45) also reported that glutamine increased ASS mRNA expression in Caco-2 cells (a human colon intestine cell line) and this effect does not involve cell swelling. However, these effects were reported at high supra physiological glutamine concentration. Glutamate derived from glutamine is an allosteric activator of carbamoyl phosphate synthetase (CPS) in the liver. This is a key regulatory enzyme of urea cycle that converts ATP, bicarbonate and ammonium ions into carbamoyl-phosphate, which subsequently enters the Urea cycle after reaction with ornithine.

3.4. Lipogenesis

Glutamine carbon is utilized as a precursor for lipid synthesis in adipocytes (22). Fatty acids produced from glutamine are incorporated into triacylglycerol in incubated adypocytes (46). Recently, Rumberger *et al.* (47) showed that glutamine (16 mM) potentiated the glucose dependent increase in fatty acid synthase (FAS) and glycerophosphate dehydrogenase (GPDH) mRNA in cultured primary rat adipocytes. These authors suggested that products of glutamine metabolism, such as glucosamine-6-phosphate, were important for glucose regulation of FAS and GPDH. Thus, glutamine is an important substrate for lipid synthesis and contributes to the regulation of expression of key enzymes of this biosynthetic pathway.

4. CELL PROLIFERATION

Glutamine plays an important role in cell proliferation. This effect of glutamine has been observed in a variety of cell types including lymphocytes (48, 49, 50, 51), enterocytes (23, 27, 9), and tumor cells (15, 52).

Yamauchi *et al.* (9) showed that the proliferation of Caco-2 cells was increased by nucleoside, nucleotide and glutamine supplementation, but not by glutamate. Arginine potentiated the effect of glutamine. The level of nucleotide synthesis from glutamine, as indicated by N15 incorporation from L-[5-N15]-glutamine, was increased by arginine supplementation and decreased by nucleoside and nucleotide supplementation. These findings suggest that the effects of glutamine and arginine on Caco-2 cell proliferation are mediated by the stimulation of nucleotide synthesis, and that the major role of glutamine in this process was not energy supply. The conversion of glutamine phosphorybosylpyrophosphate by amidophosphorybosyltransferase from ribose-5-phosphate is the first step of the *de novo* synthesis of purines. *De novo* synthesis of pyrimidines begins with the production of carbamyl phosphate from glutamine, carbon dioxide, and adenosine triphosphate. Carbamyl phosphate is then converted to carbamyl aspartate by aspartate transcarbamylase (53).

Rhoads *et al.* (54) have shown that glutamine activates ERKs and JNKs (extracellular signal regulated kinase and jun kinase, respectively), proteins involved in signal transduction pathways stimulated by growth factors in IEC-6 (epithelial cells from rat small intestine) and IPEC-J2 (porcine intestine epithelial cell line) cells, resulting in an increase in AP-1 dependent gene transcription and c-Jun mRNA levels. AP-1 and c-Jun are transcription factors that regulate the expression of genes involved in cell division and inflammation.

Supra physiological concentrations of glutamine (16 mM) can stimulate expression of adenylosuccinate synthase (ADSS-1) that can regulate cell proliferation via activation of protein kinase A and mTOR in neonatal rat cardiomyocytes (55). The latter is an important intracellular transducer of a growth-related signaling pathway, which is rapamycin-sensitive and dependent on activation of a 70kDa S6 kinase ($p70^{S6K}$) (55). The S6 phosphorylation is known to be required for the translation of the terminal oligopyrimidine family of RNAs that contain an oligopyrimidine tract upstream of their transcription-initiation site. These messengers encode proteins belonging to the protein-translation machinery (56).

5. STIMULATION OF ANABOLIC PATHWAYS

Glutamine transport into the human hepatoma cell line HepG2 occurs primarily by an ASCT2-type transporter (57, 58). Bungard and McGivan (59) found that variation in cell growth rate did not affect ASCT2 expression, but both growth rate and ASCT2 expression were significantly reduced by glutamine deprivation. Expression of a number of other proteins was shown to be unaffected under these conditions. The authors postulated that both ASCT2 promoter activity and ASCT2 protein expression in these cells are dependent on glutamine availability.

In rat hepatocytes, Na^+-co-transported amino acids such as glutamine stimulates lipogenesis and glycogen synthesis by activation of acetyl-CoA carboxylase (ACC) and glycogen synthase (GS), respectively. Krause *et al*. (60) have documented a time dependent activation of ACC and GS as well as an increase in phosphorylation of $p70^{S6K}$ in hepatocytes, and suggested that the activation of ACC, GS and the $p70^{S6K}$ resulted from an anabolic response of the liver to glutamine.

6. PROTEIN DEGRADATION

In organs such as the liver and the muscle, protein degradation results from the activity of the three major systems: the lysosomal (cathepsins), the Ca^{2+}-activated (calpains), and the ATP-ubiquitin-dependent proteolytic pathways (61) These pathways have also been reported to be important in rat intestinal mucosa. Enteral glutamine stimulates mucosal protein synthesis and attenuates ubiquitin-dependent proteolysis improving protein balance in human gut. The mRNA level of ubiquitin was significantly decreased by glutamine supplementation (0.8 $mmol.kg^{-1}.h^{-1}$) while cathepsin D and m-calpain mRNA levels were not affected (62). However, glutamine depletion resulted in a reduction in protein degradation in monocytes (63).

7. CELL DEFENSE AND REPAIR

7.1. Heat shock proteins (HSP)

Glutamine is a potent enhancer of heat shock protein 72 (HSP72) expression *in vitro* and *in vivo* (64). The induction of a heat shock response can attenuate pro-inflammatory cytokine release (65, 66). HSP may down regulate cytokine expression binding to the heat shock element present in the promoter region of interleukin-1 beta (IL-1beta) and potentially of other cytokines, a process that results in down regulation of cytokine expression (65). Additionally, glutamine in a concentration equivalent to that found in normal plasma markedly increases HSP72 expression in mononuclear cells following LPS treatment (67).

Glutathione depletion in skeletal muscle is pronounced following major trauma and sepsis in intensive care unit patients (68, 69), which can lead to excessive oxidative stress and damage. Flaring *et al* (70) have shown that intravenous glutamine supplementation attenuates glutathione depletion in skeletal muscle in humans following standardized surgical trauma. In a recent proteomics study, Eliasen *et al.* (71) demonstrated that glutamine depletion resulted in reduced HSP 72 expression in primary monocytes, an effect dependent of decreased mRNA stability (71).

7.2. Redox potential

Glutamine is required for glutathione synthesis as it can be metabolized by the gamma-glutamyl cycle to produce glutathione. Glutathione is produced from glutamate, glycine and cysteine (14). Glutathione is present in the cell in both reduced (GSH) and oxidized (GSSG) forms. The ratio of GSH to GSSG is the main regulator of the cellular redox potential (15, 72). Addition of glutamine to cells *in vitro* can lead to an increase in total glutathione concentration (15, 31). Glutamine metabolism via entry into the TCA cycle may allow action of malic enzyme ($NADP^+$ dependent), which will result in an increase in NADPH production. This will subsequently increase the GSH/GSSG ratio. Studies performed by Roth *et al.* (29) have shown that mice dietary supplemented with glutamine exhibit an increase in the cellular content of reduced glutathione (GSH).

The synthesis of a number of pro-inflammatory cytokines depends on the activation of the transcription factor NFkB, which in turn depends on the cellular redox potential and consequently is regulated by the intracellular GSH:GSSG ratio. The possible involvement of glutamine in NFkB dependent up-regulation of cytokine synthesis however remains to be clarified.

7.3. Apoptosis

Glutamine limitation has recently been shown to modulate apoptosis in a large number of cell types. In enterocytes (HT-29 cell line), apoptosis induced via the extrinsic (death receptor) pathway using TRAIL, was inhibited in a dose dependent manner by glutamine (0 to 500 μM) (73). At the molecular level, glutamine prevented nuclear condensation and activation of caspases-3 and –8 in response to TRAIL treatment. The antiapoptotic nature of glutamine was found to be independent of the intracellular glutathione (GSH) redox status.

Apoptosis can be induced in HeLa cells by treatment with anti-Fas antibody. In glutamine-free medium, HeLa cell apoptosis increases in a dose-dependent manner with anti-Fas antibody, whereas cells in the presence of

glutamine (4 mM) were not responsive to Fas ligand (74). MAPK/JNK pathways are involved in anti-Fas induced HeLa cell apoptosis. In fact, phosphorylation of ERK occurs at 10 min following anti-Fas antibody treatment regardless the presence of glutamine. However, Fas ligand does not activate JNK/SAPK cascade in the presence of glutamine. In glutamine-starved HeLa cells, JNK/SAPK activity is markedly increased by Fas stimulation (74). JNK/SAPK induction by Fas ligand is mediated through ASK1 (a critical protein kinase in apoptosis), which is activated after Fas ligand treatment only in the absence of glutamine. These observations suggest that glutamine suppresses ASK1 and JNK/SAPK activation by Fas ligand (74).

Human glutaminyl-tRNA synthetase (QRS) is one of the enzymes that utilize free glutamine (74). QRS is not only a key enzyme for cell proliferation but also plays a regulatory role in cell death through an antagonistic interaction with ASK1 (74). The authors studied the effect of glutamine on the molecular interaction of QRS with ASK1 in HEK-293 cells (human embryo kidney cell line). The expression level of QRS and ASK1 was not affected by glutamine, but the molecular interaction between these two proteins was significantly increased in cells cultured in the presence of glutamine. QRS and ASK1 interaction can also be intensified by addition of 20 mM glutamine to the immuno precipitation buffer, even when cells were cultured in the absence of glutamine. However, (71) reported that a reduction in extracellular glutamine concentration to 0.05 μM had no effect on the concentration of glutamine changed tRNAs in monocytic cells (71).

T cell death is considered to be critically important for maintenance of T-cell homeostasis and deletion of self-reactive T-cells. This pathway requires interaction between Fas and Fas ligand (FasL/CD95L) (75). On the other hand, expression of the Bcl-2 (an anti-apoptotic protein) can rescue T cells from apoptosis (75). Chang *et al.* (76) have shown that glutamine significantly down-regulates the expression of Fas and FasL but up-regulates the expression of CD45RO and Bcl-2 in Jurkat T cells (human T-lymphocyte cell line). In addition, glutamine significantly decreased both caspase-3 and caspase-8 activities in PMA-ionomycin stimulated Jurkat T cells. These results suggest that glutamine may protect activated T cells from apoptosis partially by up-regulating the expression of Bcl-2 and inhibiting Fas.

Voehringer *et al* (77) have found that T lymphocytes undergoing apoptosis are depleted of reduced glutathione coinciding with the onset of chromatin fragmentation. In contrast, augmentation of intracellular GSH is sufficient to reduce the Fas-triggered increase in apoptosis. Overexpression of Bcl-2 causes accumulation of glutathione in the nucleus, thereby altering the nuclear redox state and blocking caspase activity and other nuclear features of apoptosis.

The endogenous concentration of various metabolites was determined in human neutrophils undergoing apoptosis (78). The endogenous concentration of lactate and glutamine was reduced, whereas that of arginine, glycine, alanine, aspartate, and glutamate was not modified (78). The authors postulated that glutamine utilization might be increased in apoptotic neutrophils.

We have investigated nuclear, mitochondrial, and plasma membrane events associated with apoptosis in rat and human neutrophils cultured in the presence or absence of glutamine (20). Condensation of chromatin assessed by Hoechst 33342 staining was reduced in neutrophils cultured in the presence of glutamine. Annexin V binding to externalized phosphatidylserine was reduced in the presence of glutamine. In the absence of glutamine, neutrophils exhibited a marked reduction in the uptake of rhodamine 123, which was restored by the addition of glutamine. Rhodamine 123 uptake is used to monitor loss of mitochondrial transmembrane potential (79). Similar effect was found in human neutrophils by measuring DNA fragmentation and mitochondrial transmembrane potential. Therefore, glutamine protects from events associated with triggering and executing apoptosis in both rat and human neutrophils. This protective effect of glutamine against neutrophil apoptosis was accompanied by an increase in Bcl-2 expression (20).

We have found (80) that acute exercise leads to marked changes in expression of pro- and anti-apoptotic genes of neutrophils in mature rats (90 days old). The alterations induced by acute exercise include an increase in the expression of bax and bcl-xS expression and a significant decrease in bcl-xL expression. The effect of exercise on gene expression was not observed in neutrophils obtained from immature rats (60 days old). This suggested that the changes in the pro- and anti-apoptotic gene expression induced by exercise are dependent on sexual maturation (80). The same effect was observed after glutamine administration by gavage. Glutamine treatment (1g.kg-1 body weight) decreased bax and bcl-xS expression in neutrophils from mature rats but had not effect on cells of immature rats (80).

8. IMMUNE FUNCTION AND CYTOKINE PRODUCTION

Glutamine is known to modulate immune cell function and cytokine production both *in vitro* and *in vivo*. A requirement for glutamine was also observed for the expression of key lymphocyte cell surface markers such as CD25, CD45RO, CD71 and for the production of interferon - gamma and tumor necrosis factor (TNF)-alpha (29). This topic has been extensively reviewed by Newsholme (81). Expression and production of TNF-alpha by cultured mononuclear cells stimulated with lipopolyssacharide (LPS) can be suppressed by glutamine (2 to 10 mM) (20, 67). However, the synthesis and secretion of IL-1 beta and IL-6 in LPS-stimulated rat peritoneal macrophages are up-regulated by glutamine. Indeed, LPS induced a parallel increase in mRNA and protein synthesis of IL-1beta and IL-6 (82). Jurkat cells stimulated with PMA and ionomycin demonstrated increased IL-2 production in the presence of glutamine (76). Additionally, glutamine (10 mM) increases mRNA of alfa-2 macroglobulin in cultured hepatocytes collected from rat fetuses (83). Alfa-2 macroglobulin is the major positive acute-phase protein in adult rat, and its expression is under the control of interleukin-6. Glutamine depletion also altered protein expression and function of monocytes (7).

9. PRODUCTION OF REACTIVE OXYGEN SPECIES

The superoxide anion ($O_2^{\cdot-}$) generated by NADPH oxidase serves as the starting point for the production of a number of reactive oxidants, including oxidized halogens, free radicals (such as superoxide) and singlet oxygen (84). These oxidants are used by neutrophils to kill invading microorganisms, but they also cause damage to nearby tissues. Therefore, oxidant production has to be tightly regulated to ensure that they are only generated when and where required. Glutamine increases superoxide anion generation stimulated by PMA in rat neutrophils, which had previously deprived of glutamine for 3 h (20). DON (6-diazo-5-oxo-L-norleucine), an inhibitor of phosphate-dependent glutaminase and thus of glutamine metabolism, caused a significant decrease in superoxide anion production by neutrophils stimulated with PMA both in the absence and in the presence of glutamine. PMA markedly increased the expression of $gp91^{phox}$, $p22^{phox}$ and $p47^{phox}$ mRNAs. Glutamine at 2 mM increased the expression of these three proteins both in the absence and in the presence of PMA. Therefore, glutamine increased superoxide anion production in neutrophils, was partially due to regulation of the expression of the components of NADPH oxidase (20).

10. MODULATION OF INSULIN ACTION

Traxinger and Marshall (85) postulated that desensitization of the glucose transport system requires three components: glucose, insulin, and selected amino acids. Overall, these studies revealed that amino acids play an important role in modulating insulin action at the cellular level and provided new insights into the metabolic mechanisms mediating insulin resistance in the glucose transport system (85). The primary amino acid modulating the glucose-induced loss of maximal insulin responsiveness was glutamine (85, 86, 87, 88).

A later study demonstrated that hexosamine, a product of glucose and glutamine metabolism, was involved in the induction of insulin resistance. Azaserine and 6-diaxo-5-oxo-norleucine, the glutamine analogs that irreversibly inactivate glutamine-requiring enzymes, such as glutamine:fructose-6- amidotransferase (GFAT), the first and the rate-limiting enzyme of hexosamine biosynthesis, inhibit insulin-desensitization in cultured adipocytes. Glucosamine, an agent known to preferentially enter the hexosamine pathway at a point distal to enzymatic amidation by glutamine: fructose-6- amidotransferase (GFAT), effectively desensitizes the glucose transporter system in adipocytes in a dose-dependent manner. The authors also found that glucosamine was 40 times more potent than glucose in mediating desensitization and did not require glutamine for its action being able to induce desensitization even in the presence of azaserine (86).

Glutamine in association with glucose and insulin can increase activity and mRNA levels of pyruvate kinase (88). Azaserine was able to prevent the increase in pyruvate kinase in a dose-dependent manner. However, azaserine was unable to prevent glucosamine-induced increase in pyruvate kinase activity, which is expected since glucosamine enters the hexosamine pathway at a point distal to the action of GFAT (88).

Wu *et al.* (89) have shown that GFAT is present in endothelial cells and that the hexosamine synthesis increased with the extracellular concentration of glucose and glutamine. In addition, high concentrations of glucose and glutamine increased GFAT activity. These findings may have implications for poorly controlled patients with diabetes mellitus as these patients display elevated plasma concentrations of both glucose and glutamine. Glucosamine, the main bioproduct of the hexosamine biosynthetic pathway, inhibits nitric oxide synthesis in endothelial cells. Moreover, increasing extracellular concentrations of both glutamine and glucose resulted in decreased nitric oxide production by endothelial cells (90, 91, 92), suggesting that the glucosamine biosynthetic pathway mediates the inhibition of endothelial nitric oxide synthesis induced by hyperglycemia and high plasma glutamine levels (89).

We have previously reported that glutamine increased mouse mesangial cell proliferation (93). Our data suggested that glutamine potentiated the level of glucose-induced mesangial cell proliferation via activation of the GFAT metabolic and MAPK signaling pathways. As several complications of diabetes mellitus including those leading to diabetic glomerulosclerosis appear to be mediated via the GFAT and MAPK pathways, we suggest that glutamine acting synergistically with elevated glucose may impact on to the development and progression of diabetic nephropathy (94). Accordingly, Schleicher and Weigert (95) have shown that the increase in transforming growth factor-beta (TGF-beta1) production in mesangial cells induced by high glucose levels was abolished following inhibition of the GFAT pathway. This suggests that the GFAT pathway at least in part mediates the hyperglycemia-induced production of TGF-beta, a prosclerotic cytokine involved in the development of diabetic nephropathy.

Recently, Weigert *et al.* (96) reported that the hexosamine pathway-mediated induction of TGF-beta1 synthesis in mesangial cells is dependent on GFAT enzyme activity. This study suggested that the hexosamine pathway increases transcriptional activity of nuclear proteins leading to an enhancement of cytokine biosynthesis. It has been observed that a stable overexpression of GFAT increased the levels of TGF-beta1 protein, mRNA and promoter activity and that these effects appear to be transduced by PKC. Involvement of the hexosamine pathway in hyperglycemia-induced production of cytokines (TGF-α and basic fibroblast growth factor-bFGF) has also been demonstrated in vascular smooth muscle cells (96). These studies revealed a rapid increase in GFAT activity following treatment with agents that elevate the levels of cyclic adenosine 3',5' monophosphate (cAMP), thus indicating that GFAT activity is tightly regulated by cAMP-dependent phosphorylation. Using immuno-histochemistry and *in situ* hybridization techniques, high

expression of GFAT was detected in human adipocytes, skeletal muscle, vascular smooth muscle cells, and renal tubular epithelial cells (96). Significant immunostaining for GFAT was found in glomerular cells of patients with diabetic nephropathy. These findings support the proposition that an increased flow through the hexosamine pathway, regulated by GFAT, may be causally involved in the development of diabetic vascular disease, and in particular diabetic nephropathy (96).

11. INSULIN SECRETION

Acute effects of nutrient stimuli, including the insulinotropic amino acids, on pancreatic beta cell function are widely reported. However, the chronic effects of normally non-insulinotropic amino acids, such as L-glutamine, on pancreatic beta-cell function and integrity are only now being studied. It is established that pancreatic islet cells and clonal beta-cells can metabolize L-glutamine at high rates. The pathway of L-glutamine metabolism has traditionally been described as L-glutamine → L-glutamate → 2-oxoglutarate → oxidation in TCA cycle. L-glutamate has been proposed to be a stimulation-secretion coupling factor and a precursor for glutathione synthesis via the gamma glutamyl cycle (31). We have recently reported the effects of prolonged exposure (24 hr) to the amino acid L-glutamine, on gene and protein expression using clonal BRIN-BD11 beta cells (97). Expression profiling of BRIN-BD11 cells was performed using oligonucleotide microarray analysis. Culture for 24 hours with 10 mM L-glutamine compared to 1mM resulted in substantial changes in gene expression with 148 genes up-regulated (>1.8-fold), 18 down-regulated (more than 1.8-fold), including many genes involved in cellular signaling, metabolism, gene regulation and the insulin secretory response. Subsequent functional experiments confirmed that L-glutamine increased the activity of the Ca^{2+} regulated phosphatase calcineurin and the transcription factor Pdx1 (which is essential for insulin gene transcriptional regulation). We additionally demonstrated that beta cell derived L-glutamate was released into the extracellular medium at significant rates. As calcineurin is a regulator of the glutamate NMDA receptor activity we investigated the action of NMDA on nutrient induced insulin secretion, and demonstrated suppressed insulin release. These observations indicate important long-term effects of L-glutamine in regulating beta cell gene expression, signaling and secretory function.

12. EXTRACELLULAR MATRIX FORMATION

Bellon *et al* (98) have shown that glutamine increases the synthesis of collagen in human fibroblasts by a direct stimulatory effect and as a precursor of proline and hydrooxyproline residues. The direct effects of glutamine on collagen biosynthesis included a dose-dependent increase in transcription and mRNA steady-state of alpha 1(I) and alpha 1(III) collagen (99). The response of fibroblasts with respect to collagen synthesis and mRNA reached a maximal level at glutamine concentrations between 0.15 and 0.25 mM, and did not change further up to 10 mM. The authors postulated that the selection of glutamine for protein synthesis takes place in close conjunction with the amino acid transport system. The effect of glutamine on collagen gene expression appeared to be specific as analogues and/or derivatives of glutamine, such as acivicin, 6-diazo-5-oxo-L-norleucine (DON), homoglutamine, ammonium chloride and glutamate were unable to produce the same effect. Karna *et al.* (100) showed that intermediates of glutamine interconversion, glutamate and pyrroline-5-carboxylate (P5C), stimulate collagen biosynthesis in cultured skin fibroblast cells. P5C was found to be a potent stimulator of collagen biosynthesis, whereas glutamate stimulated type I procollagen expression.

Routh *et al.* (101) studied the effect of troglitazone, a peroxisome proliferator-activated receptor-gamma agonist that has been shown to inhibit mesangium expansion in experimental type 2 diabetes. They found that the reduction in glutamine utilization and alanine formation induced by troglitazone is associated with a decrease in monolayer collagen-glycosaminoglycan content. In spite of the reduced glutamine uptake, ammonium formation did not decrease. This is consistent with an increased glutamate flow through the deamination pathway catalysed by GDH.

Pithon-Curi *et al.* (102) have investigated the role of glutamine in the synthesis of extracellular matrix (ECM) proteins in cultured mesangial cells. Glutamine at 2 mM elicited an increase in α_1-type IV collagen and fibronectin transcripts compared to control cells in absence of glutamine. A concomitant marked increase in smooth muscle cell alpha-actin (alpha-SMA) transcripts accompanied by an increase in alpha-SMA stress fibers was detected. Elevated expression of alpha-SMA in mesangial cells has been proposed as a marker of cell activation, and frequently precedes the increase in ECM protein production. These findings support the hypothesis that glutamine leads mesangial cells to produce pro-sclerotic markers.

Hyperglycemia induces marked changes in mesangial cell function and extracellular matrix protein accumulation as seen in diabetic glomerulopathy (103, 104). The hexosamine biosynthesis pathway is implicated in mediating several metabolic effects of high glucose and glutamine in cells. Singh *et al.* (105) showed that metabolism of glucose through the hexosamine biosynthesis pathway mediates the effects of glucose on extracellular matrix (fibronectin) synthesis and transcription factor phosphorylation in SV-40-transformed rat mesangial cells. UDP-N-acetyl-glucosamine is the end product of the hexosamine biosynthesis pathway and serves as a precursor for O-linked serine/threonine glycosylation of cytoplasmic and nuclear proteins.

Mesangial cells cultured in the presence of high glucose and glucosamine show high levels of O-N-acetylglucosamine in several cytoplasmic and nuclear proteins (106). Inhibition of O-glycosylation by benzyl-2-acetamido-2-deoxy-alpha-D-galactopyranoside blocks both high glucose and glucosamine-induced fibronectin synthesis and CREB phosphorylation. In addition,

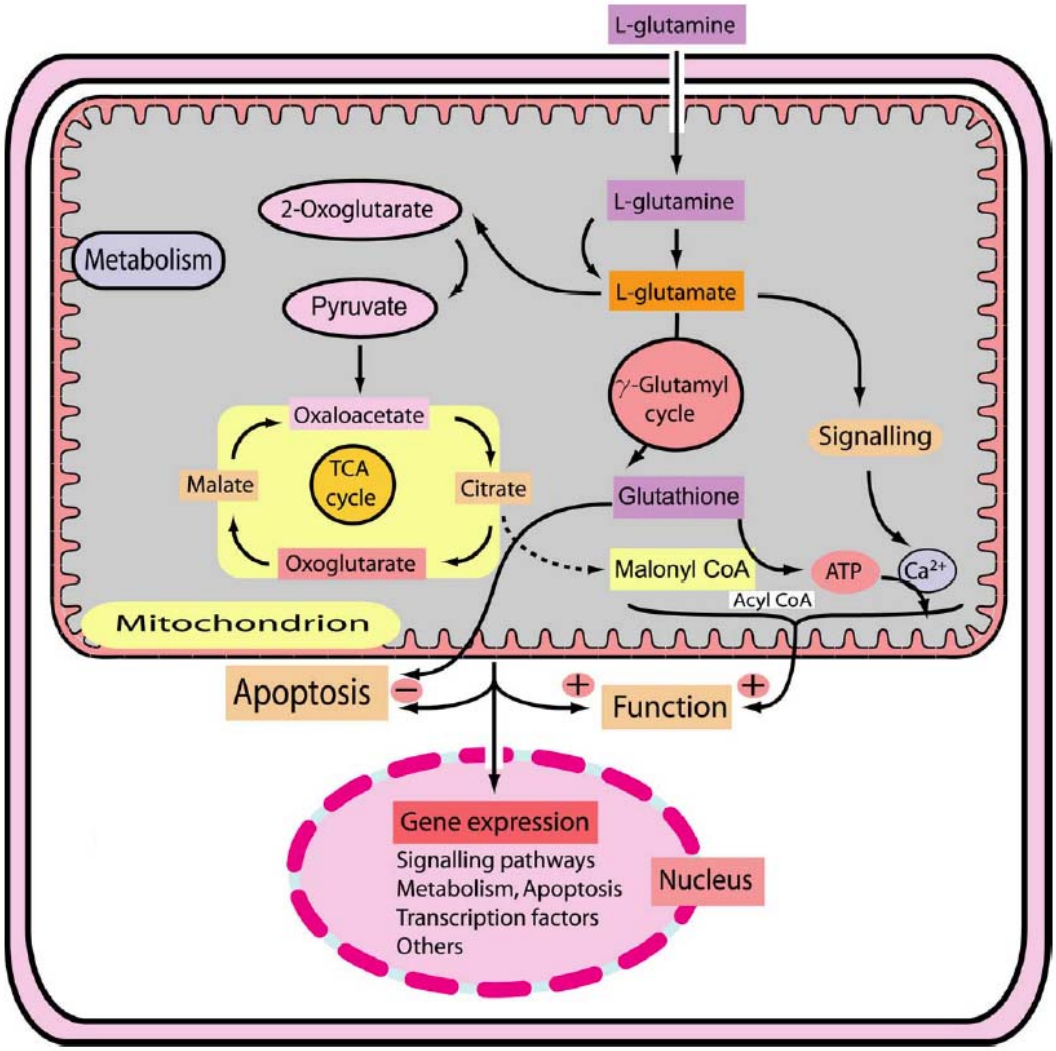

Figure 3. Molecular mechanisms by which glutamine or glutamine metabolism may regulate gene expression. The changes in gene expression by treatment with glutamine may occur by a direct effect of the amino acid or through products of its metabolism. Glutamine metabolism generates potentially active metabolites that may control gene expression. These active metabolites can be: glutamate, glutathione, ATP and TCA cycle originated metabolites.

mesangial cells exposed to high glucose exhibited an elevated GFAT expression, and became more sensitive to the glucose effects on fibronectin expression and CREB phosphorylation than control cells. The authors then postulated that the hexosamine biosynthesis pathway may act as a glucose sensor and mediates at least some of the effects of hyperglycemia in the diabetic kidney (106).

On consideration of the major findings discussed in this review, we now highlight the possible molecular mechanisms by which glutamine may regulate gene expression in a cell which consumes glutamine at high rates (Figure 3). Figure 3 describes possible pathways of glutamine metabolism and the generation of key signaling metabolites (eg. Glutamate, Glutathione, ATP and Acyl CoA) which subsequently regulate gene expression, signal transduction, metabolism and apoptosis.

13. PERSPECTIVE

Evidence is presented herein that glutamine is involved in many processes vital to cell function and integrity. The precise molecular mechanisms of glutamine action remain to be elucidated but undoubtedly involve changes in gene and protein expression, protein activity and changes in intracellular metabolite concentrations. This is best illustrated by recent published and unpublished studies by some of the authors of this review who assessed the effect of glutamine on pancreatic beta-cell metabolism and function. Glutamine metabolism results in the generation of key stimulus secretion coupling factors including glutamate and glutathione, which indirectly stimulate ATP production and enhance insulin secretion (31). Glutamine additionally differentially regulates the expression of genes involved in the regulation of insulin secretion including ion-channels,

metabolic enzymes and protein kinases and phosphatases. Thus, glutamine may have both acute and chronic effects on cell metabolism and function.

Therapeutically the parenteral or enteral administration of glutamine has been recommended for critically ill patients where it is known to have beneficial effects on recovery. However, this amino acid and protein hydrolyzates enriched with glutamine have been widely used by healthy individuals, in particular by athletes, to maintain immune function. Glutamine regulates the synthesis and activation of important proteins including those of the mesangial extracellular matrix, a key element in the development of glomerulosclerosis. Thus although glutamine supplementation brings about clear benefits in many situations, such as when depletion may negatively impact on cell function, problematic adverse effects of the use of high concentrations for a prolonged period of time cannot be fully ruled out.

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Abbreviations: ACC: acetyl-CoA carboxylase; ADSS: adenylosuccinate synthase; alpha-SMA: smooth muscle cell alpha-actin; ASCT: sodium-dependent neutral amino acid transporter; ASK: Apoptosis signal-regulating kinase; ASS: argininosuccinate synthase; cAMP: cyclic adenosine 3', 5' monophosphate; CPS: carbamoyl phosphate synthetase; CREB: cAMP response element binding protein; DON: 6-diazo-5-oxo-L-norleucine; ECM: extracellular matrix; ERK: extracellular signal regulated kinase; FAS: fatty acid synthase; GFAT: glutamine:fructose-6- amidotransferase; GPDH: glycerophosphate dehydrogenase; GS: glycogen synthase; GSH: glutathione reduced; GSSG: glutathione oxidized; HSP: heat shock protein; IEC: epithelial cells from rat small intestine; IL: interleukin; IPEC: porcine intestine epithelial cell line; JNK: c-jun NH(2)-terminal kinase; LPS: lipopolyssacharide; MAPK: mitogen-activated protein kinase; NMDA: N-methyl-d-aspartate; PEPCK: phosphoenolpyruvate carboxykinase; PKC: protein kinase C; PMA: Phorbol 12-myristate 13-acetate; QRS: glutaminyl-tRNA synthetase; SAPK: stress-activated protein kinase; TCA: tricarboxylic acid; TGF: transforming growth factor; TNF: tumor necrosis factor; TRAIL: tumor necrosis factor-related apoptosis-inducing ligand

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