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REVIEW ARTICLE

# Glutamine, arginine, and leucine signaling in the intestine

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**Abstract** Glutamine and leucine are abundant constituents of plant and animal proteins, whereas the content of arginine in foods and physiological fluids varies greatly. Besides their role in protein synthesis, these three amino acids individually activate signaling pathway to promote protein synthesis and possibly inhibit autophagy-mediated protein degradation in intestinal epithelial cells. In addition, glutamine and arginine stimulate the mitogen-activated protein kinase and mammalian target of rapamycin (mTOR)/p70 (s6) kinase pathways, respectively, to enhance mucosal cell migration and restitution. Moreover, through the nitric oxide-dependent cGMP signaling cascade, arginine regulates multiple physiological events in the intestine that are beneficial for cell homeostasis and survival. Available evidence from both in vitro and in vivo animal studies shows that glutamine and arginine promote cell proliferation and exert differential cytoprotective effects in response to nutrient deprivation, oxidative injury, stress, and immunological challenge. Additionally, when nitric oxide is available, leucine increases the migration of intestinal cells. Therefore, through cellular signaling mechanisms, arginine, glutamine, and leucine play crucial roles in intestinal growth, integrity, and function.

**Keywords** Amino acids · Cellular signaling · Intestine · Nutrition

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**Abbreviations**

| | |
|---|---|
| ARG | L-Arginine |
| ERK | Extracellular signal-related kinase |
| GLN | L-Glutamine |
| HO | Heme oxygenase |
| hsp | Heat shock proteins |
| LEU | L-Leucine |
| MAPK | Mitogen-activated protein kinase |
| MEK | MAPK kinase |
| mTOR | Mammalian target of rapamycin |
| NO | Nitric oxide |

## Introduction

All plant and animal proteins contain relatively large amounts of L-glutamine (Gln) and L-leucine (Leu) (Kim and Wu 2008; Wu et al. 2007a). In addition, free Gln is abundant in plasma (0.5–1 mM depending on species), fetal fluids (e.g., 2–4 mM in porcine amniotic and allantoic fluids; 24 mM in ovine allantoic fluid) (Kwon et al. 2003; Wu et al. 1996a), and milk (e.g., 3–4 mM in porcine milk) (Wu and Knabe 1994). However, the content of L-arginine (Arg) in foods and physiological fluids varies greatly, with high abundance in seafood, seeds, nuts and fetal fluids (e.g., 2–4 mM in porcine allantoic fluid) but relative deficiency in the milk of most species (including humans, cows, and pigs) (Wu et al. 2008a, b). Interestingly, in both suckling neonates and post-weaning animals, the epithelial absorptive cell of the small intestine (enterocyte) extensively degrades both Gln and Leu (Chen et al. 2007; Wu 1998). In contrast, Arg is actively catabolized by enterocytes of weaned mammals, but not preweaning ones (Wu et al. 2007b). In adults, nearly all of the Gln and 40% of Arg and Leu in the diet do not enter the portal

circulation (Stoll and Burrin 2006). The active intestinal metabolism of these amino acids has important implications for growth and health (Flynn et al. 2008).

Of particular interest, recent years have witnessed exciting developments in the roles for amino acids in cellular signaling (Liao et al. 2008). Arg, Gln, and Leu are prototype amino acid signals for enterocytes. Leu and Arg activate the mammalian target of rapamycin (mTOR) signaling pathway to promote protein synthesis and possibly inhibit autophagy-mediated protein degradation in intestinal epithelial cells (Blommaart et al. 1995). Additionally, Gln stimulates the mitogen-activated protein kinase (MAPK) pathway and the synthesis of heat shock proteins (hsp) (Rhoads et al. 1997, 2000; Wischmeyer et al. 1997). The objective of this article is to review recent advances in the amino acid signaling of the intestine.

## Glutamine signaling

### Gln as an essential nutrient for the small intestine

Traditionally, Gln has been viewed as a nutritionally nonessential amino acid. However, the work of Windmueller and Spaeth in the 1970s (Windmueller and Spaeth 1978, 1980) showed that Gln was the primary metabolic fuel of the small intestine, greatly surpassing glucose and fatty acids. This seminal discovery led to large growth in Gln research during the 1980s and 1990s. Furthermore, Gln levels were found to be regulated within the bloodstream, liver, and intestine by elaborate inter-organ metabolism and transport mechanisms (Fischer et al. 1995). By the 1990s, virtually all clinicians and investigators recognized that a certain level of Gln (>0.5 mM) was not only desirable, but was also necessary for maximal function of the intestine. Others, mainly intensive care unit investigators, surgeons, gastroenterologists, and oncologists, wondered if higher levels of Gln could produce therapeutic effects in various disease states.

The 1990s witnessed international fervor in defining the role of Gln in nutritional diseases such as short bowel syndrome, multiorgan system failure, chronic diarrhea, and inflammatory bowel disease (Wang et al. 2008a). International symposia were held to discuss Gln and glutamate metabolism, and numerous topic symposia have been held at nutrition and gastroenterology meetings dedicated to understanding this unique amino acid. Gln was an ideal amino acid to study for treatment of diarrheal disease, because (1) its transport was coupled to sodium and (2) its effect on sodium absorption was additive to that of glucose even in conditions associated with significant intestinal injury, such as rotavirus enteritis (Rhoads et al. 1991) and cryptosporidiosis (Argenzio et al. 1994) (Fig. 1). Subsequent studies showed that Gln enters cells via several transporters, the most significant being sodium-dependent broad amino acid transporter $ATB^0$/ASCT2, and the transporters were regulated to maintain a high level of entry into enterocytes (Avissar et al. 2004). Work spearheaded from the laboratory of Wilmore was the first to document trophic effects at the mucosal level, including mitogenesis in the crypt (Jiang et al. 1993; O'Dwyer et al. 1989). Furthermore, Ko et al. (1993) reported that ~1 mM Gln was required for enterocytes to proliferate in response to epidermal growth factor (Papaconstantinou et al. 2000).

Our laboratory encountered an unusual finding, that Gln at doses ~10 mM could signal to the nucleus to initiate mitogenic pathways in the intestinal cell (Rhoads et al. 1997). Any nutrient of such vital function might have the capacity to regulate cellular metabolism, but Gln was the first amino acid that was found to be capable of activating intestinal cell signaling-related kinases which phosphorylated their downstream targets (within minutes). Thus, because Gln improved the efficacy of nutrient infusions and signals within the cell, we and others have suggested that Gln may function as a "primitive hormone" for the gut.

The processes best studied for Gln were: (1) a signal to intestinal cells to proliferate, predominantly via MAPKs; (2) a signal to enhance cell survival in the intestine and other vital organs, best exemplified by hsp; and (3) a signal to block enterocyte apoptosis. A paradigm emerges that the intestinal cell is like a Ferrari, with fuel "infusion" producing a rapid start on the freeway, the gasoline burning smoothly during the cruise. If the car were to run out of fuel, the effect would be analogous to idling, and the car would come to a stop. Using this paradigm, acceleration would be analogous to activating mitosis via MAPKs (Rhoads et al. 2000), coasting analogous to maintaining serum Gln at levels for gut homeostasis, and braking analogous to Gln withdrawal. Gln deprivation activates stress pathways, including Jun nuclear kinases (Ko et al. 2001), hsp and apoptosis (Larson et al. 2007; Phanvijhitsiri et al. 2006; Wischmeyer et al. 1997).

During the past 20 years, more than 1,900 scientific articles have been published which focused on Gln. However, these studies have not consistently demonstrated beneficial effects in humans. The most convincing studies that showed efficacy of Gln were conducted with severe stress models, including multiorgan system failure, endotoxemia, skin burns, weaning, and cancer. Additionally, findings in rodent and pig models have been relatively consistent. For example, dietary supplementation with 1% Gln prevented jejunal atrophy and enhanced growth performance in weaned pigs (Wang et al. 2008b; Wu et al. 1996b). However, as noted above, human research on glutamine has not yet "delivered the knockout punch" to

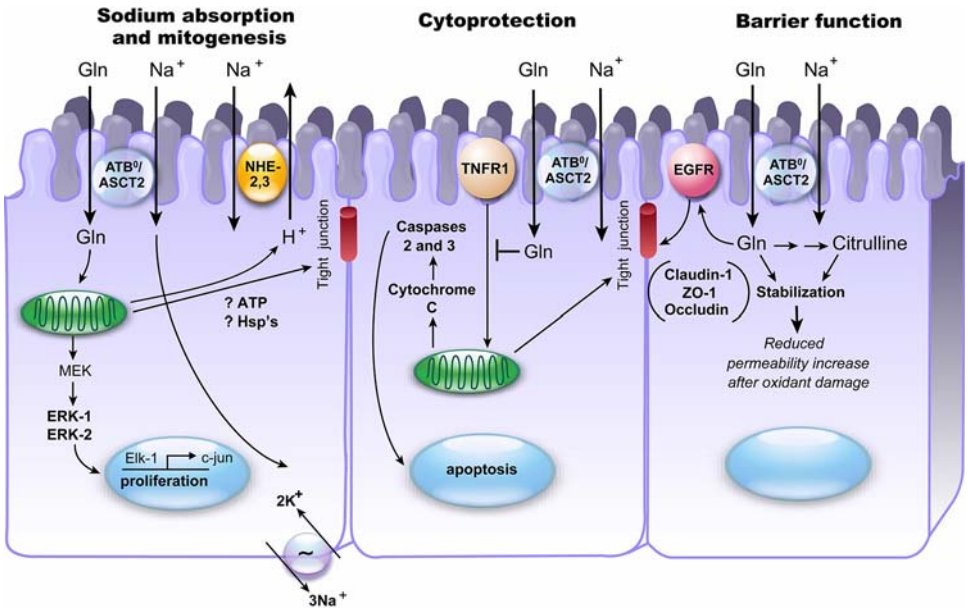


**Fig. 1** Role of glutamine signaling in the function of intestinal mucosal cells

produce widespread changes in therapy. Nonetheless, many active clinicians use Gln for certain conditions, for example to treat diarrhea in patients with small bowel transplants, patients on chemotherapy, or patients with extensive skin burns.

In an editorial review, an expert on parenteral nutrition concluded that "on the basis of currently available clinical data, it is inappropriate to recommend glutamine for therapeutic use in any condition" (Buchman 2001). It is an enigma that its major popular use in the US is for bodybuilders who consume Gln powder to enhance muscle mass. Perhaps the lack of efficacy in human treatment trials relates to the dose studied or to the heterogeneity of the subjects. Consider the effects of three levels of administration: "*serum* physiological levels" (500–800 $\mu$M); "*lumen* physiological levels" (mimicking post-prandial levels as measured in the intestine of suckling pigs, the 95% confidence interval spanning 1–4 mM) (Wu and Knabe 1995; Wang et al. 2008b); or *pharmacological* levels (>4 mM). A well-powered, multicenter, placebo-controlled trial was recently conducted to determine if Gln would influence the time to achieve feeding tolerance in premature infants (Poindexter et al. 2003). Unfortunately, this study was designed in such a way that rather than supplementation with Gln, nutritionally essential and nonessential amino acids were removed from the amino acid solution and replaced with Gln. Consequently, serum Gln levels increased only from a mean value of 291 to 381 µM, and there was no beneficial clinical effect. In the adult studies that have shown beneficial effects (generally in reducing infections, possibly by enhancing the intestinal barrier) in trauma victims and surgical patients, serum Gln levels have always exceeded 530 µM (Beale et al. 2008; Boelens et al. 2001; Novak et al. 2002). In 2009, Gln is currently available for parenteral use but is rarely added to parenteral nutrition solutions, largely because of concern about Gln stability at room temperature. This potential problem can be ameliorated by the use of a stable Gln dipeptide.

## Gln is a signal to intestinal cells to proliferate via MAPKs

Researchers have known from the earliest days of cell culture that Gln must be present in relatively high concentration (>2 mM) in the nutritive media in order for cells to proliferate. However, Gln also has the unique ability to "spark" proliferation in intestinal cells. We aimed to mimic what would happen if intestinal cells bathed in plasma-like media containing only 500 µM Gln were exposed to high-dose luminal Gln, for example in an oral rehydration solution. Porcine IPEC-J2 cells (a nontransformed cell line from the mid-jejunum) were shifted from 0.7 mM Gln to 2.5 mM Gln in the absence of serum or other growth factors (Rhoads et al. 1997, 2000). The Gln "sparked" a 50% increase in $^{3}$H-thymidine incorporation into DNA, representing a proliferative response. When cells were Gln-starved and then exposed to 2.5 mM Gln, there was a 20-fold increase in thymidine incorporation! The increases were associated with activation of extracellular signal-related kinases (ERKs) 1 and 2 via MAPK kinase (MEK) (Rhoads et al. 2000) and phosphorylation of nuclear transcription factors Elk-1 and c-Jun, concomitant with activation of activator protein-1 (AP-1) dependent-gene transcription (Rhoads et al. 1997). Notably, maximal ERK activation occurred within 20 min. Gln signaling was

not redundant with growth factor signaling, because in the rat cell line IEC-6, Gln was additive to either insulin-growth factor-1 (IGF-1) or epidermal growth factor (EGF) in promoting cell proliferation (Rhoads et al. 1997). Thus, Gln could be viewed as a growth signal, and not just a required nutrient.

Recently, ERK activation by Gln was shown to provide an essential mechanism for intestinal cell survival (Larson et al. 2007). In those studies of the rat intestinal cell line RIE-1, ERK inhibition with UO126 or PD98059 blocked its ability to prevent apotosis. Contrary to our findings that Gln stimulation of thymidine incorporation was strongly inhibited by ERK inhibition (Rhoads et al. 2000), they observed that ERK inhibition in the presence of Gln did not block Gln-stimulated increase in cell number. This discrepancy may represent an example of physiological (serum level) Gln versus pharmacological Gln, because we studied 10 mM Gln while 1 mM Gln was used by Larson et al. (2007). Other possible explanations include the use of different cell lines and a different index of proliferation (thymidine incorporation versus the coversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan).

Are these mitogenic and anti-apoptotic effects of Gln on cultured cells relevant to whole animal physiology? Blikslager et al. (1999) found that after severe ischemic injury to porcine isolated Thiry-Vella loops of ileum, instillation into the loop of Gln or transforming growth factor-α (TGF-α) alone did not produce an acceleration of villous regrowth. However, addition of both TGF-α and Gln together to the loop increased recovery rate by more than 3 days (Blikslager et al. 1999). Perhaps the best evidence that Gln can promote intestinal villus growth comes from studies of rats with massive bowel resection. For example, in rats with short bowel syndrome resulting from a 80% gut resection, Gln supplementation (2.2 g/kg enterally, compared to 0.4 g/kg in the control diet) increased intestinal mucosal DNA content, by 40% (Ziegler et al. 1996). A relatively straightforward way to examine Gln effects in newborn rat pups is to artificially feed (by gastrostomy) standard formula or formula with supplemental Gln. Neu's group added Gln at 4 g/kg/day or about 1/3 of total protein, given by gastrostomy tube, in the presence of an inhibitor of de novo Gln synthesis (Potsic et al. 2002). When they compared the impact of Gln-supplemented versus standard formula on intestinal microstructure, Gln-supplemented formula was found to produce a highly significant (68%) increase in intestinal villus height. These studies do not rule out a synergistic effect of hormones and Gln on villus regrowth contribution such as in short bowel syndrome, because a number of gastrointestinal hormones are increased after bowel resection (Lentze 1989).

## Gln is a signal to enhance cell survival in the intestine and in other critical organs

Originally found to induce hsp in other cell types, such as renal cells and fibroblasts, Gln was subsequently shown to upregulate hsp expression in intestinal cells. Chang's group has investigated the mechanisms by which Gln induces hsp expression in intestinal cell lines (Phanvijhitsiri et al. 2006; Ropeleski et al. 2005; Wischmeyer et al. 1997, 2001). They found that *luminal physiological* Gln doses (1–5 mM), when applied to Gln-starved cells, increased their resistance to oxidant-mediated cell death. The cytoprotection was associated with induction of the molecular chaperone hsp70. The nonmetabolizable analogue of L-Gln, D-Gln, was ineffective. To demonstrate that Gln cytoprotection was not an in vitro cell culture artifact, they injected rats with Gln to raise serum levels to supraphysiological serum levels (3–5 mM) transiently. Wischmeyer et al. (2001) showed that the Gln "blast" activated hsp25 (another smaller molecular weight hsp) in multiple organs, including the lung, heart, liver, and colon. Associated with this induction was complete protection of the rats from a subsequent systemic challenge with bacterial endotoxin, which was lethal in control rats (Wischmeyer et al. 2001).

Gln potentiation of hsp72 expression is associated with increased gut epithelial cell resistance to apoptotic injury (Ropeleski et al. 2005). In many cell types, nuclear activation of hsp transcription has been shown to be due to trimerization of heat shock factor-1 (hsf-1) and subsequent binding to target gene promotors. Indeed, hsf-1-deficient rat embryonic fibroblasts lack the expected protective effects of Gln (Peng et al. 2006). In two separate intestinal cell lines, Ropeleski et al. (2005) investigated the mechanism responsible for the action of Gln vis á vis hsf-1 and the induction of hsp72. No Gln-dependent differences in hsf-1 phosphorylation, trimerization, or nuclear localization was found during heat shock. HSF-1 minimal promoter activity was unchanged by the presence of Gln. Nevertheless, the presence of Gln was an important determinant of wild-type hsp72 promoter transcriptional activation. Results of these experiments indicate that Gln does not affect the classical pathway of hsf-1 activation, but that Gln-dependent upstream transfactor binding may be elsewhere in the hsp72 promoter or alternatively that a coactivator may enhance hsp72 transcription.

Gln treatment of intestinal and other epithelial cells increases both hsp72 and hsp27 protein levels. In several disease models, Gln protection requires induction of a similar protective factor, heme oxygenase-1 (HO-1), the master regulator of the hypoxic response (Bilban et al. 2008). For example, in a model of radiation-induced intestinal damage, inhibition of HO-1 with zinc protoporphyrin blocked the protective effect of Gln (Giris et al.

2006). Similar findings were observed in rats injected with endotoxin. Interestingly, Uehara et al. (2005) reported that Gln treatment markedly induced HO-1 mRNA and protein in colonic epithelial cells, as well as the lamina propria cells of the ileum and the colon. Gln administration before lipopolysaccharide administration ameliorated lipopolysaccharide-induced mucosal injury, inflammation, and apoptotic cell death in the ileum and the colon. In addition, Gln treatment markedly decreased lipopolysaccharide-induced mortality. As with the radiation enteritis studies, treatment with tin mesoporphyrin abolished the beneficial effect of Gln pretreatment, suggesting that HO-1 mediates the cytoprotection (Uehara et al. 2005).

### Gln inhibits apoptosis in intestinal cells

A number of groups have investigated the effects of Gln on apoptosis in intestinal cells. Identified mechanisms are summarized in Fig. 1. To our knowledge, all investigations have concluded that Gln at physiological doses is effective in preventing intestinal cell apoptosis. Ko et al. (1993) showed that cultured rat intestinal cell line RIE-1, when deprived of either Gln for 24 h, underwent apoptosis. These results suggest that Gln serves as a specific survival factor in enterocytes.

In investigations by Evans et al. (2003), tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in HT-29 colonic epithelial cells was characterized by nuclear condensation and the activation of caspase-8 and caspase-3. TRAIL-induced apoptosis was completely prevented by Gln pretreatment, but was not inhibited by other amino acids, including the glutathione (GSH) constituents (glutamate, cysteine and glycine). Cellular GSH was oxidized during TRAIL-induced apoptosis, an effect which was completely blocked by Gln. However, inhibition of GSH synthesis did not alter Gln antiapoptotic effects. Thus, Gln protection against cytokine-induced apoptosis occurs by a mechanism that is distinct from the protection against oxidative stress mediated by cellular GSH.

Larson et al. (2007) recently studied apoptosis and its relationship to the ERK pathway. This pathway has traditionally been associated with mitogenesis and carcinogenesis. Apoptosis was increased following ERK inhibition. Gln starvation increased phosphorylated protein kinase B (PKB or Akt); inhibition of Akt enhanced intestinal cell DNA fragmentation. These studies suggested an essential role for a functional ERK signaling pathway in Gln-mediated cytoprotection. The authors proposed that the activation of phosphoinositide 3-kinase (PI3 K)/Akt during periods of Gln deprivation likely occurs as a protective mechanism to limit apoptosis associated with cellular stress.

Growing evidence shows that Gln signaling is necessary to limit cell death. However, Gln transport across the plasma membrane may also be required for cells that have inadequate synthesis of Gln. For example, Fuchs et al. (2004) reported that when hepatoma cells were transfected with the an antisense RNA expression plasmid to knockdown the Gln transporter, ATB(0)/ASCT2, the cells had increased activities of caspases-2 and -9, as well as dramatically increased ASCT1 mRNA levels, presumably as a futile compensatory response. Remarkably, the cell number declined by 98% after knocking down the Gln uptake transporter (Fuchs et al. 2004). This finding further supports the view that adequate availability of intracellular Gln plays an important role in the prevention of apoptosis.

### Gln is necessary for tight junction stabilization

Studies of intestinal cell monolayers have provided a mechanistic explanation for how Gln deprivation and supplementation may impact the ability of the intestine to withstand injury at the epithelial level. Li et al. (2004) demonstrated that Gln deprivation in Caco-2 cell monolayers (via exposure to the Gln synthetase inhibitor, methionine sulfoxime) caused a dissolution of membrane-bound components of the tight junction (zonula occludens, including claudin-1 and zonulae occludens-1). Additionally, electron microscopic evaluation revealed that the reduction in electron-dense material at the zonulae occludens could be rescued by the addition of 0.6 mM Gln to the Gln-free medium. Simultaneous work from Seth et al. (2004) showed that enterocyte monolayer damage produced by acetaldehyde produced a dissolving effect on tight junction ZO-1, E-cadherin, and beta-catenin, which was completely rescued by 2 mM Gln. Interestingly, the tight junction effect was not blocked by inhibiting Gln metabolism. This finding is surprising, as the ATP level is known to affect tight junction stability (Hayashi et al. 1999); however effects of non-metabolized Gln on protective mechanisms such as hsp may be responsible for its protective effect.

If Gln metabolism and ATP generation is not the mechanism for barrier stabilization, what other mechanisms might be involved? In the setting of barrier disruption with intestinal monolayer exposure to acetaldehyde, Gln induced a rapid increase in the tyrosine phosphorylation of EGF-receptor, and the protective effect of Gln was prevented by AG1478, an EGF-receptor tyrosine kinase inhibitor (Seth et al. 2004).

### Glutamine has anti-inflammatory effects in the intestine

Gln is a primary fuel for white blood cells, a feature which has led to the concept that cytokine levels may be reduced

in the intestine when Gln is supplemented enterally or parenterally. Human studies have supported a role of Gln in reducing proinflammatory IL-6 and IL-8 production by intestinal biopsies, while enhancing anti-inflammatory IL-10 levels in the gut (Coeffier et al. 2001, 2003). This immunological response may contribute to the effect of Gln on morbidity and gut permeability in patients with multiorgan system trauma (Houdijk et al. 1998). Although it is beyond the scope of this review to cover the effects of Gln on the immune system, it may be worthwhile to describe a representative study involving the inflammatory cytokine response to traumatic brain injury. In a rat model of puncture-style brain trauma, Gln administration following injury reduced gut damage (measured histologically) and intestinal cytokine expression (Chen et al. 2008). The underlying mechanism may be reduction of NF-KB protein expression in the intestine.

## Arginine signaling

Arg has long been viewed as an essential nutrient to tissue healing and a potentially important component of immunonutrition (Barbul 1986). Attraction to Arg in particular has stemmed from the knowledge that its product proline is an essential component of collagen (Krane 2008). Arg is a key trigger for the release of specific hormones (such as growth hormones) (Wu et al. 2007a), and the major amino acid precursor of polyamines which are essential for gut healing (McCormack and Johnson 1991). Most importantly, Arg is the substrate for the synthesis of nitric oxide (NO), the major non-adrenergic, non-cholinergic vasodilator (Alican and Kubes 1996). This review will not cover the important motility effects of Arg-derived NO in relaxing intestinal smooth muscle, such as during defecation, when NO mediates relaxation of the rectal sphincter (Stebbing et al. 1996). Arg conversion to NO may also be a key to the abnormal vasoregulation which underlies the pathogenesis of necrotizing enterocolitis (NEC), the most important disease of newborn infants, affecting $\sim$7% of infants with a birthweight <1,500 g (Ito et al. 2007). Two independent groups found that premature infants who subsequently develop NEC have extremely low Arg levels (<30% of that of full-term infants) (Becker et al. 2000; Zamora et al. 1997).

Inhibition of NO synthesis has been shown to have injurious effects on the intestine of sheep (Alican and Kubes 1996), pigs (Di and Krantis 2002) and rats (Luo et al. 2001), although overproduction of NO is also injurious (Zamora et al. 2005). While vasoconstriction may be a contributory mechanism, research in our laboratory focused on cultured intestinal cells. This work was based on the elegant studies with renal cells, showing that Arg conversion to NO is essential for cell migration to cover ulcerated surfaces (Noiri et al. 1996) and that NO enables scalar (up-down) movement of the cell matrix, while growth factors promote vectorial locomotion (Noiri et al. 1998). We found that NO synthase is increased at the leading edge of migrating intestinal cells and that NO donors can partially mimic the effect of Arg in enhancing cell motility (Rhoads et al. 2004). In addition, Arg can increase cell proliferation, but does so less effectively than Gln (Rhoads et al. 1997).

Our current view of Arg is that it is a "two-edged sword." Doses in the "lumen physiological level" ($\sim$4 mM) in our studies were beneficial to cell migration, while doses >10 mM were deleterious in our studies (Rhoads et al. 2004). Similar findings were presented recently by other investigators who showed that after ischemia/reperfusion injury, 10 or 60 mM Arg accentuated ROS production and nitrotyrosylation of the intestinal mucosa (Kozar et al. 2004; Sato et al. 2006). One might propose that such high levels of Arg intraluminally might be hard to achieve with conventional nutrition and even "immunonutrition" (Wu et al. 2007b). In a parasitic infection model in piglets (cryptosporidiosis), Gookin et al. (2008) reported that 10 mM Arg potentiated intestinal epithelial injury. Interestingly, inducible NO synthase was massively induced at the villus tips, the exact site of maximal parasitic infestation.

Despite these limitations of high-dose Arg, its concentrations in the "lumen physiological range (2–4 mM)" are potent stimulators of intestinal cell migration and epithelial restitution (Fig. 2). In cultured rat (cdx2-transformed IEC-6) and piglet (IPEC-J2) intestinal cell lines, we found that protein synthesis was required for intestinal cell migration and that inhibitors of the phosphoinositide 3-kinase/mTOR pathway also inhibited cell migration. Conversely, Arg enhanced cell migration and activated the immediately downstream mTOR target, p70(s6k) (but not ERKs) in enterocytes (Rhoads et al. 2006). Immunocytochemical staining of the migrating cells revealed abundant p70(s6k) staining in cytoplasm, whereas the activated form, phospho-p70(s6k), was localized entirely in the nucleus of resting cells, partially redistributing to the cytosol upon activation by Arg. These studies of cell signaling effects of Arg were complemented by investigations in a piglet model of viral diarrhea, made possible by the collaboration with Jack Odle (Rhoads et al. 2007). In severe piglet rotavirus enteritis, in vitro jejunal protein synthetic rate increased twofold, concomitant with a 4-fold increase in jejunal p70(s6k) phosphorylation in association with increased total p70(s6k) level (Rhoads et al. 2007). Immunolocalization revealed that the rotavirus infection produced a major induction of cytoplasmic p70(s6k) and nuclear phospho-p70(s6k), the activated form, mainly in the crypt zone. Although there was profound activation in

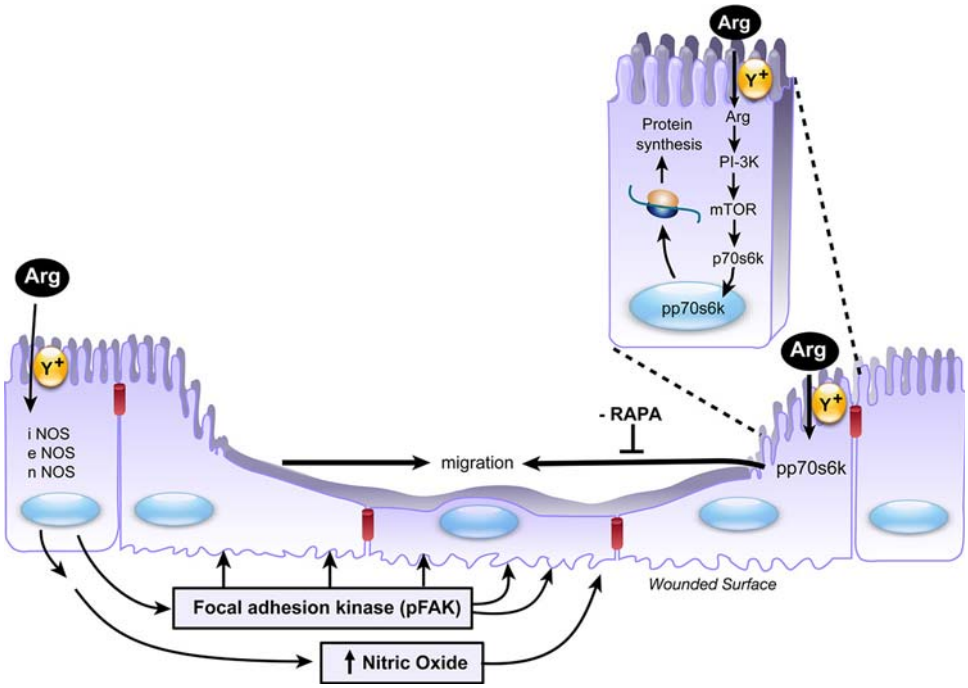


**Fig. 2** Role of arginine signaling in the function of intestinal mucosal cells

the *intestinal* crypts, downregulation of semitendinosus *muscle* p70(s6k) phosphorylation was seen at days 1–3 postinoculation. We concluded that there is coordinate tissue-specific regulation of the mTOR pathway during viral enteritis, with *intestinal* activation of p70(s6k) during an active state of mucosal regeneration, concomitant with inactivation of *muscle* p70(s6k) signaling, in order to reduce muscle protein synthesis in diarrheal disease.

In a subsequent study of pig rotavirus enteritis, we treated a group of infected animals with supplemental Arg in their feeds, aiming to increase p70(s6k) activation (Corl et al. 2008). It should be noted that Arg was supplemented at a relatively low dose of 0.4 g/kg per day. We found that piglets consuming milk with supplemental Arg had strong intestinal p70(s6k) phosphorylation, occurring not only in the crypts, but over the entire villus. Jejunal villi of a rapamycin-treated group showed complete inactivation of p70(s6k) and a decrease in mucosal transepithelial electrical resistance (reflecting increased permeability). The intestinal dysfunction was reversed by Arg. However, Arg treatment did not adversely impact the nutritional status of the piglets or the severity of the diarrhea. Although Arg had no impact on the viral infection itself, Arg did augment intestinal protein synthesis in part by p70(s6k) stimulation. Additionally, Arg supplementation improved intestinal permeability via a mTOR/p70(s6k)-independent mechanism that has yet to be identified. Because of the propensity of Arg to cause diarrhea, perhaps by increasing intestinal levels of NO, there has been considerable interest in citrulline (Cit) as an Arg precursor that could be given to humans (Wu and Meininger 2000; Curis et al. 2007). Cit is available as a nutritional supplement at most health foods stores and is converted to Arg in the kidneys and other extrahepatic cell types (Wu and Morris 1998).

## Leucine signaling in intestinal cells

Amino acids regulate the translation of mRNA on a global scale and also act to cause preferential changes in the translation of mRNAs encoding particular families of proteins. However, amino acids do not directly regulate translation initiation and elongation factors, but instead modulate signaling through pathways traditionally considered to be controlled by hormones. The best-characterized example of amino acid-induced regulation of a signal transduction pathway is mTOR, which modulates the function of proteins engaged in both global and specific mRNA translation (Kimball and Jefferson 2006). The branched-chain amino acids are the prototype stimulators of muscle protein synthesis via the mTOR pathway. In intestinal cells, an excellent readout of mTOR activation is the phosphorylation of p70(s6k). Arg and Leu are the two most effective amino acids for stimulating this activation event (Ban et al. 2004).

In our own model, we compared the effects of Leu and Arg on intestinal cell migration and p70(s6k) phosphorylation. In contrast to Arg, Leu activated pp70(s6k), was a weaker stimulator of migration, and did not increase NO release (Rhoads et al. 2008). The NO donor

DETA-NONOate (DETA/NO) did not affect phosphorylation of p70(s6k) but enhanced the rate of cell migration modestly. Wound coverage with Leu plus DETA/NO was greater than coverage with DETA/NO alone. These studies led to a model of Arg-stimulated intestinal restitution, wherein Arg stimulates NO at the leading edge of the migrating front to facilitate its release from the substratum via focal adhesion kinase phosphorylation (Rhoads et al. 2004), while increased protein synthesis, driven by p70(s6k), enhances cytoskeletal remodeling necessary for coverage of the ulcerated surface (Liu et al. 2008).

Growing interest has focused on an important role of branched-chain amino acids in regulating intracellular proteolysis (Kadowaki and Kanazawa 2003). The two major systems, autophagic-lysosomal pathway and ubiquitin-proteasome pathway, function to produce free amino acids and maintain control during catabolism. Leu is one of several amino acids that serve as a direct regulator (inhibitor) of this process in hepatic cells. In skeletal muscle, the effect of Leu on proteolysis is mediated by its transamination product (α-ketoisocaproate), whereas in liver, Leu interaction with a receptor on the liver cell membrane is directly implicated. Interestingly, there is a relationship between the percentage inhibition of proteolysis and the degree of S6 phosphorylation which has led to the fascinating concept that inhibition of autophagy and phosphorylation of S6 are under the control of the same signal transduction pathway, the mTOR pathway (Blommaart et al. 1995). To our knowledge, autophagic proteolysis and its regulation by branched-chain amino acids has not yet been investigated in the intestine.

## Interactions among glutamine, leucine, and arginine signaling

Leu and Arg increase cell size and protein synthesis, while Gln stimulates cell division (Naomoto et al. 2005). Thus, Gln and Arg have entirely different cellular effects and signaling pathways. However, their effects are not additive or complementary. Gln dose-dependently inhibits Arg-derived NO release from endothelial cells and aortic rings (without changing Arg levels) via a glucosamine-dependent mechanism (Wu et al. 2001). Similarly, Gln has been reported to inhibit Arg- or Leu- enhanced phosphorylation of p70(s6k) and 4E-BP1 in cultured intestinal cells (Nakajo et al. 2005). However, such an effect is unlikely to occur in animals, as glutamine is an anabolic nutrient for intestinal mucosal cells of piglets that consume a protein-adequate diet (Wang et al. 2008b). It is now clear that some in vitro observations on regulation of the mTOR signaling are not physiologically relevant in vivo (Suryawan et al. 2008). Thus, there are complex interactions among Arg, Gln and Leu in intestinal cell signaling that warrant further exploration.

## Further questions and future directions of research

There are a number of basic science and clinical questions that must be answered before Gln or Arg becomes a part of conventional medical practice. Some questions worthy of consideration are summarized as follows:

a. Is intestinal signaling unique to a few key amino acids, or does each nutrient have its own unique signature in the intestine? As noted above, intestinal cells react very differently to Gln versus Arg. Amino acid levels are sensed by unique elements in the promoter of certain genes called nutrient-sensing response elements (Palii et al. 2008). When nuclear ATF4 binds to these elements, L-asparagine synthetase is transcribed (Siu et al. 2002). Thus, because of the vast array of responses to the different amino acids, there may be different signals from different amino acids and also from other fuels, such as glucose, ketone bodies, and fatty acids.
   Some of the answers may be reached by a proteomics approach (Wang et al. 2008c), which can reveal the proteome of the intestinal cell (Yang et al. 2007). For example, a recent study of human Caco-2 cells showed that Arg deprivation decreased cell proliferation and hsp expression and enhanced cell susceptibility to apoptosis (Lenaerts et al. 2007). There were approximately 30 proteins (including cyclin B-CDK1 and eukaryotic translation initiation factor 2 subunit 1) that were down-regulated within 48 h of Arg deprivation. A proteomics-based study of Gln by another group in the human cell line HCT-8 exposed to experimental apoptotic conditions showed that Gln withdrawal followed by supplementation of 2 mM Gln differentially regulated 28 proteins at least twofold (Deniel et al. 2007). Most proteins were involved in cell cycle progression and apoptosis, but 17% were involved in signal transcduction. Most of the proteins regulated by Gln have not been previously investigated, for example, mitogen-activated protein kinase kinase kinase 7, glucosidase II beta subunit, and cell death regulater Aven.
b. Many trophic and anti-apoptotic genes are affected by amino acids, especially Gln, as noted above. Could obesity be related to repetitive "trophic signaling events" in the gut (e.g. ERK, phosphoinositide 3-kinase, and p70(s6k) activation)? Given the current obesity epidemic worldwide (Jobgen et al. 2006), this is an important issue to be addressed. Chronic

activation of these trophic pathways could lead to intestinal hypertrophy and increased absorptive efficiency.

c. Theoretically, such activation could also be related to carcinogenesis in the intestine. Tumor cells have a preferential metabolism and requirement for Gln (Blachier et al. 2007). Therefore: are there strategies whereby Gln uptake or metabolism could be selectively inhibited in tumor cells via the use of antisense constructs or knockdown techniques (Martín-Rufián et al. 2006)?

d. Could high (or low) amino acid intake influence the microbiome of the intestine, thereby contributing to weight problems, allergies, or inflammatory bowel disease? Recent studies have shown that dietary supplementation with Arg reduced white-fat mass in genetically obese rats (Fu et al. 2005) and diet-induced obese rats (Jobgen et al. 2008), as well as growing-finishing pigs (Tan et al. 2008). Interestingly, results of the metabolomic analysis indicated that the Arg treatment altered serum concentrations of microbial metabolites (He et al. 2008), due to changes in the activity and/or population of intestinal bacteria.

e. In a particular disease, what is the effect of optimal dose amino acid treatment? Will Gln or Arg supplementation: (1) impact the primary disease process and promote healing; (2) allow improved protein synthesis and provide a nutritional benefit; (3) have no effect at all, representing a waste of resources spent on amino acid research; or (4) "fuel the fire" and increase gut injury, for example by potentiating an aberrant immune response or enhancing oxidative injury to the intestine?

f. What is the optimal delivery system for Gln, Arg and Leu? Although they can be given orally or intravenously, which route would most likely lead to optimal levels in the intestinal epithelium or lamina propria immune cells, respectively? Additionally, they can be given as mono-amino acids or dipeptides, the latter having increased rates of intestinal absorption. Finally, recent evidence shows that di- and tri-peptides are transported by the brush border PepT1 transporter which is linked to toll-like receptor-2 (Dalmasso et al. 2008). This is of major importance, because synthetic tripeptides (such as Lys-Pro-Val) have potent anti-inflammatory effects in vivo and in vitro.

g. What is the best clinical measure of intestinal homeostasis and maintenance of barrier function in humans? Previoius outcome measures have included survival, infection (sepsis) rate, recovery time from multiorgan system failure, odds ratio of developing necrotizing enterocolitis, and cost of hospitalization. Few laboratories are employing molecular techniques to assess intestinal homeostasis, but the technology is available for measuring nitrogen balance, gut permeability, and systemic cytokine levels. It also seems likely that there will be circulating markers of intestinal cell death that can be measured in the near future. For example, a recent study used circulating levels of intestinal fatty acid binding protein (I-FABP) and liver (L)-FABP as markers for intestinal and hepatic cellular damage, respectively (Derikx et al. 2007). Splanchnic hypoperfusion correlated with intestinal mucosal damage, and elevated plasma levels of I-FABP and L-FABP were associated with a poor outcome in critically ill patients with abdominal sepsis.

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