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Proteomic-based discovery of nutrient-gene regulations: implications for gut health

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Proteomic-based discovery of nutrient-gene regulations

Implications for gut health

The studies presented in this thesis were performed at the Nutrition and Toxicology Research Instititute Maastricht (NUTRIM), which participates in the graduate school VLAG (Food Technology, Agrobiotechnology, Nutrition and Health Sciences), accredited by the Royal Netherlands Academy of Art and Sciences.

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Proteomic-based discovery of nutrient-gene regulations

Implications for gut health

PROEFSCHRIFT

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Promotor

Prof. dr. E.C. Mariman

Copromotor

Dr. J. Renes

Beoordelingscommissie

Prof. dr. ir. W.H.M. Saris (voorzitter)
Prof. dr. R-J.M. Brummer
Prof. dr. W.A. Buurman
Prof. dr. ir. R.P. Mensink

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The gut plays a critical role in nutrition being the site of food digestion and nutrient absorption. For this purpose, the intestinal cells are in close contact with the gut luminal content. Consequently, the epithelial lining of the intestine should provide an efficient mucosal barrier that prevents the entry of foreign antigens, such as toxins, food proteins, commensal gut flora and invading pathogens in the human body. Dysfunction of the intestinal tract has a significant impact on the health of the individual. Thus, the maintenance and improvement of gut health are essential for human well-being.

Some nutrients have the ability to support gut function and integrity under certain conditions, and one of them is the amino acid glutamine. Glutamine is an important fuel source for cells rapidly turning over, such as intestinal epithelial cells. It is a nonessential amino acid under physiological conditions. Several *in vitro* and *in vivo* studies have shown an important role for glutamine in the maintenance and repair of gut mucosa in critically ill patients, and therefore it may be a conditionally essential nutrient. Another conditionally essential amino acid, required exogenously during catabolic disease states or periods of rapid growth, is arginine. Arginine and its metabolites, under which nitric oxide, are thought to have a role in important cellular processes such as proliferation and apoptosis.

Since the underlying mechanisms of the health-promoting potential of glutamine and arginine are unclear, more molecular-based investigations are required. Both amino acids are metabolized extensively by the intestine, and during periods of deficiency of these amino acids or of their metabolites, cellular processes are likely to be influenced. It is of high interest to unravel these regulated processes and find out how they impair intestinal epithelial cell function.

THE GASTROINTESTINAL TRACT – STRUCTURE AND FUNCTION

The gastrointestinal (GI) tract is the system of organs which is responsible for the digestion of food into smaller compounds. These can be absorbed for obtaining energy, or serve as building blocks for larger biomolecules. In addition, the GI tract absorbs vitamins and minerals, and expels the remaining waste. The upper GI tract consists of the mouth, the pharynx, the esophagus, and the stomach. The lower GI tract comprises the small intestine with duodenum, jejunum, and ileum, and the large intestine with caecum, colon, and rectum, and ends with the anus. The whole system has a uniform histology with differences reflecting specialization to a dedicated function. The mucosa is the innermost layer which is exposed directly to the ingested food compounds, and consists of epithelium, lamina propria, and the muscularis mucosae. In the small intestine, this layer is specialized

in absorbing a multitude of different compounds. The mucosa and submucosa (which consists of vascular connective tissue) are folded to increase the absorptive area in the small intestine. This area is further increased by the complex structure of villi, which are finger-like structures of epithelium and lamina propria that protrude into the intestinal lumen and crypts. The epithelium of the villi is of the simple columnar type, and most of the epithelial cells are absorptive cells, some are goblet cells. The absorptive cells have a brush border domain with microvilli at their apical surface. The hydrolytic enzymes of the intestinal brush border membrane are essential for the degradation of nutrients to absorbable units. The structure of the small intestine is depicted in Figure 1.

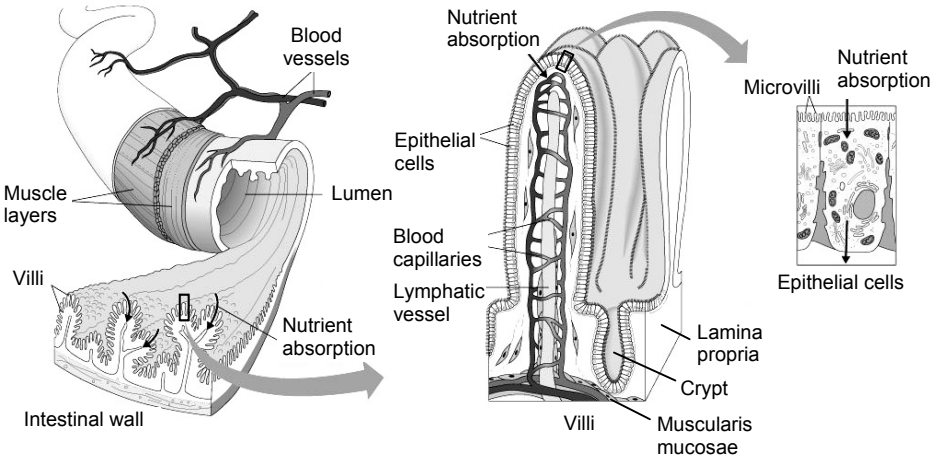

Figure 1. The structure of the small intestine (adapted from Marieb EN (2004) The digestive system in Human Anatomy & Physiology. Pearson Benjamin Cummings, San Francisco).

Next to nutrient absorption, the intestine has another vital role in the human body. The epithelial cell layer of the intestinal tract is interconnected by tight junctions, adherence junctions and desmosomes surrounding the apical region of the cells [1], and restricts transcellular and paracellular transport of ions, small molecules and macromolecules, thereby being the main component of the intestinal barrier. Hence, this barrier protects the human body against pathogens by preventing the translocation of bacteria and other foreign antigens to extra-intestinal sites, such as the mesenteric lymph node complex, liver, spleen, kidney, and bloodstream [2], and thus, is a part of the innate immune system. Interaction of microorganisms with epithelial cells triggers a host response by activating specific transcription factors which control the expression of chemokines and cytokines. This host

response is characterized by the recruitment of immune system cells at the site of infection [3]. Hence, both innate and adaptive defense mechanisms protect the mucosal layers of the GI tract against pathogens [4, 5].

THE ROLE OF THE SMALL INTESTINE IN CRITICAL ILLNESS AND STARVATION

Critical illnesses are characterized by the presence of several factors, such as injury, ischemia, sepsis, and starvation, that can cause marked alterations in the structure and function of multiple organ systems in the human body, including the intestine [6, 7]. The specialized structure and the high energy demands of the small intestine [8] make this tissue vulnerable for the changes observed in critical illness.

As mentioned before, the barrier function of the intestine has a great impact on human health. The intestinal mucosal barrier may become compromised following a variety of systemic and local gut insults. Gut insults of systemic origin include shock, sepsis, major injury, advanced malignant diseases and malnutrition, and insults of local origin include radiation injury, chemotherapy, inflammation, infection, and severe diarrhea [9]. In critically ill and surgical patients, these insults occur often simultaneously, contributing to villous injury, breakdown of the gut mucosal barrier and increased mucosal permeability [10]. In rats, adaptive mechanisms following starvation influenced gut mucosal integrity leading to increased permeability [11]. In addition, fasting and other forms of protein malnutrition are associated with gut mucosal cell impairment marked by decreased levels of reduced glutathione, increased bacterial translocation from the lumen, and stimulation of epithelial cell apoptosis [12].

The gut is thought to have a role in the genesis of septic complications and multiple organ failure. When the mucosal barrier is compromised, microorganisms and their toxic products gain access to the portal and systemic circulations. Subsequently, translocated bacteria and toxins may provoke a systemic response which accounts for the hypermetabolism and hypercatabolism that characterizes systemic sepsis [9]. Under these circumstances, systemic inflammatory response syndrome and multiple organ dysfunction syndrome develop, which can lead to deterioration and death of the patient in the intensive care unit [13]. Indeed, studies have shown that bacterial translocation occurs in human [14], and that these events are correlated with an increased incidence of septic complication [15]. These data point to the value of (nutritional) factors that can support gut integrity and function in critically ill patients. Emerging studies are defining potential therapeutic roles for specific nutrients, under which glutamine and arginine, in gut mucosal turnover, repair and barrier function [12].

GLUTAMINE

Glutamine is a polar amino acid that is uncharged at physiological pH. It contains five C-atoms and bears a 5-carboxamide in its R-group that can be deaminated for nitrogen release. The C_5-structure makes glutamine convertible to the C_5-dicarboxylic acids of the tricarboxylic acid (TCA) cycle. Therefore, glutamine plays a central role in nitrogen as well as carbohydrate metabolism [16]. Since glutamine can be synthesized by most tissues, it has been initially classified as a nonessential amino acid. However, during catabolic states such as trauma, major surgery and sepsis, the amino acid may become essential which has led to the classification of glutamine as a conditionally essential amino acid [17].

Intestinal glutamine metabolism in health and disease

Glutamine is an important substrate for the intestine and plays a central role in the intestinal amino acid metabolism. The majority of the glutamine uptake by the GI tract occurs in the epithelial cells that line the villi of the small intestine [18]. The intestinal cells obtain glutamine from several sources. First, protein intake and digestion in the lumen of the intestine releases glutamine as a free amino acid or included in small peptides. These amino acids and peptides are taken up by the intestine via transporters in the apical membrane of the enterocytes [19]. Glutamine can also be taken up from the systemic circulation by the basolateral membrane of intestinal epithelial cells [20]. Endogenous glutamine arises from the conversion of glutamate and ammonia by glutamine synthetase [21] and from protein breakdown. The capacity of glutamine synthesis in the human small intestine is limited in comparison with the high glutaminase activity observed in this tissue [21].

Glutaminase hydrolyses glutamine to yield glutamate, and this is the major intestinal pathway for glutamine metabolism under physiological conditions. Glutamate is subsequently converted to alpha-ketoglutarate, which enters the TCA cycle for the production of energy. *In vitro* experiments have shown that glutamine and glucose provide similar amounts of ATP in intestinal epithelial cells [22]. Alternatively, glutamate can be metabolized via glutamyl-gamma-semialdehyde to proline, ornithine and citrulline [23]. As amino acid, glutamine is incorporated during peptide and protein synthesis, but it is also directly involved in purine nucleotide synthesis via glutamine phosphoribosyl pyrophosphate amido-transferase and in pyrimidine nucleotide synthesis via carbamoyl phosphate synthetase II [24]. Glutamine is a precursor for glutathione via glutamate. In addition, glutamine is the donor of nitrogen for the synthesis of amino sugars, such as glucosamine-6-phosphate, galactosamine, and N-acetylgalactosamine [25], which

are found in glycoproteins and proteoglycans. Furthermore, glutamine is a precursor of polyamines via ornithine, and is known to stimulate ornithine decarboxylase activity [26], which catalyzes the rate-limiting step in the biosynthesis of polyamines, molecules important for gastrointestinal mucosal growth [27]. Thus glutamine plays an important role in cellular metabolism. An overview of the major pathways involved in glutamine metabolism in the intestine is shown in Figure 2.

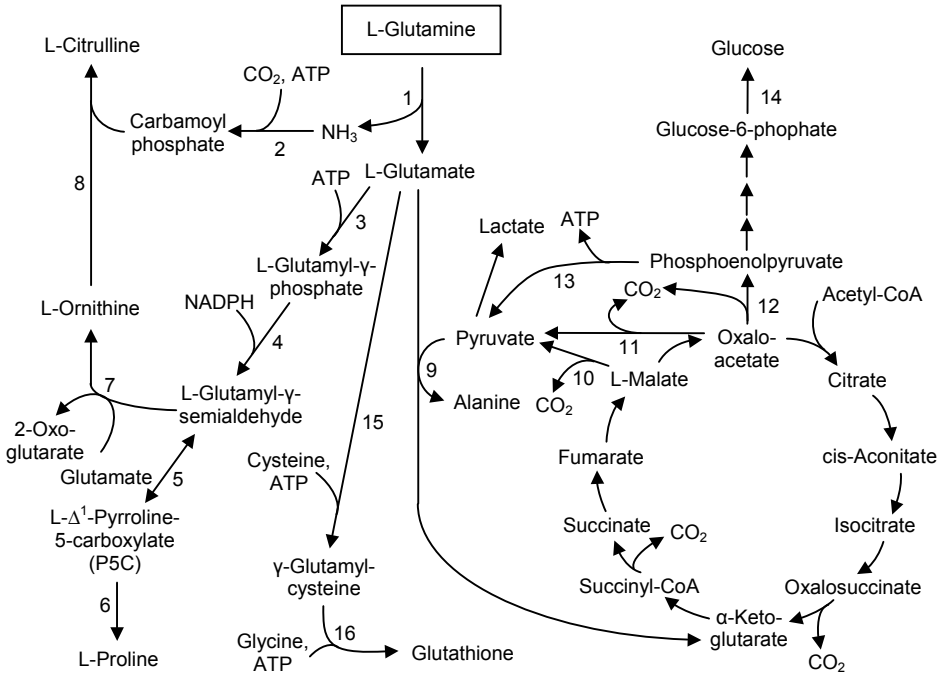

Figure 2. Pathways of intestinal glutamine metabolism in the intestine, except nucleotide and amino sugar synthesis. Enzymes that catalyze the indicated reactions are: 1, phosphate-dependent glutaminase; 2, carbamoyl phosphate synthase I (ammonia); 3 and 4, L-Δ^1-pyrroline-5-carboxylate (P5C) synthetase; 5, spontaneous, nonenzymatic reaction; 6, P5C reductase; 7, ornithine aminotransferase; 8, ornithine carbamoyltransferase; 9, alanine aminotransferase; 10, NADP-dependent malic enzyme; 11, oxaloacetate decarboxylase; 12, phosphoenolpyruvate carboxykinase (GTP); 13, pyruvate kinase; 14, glucose-6-phosphatase; 15, glutamate-cysteine ligase; 16, glutathione synthetase (adapted from Windmueller, HG (1982) Glutamine utilization by the small intestine. Adv Enzymol Relat Areas Mol Biol. 53, 201-37).

Glutamine is the most abundant amino acid in the human body but is also the amino acid that is mostly affected by catabolic disease states such as trauma, sepsis, major surgery, burns and malnutrition [28]. Uptake of glutamine by the gut, kidneys, and lymphocytes during stress generally exceeds the release, leading to a

depletion of total body glutamine stores [9, 29]. As a consequence, marked decreases in free glutamine concentrations have been reported in a variety of catabolic states [17], suggesting that during serious illness a deficiency in glutamine availability may develop.
Several effects are described with respect to intestinal glutamine use during stress states. In endotoxemic rats, a diminished uptake of circulating glutamine by the gut was observed [30]. Despite no reduction in arterial glutamine concentration in septic patients, gut glutamine extraction was diminished by 75%, and in endotoxemic animals by 46%, and this was associated with a fall in luminal glutamine transport activity and a mucosal glutaminase activity [31]. In pigs, the first days after trauma, coinciding with relative starvation, glutamine levels dropped and the uptake by the intestine decreased substantially [32]. Surprisingly, studies in another rat model of sepsis showed that glutamine utilization in enterocytes of the villi, but not of the midportion of the villi and crypts, increased during sepsis, and glutaminase activity was reduced [33]. It has been suggested that this is caused by increased glutamine utilization in alternative metabolic pathways, since glutamine transaminase activity and DNA synthesis were increased in the jejunal mucosa. Protein synthesis was also demonstrated to be increased [34]. One study investigated gut glutamine extraction at different stages of sepsis in rat, and it was demonstrated that glutamine extraction was increased in the early phase, *i.e.* after 5 h, whereas it was decreased after 20 h [35].
Hence, data suggest that the GI response to stress can differ depending on the situation. Generally, in catabolic disease states and injury, glutamine concentration in the tissues and blood declines markedly, whereas the requirement for glutamine in the intestinal mucosa is increased [9, 36].

The effects of glutamine on the intestine

There is evidence accumulating that glutamine plays an important role in the maintenance of intestinal integrity, characterizing a healthy gut. It was shown in an *in vitro* model of the intestine, namely human Caco-2 cells, that glutamine has the ability to prevent TNF-alpha-induced bacterial translocation [37]. This process was dependent on the preservation of ATP levels under these circumstances, and not on the formation of glutathione or polyamines. The translocation event occurred via a transcellular way as the bacteria appeared within intracellular vacuoles [37]. In another study with Caco-2 cells, paracellular permeability was shown to be reduced by glutamine in case of 'luminal fasting', regardless of the route of delivery, being via apical or basolateral side [38]. In an experimental animal model of gut-origin sepsis induced by burn injury, the bacterial translocation was shown to be effectively reduced when animals were fed a glutamine-enriched diet for 15 days

before injury [39]. An ischemia/reperfusion injury in the rat small intestine was shown to be beneficially influenced by addition of glutamine to the enteral feeding. Glutamine maintained barrier function by preserving cytoskeleton integrity and facilitating incorporation of G-actin into F-actin, and this effect was suggested to depend partly on cellular ATP levels [40].
Several studies performed in animals showed a beneficial effect of glutamine on the mucosal condition of the intestine. Intestinal mucosal damage induced by indomethacine or surgical manipulation could be attenuated by pretreatment with oral glutamine [41, 42]. Endogenous and exogenous glutamine deprivation in rat pups, induced by the glutamine synthetase inhibitor methionine sulfoximine and a glutamine-free diet resulted in breakdown of the epithelial junctions, deteriorated microvilli, decreased actin cores and a degradation of the terminal web [43]. Supplementation of total parenteral nutrition with glutamine in animals preserved gastric and colonic mass but did not preserve small-bowel mucosal height [44]. The supplementation of glutamine to parenteral nutrition after starvation resulted in a decrease of intestinal atrophy compared with the standard formula in rats, however oral intake of chow was shown to be most effective in reversing intestinal wasting [45]. Rats, supplemented with glutamine by gavage, showed a higher fractional release of reduced intestinal glutathione [46], which is critical for the cellular redox state. In addition, intravenous glutamine supplementation before ischemia/ reperfusion injury in rats resulted in higher mucosal levels of reduced glutathione compared to levels in mucosal cells not exposed to glutamine [47].
In hospitalized patients, two weeks of standard parenteral nutrition resulted in a slightly decreased duodenal villus height and increased intestinal permeability, whereas this was not observed in patients receiving glutamine-supplemented parenteral nutrition [48]. These results were, however, not confirmed in another, similar study [49]. Glutamine dipeptide-supplementation via parenteral nutrition in critically ill patients prevents increased intestinal permeability, associated with glutamine-free parenteral nutrition [50]. A meta-analysis regarding glutamine supplementation to enteral and parenteral nutrition in critical illness showed that dose and route of administration influence the benefit observed from glutamine administration, with high-dose, parenteral glutamine demonstrating an advantage over low-dose, enteral glutamine [51].

Glutamine as a regulator of gene expression

Glutamine is a substrate for protein synthesis. In Caco-2 cells it was shown that, under glutamine-starved conditions, the cells decreased their (global) protein synthesis rate [52]. This decrease in synthesis was however not apparent for all

proteins, for instance, IL-8 production was increased after lipopolysaccharide (LPS) stimulation under similar glutamine-deprived culture conditions [53].
It has been recognized that glutamine affects the degree of inducible heat shock protein (HSP) expression in various *in vitro* cell systems. Heat shock proteins (HSPs) belong to a highly conserved family of rapidly inducible proteins that are preferentially synthesized during several types of physiological stress, and they are essential to cell survival under such conditions [54]. HSP72 mRNA was induced by a stress event (*i.e.* heat shock) only in intestinal cell cultures (IEC-6) supplemented with glutamine, and glutamine was able to reduce cell death under these circumstances [55]. In addition, glutamine at a concentration of 8 mM was found to have maximal protective effects in these cells [55]. A recent study revealed that in the human fetal nontransformed primary small intestinal epithelial-derived H4 cell line, treated with enterotoxin B from Staphylococcus aureus, HSP72 expression was increased by 40%, but only when glutamine was present in the culture medium [56].
Other regulations of glutamine are known in intestinal cells. For example, glutamine could increase mRNA levels of argininosuccinate synthase (ASS) in Caco-2 cells [57]. Furthermore, glutamine activated both extracellular signal-regulated kinases and Jun N-terminal kinases in IEC-6 and IPEC-J2 intestinal cells, which resulted in increased gene transcription, depending on the transcription factor activating protein-1 [58].
Since glutamine is an important energy provider for intestinal cells, ATP levels are likely to decrease when cells are subjected to glutamine starvation. ATP is required for all energy-consuming pathways in the cell, and ATP depletion leads to an increase in AMP-activated protein kinase (AMPK) activity, which subsequently activates catabolic processes, and inactivates anabolic pathways in the cell with the ultimate goal to preserve ATP. It is still unclear whether the AMPK pathway becomes activated by glutamine starvation in intestinal cells, but a recent study in monocytic cells, which also use glutamine as fuel, showed an adaptive response to glutamine starvation by increasing AMPK activity [59].

ARGININE

Arginine is a basic amino acid and carries a guanidino group in its R-chain. It is positively charged at physiological pH. Under normal conditions, the amount of arginine produced by the human body is sufficient. In periods of rapid growth or under conditions of severe stress or injury, the amino acid becomes essential, because the endogenous synthesis does not fulfill the high demands. Exogenous arginine supplementation may be required and, therefore, arginine is classified as a conditionally essential amino acid.

Intestinal arginine metabolism in health and disease

Arginine comes from different sources in the intestine. During the postprandial period, arginine can be taken up by the luminal side of the enterocytes as free amino acids or in their peptide-bound form. Dietary arginine is derived from several sources. A typical Western-type (United States) diet provides approximately 5.4 g of arginine per day [60]. A study in an elderly population (about 70 years of age) in The Netherlands showed an average arginine intake of 4.4 g per day, of which meat contributed the most to the total arginine intake, followed by bread and milk products [61]. Other arginine-rich foods are fish and nuts. In adult humans and rats, approximately 40% of dietary arginine is removed in the first pass by the splanchnic bed, and most of the arginine utilization is accounted for by the small intestinal mucosa [62, 63]. The remainder enters the portal vein for use by the body. These results indicate that a substantial amount of dietary arginine is not available to tissues other than the intestine.

Arginine can be synthesized endogenously by most mammals, including humans. Arginine synthesis occurs via the intestinal-renal axis [64], and reactions from that pathways are shown in Figure 3. The metabolic pathway for arginine synthesis is illustrated in Figure 3. Glutamine, glutamate and proline coming from the diet, and glutamine arising from uptake out of the arterial blood are substrates for the synthesis of citrulline in the intestine, which is the major source of circulating citrulline for the endogenous synthesis of arginine [62]. Glutamine and glutamate are converted to L-Δ^1-pyrroline-5-carboxylate (P5C) via P5C synthetase, and proline is converted to P5C via proline oxidase. P5C synthetase is almost exclusively located in the small intestinal mucosa, whereas proline oxidase is present mainly in the small intestine, liver and kidneys [65]. P5C is the common intermediate in the pathway of arginine synthesis. From P5C only two steps are needed for citrulline production, by ornithine aminotransferase (OAT) and ornithine carbamoyltransferase (OCT). Citrulline is then released into the blood circulation, where it is extracted primarily by the kidneys for conversion to arginine. Two enzymes, argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) are needed for this conversion [66]. In intestines of neonates, the complete pathway of arginine synthesis is present [67]. In healthy adults, the endogenous synthesis of arginine is not markedly responsive to acute alterations in arginine intake [68].

Arginine can be catabolized via multiple pathways and is an amino acid with remarkable metabolic and regulatory versatility. Many of the catabolic pathways are co-expressed in the same cell [64]. First, the metabolism of arginine in human is described, after which the most important metabolic processes of arginine in the intestine are highlighted.

Arginine transport across the plasma membrane of various cells is the starting point in the catabolism of arginine, and can be regulated by various stimuli. For example, lipopolysaccharide increases arginine transport in intestinal epithelial cells [69], and tumor necrosis factor can induce the uptake of arginine in liver cells [70]. Once inside the cell, arginine can be metabolized via several enzymes thereby producing essential molecules for proper cell function and cell growth.

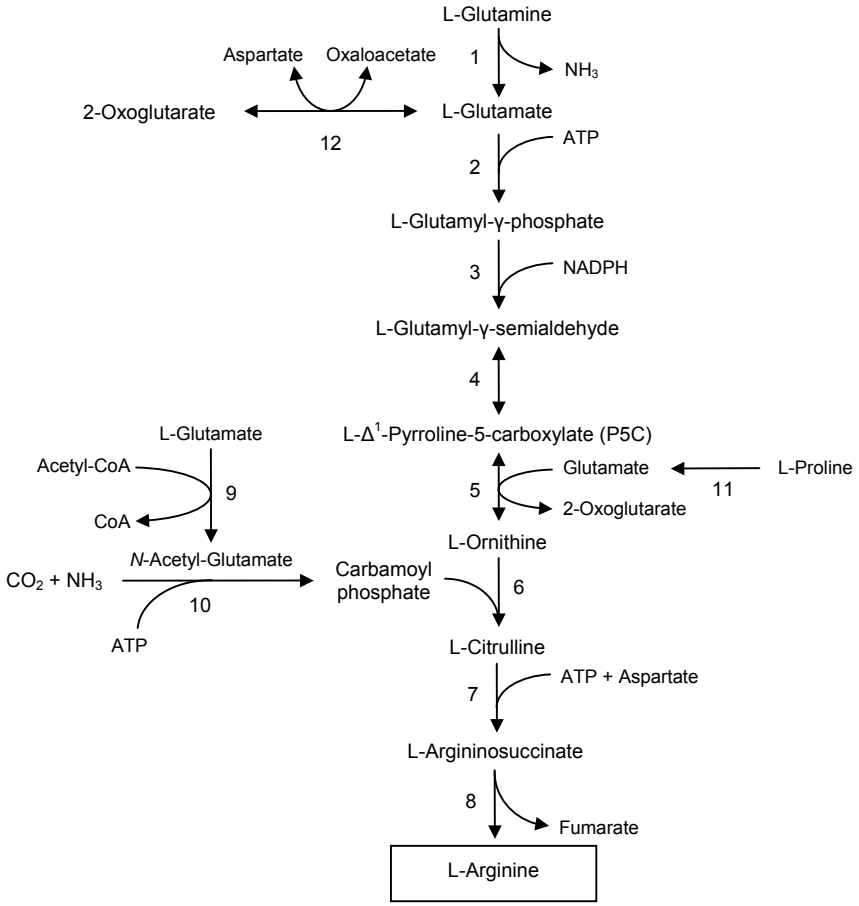

Figure 3. Pathways of arginine synthesis. Enzymes that catalyze the indicated reactions are: 1, phosphate-dependent glutaminase; 2 and 3, P5C synthetase ; 5, ornithine aminotransferase; 6, ornithine carbamoyltransferase; 7, argininosuccinate synthase; 8, argininosuccinate lyase; 9, N-acetylglutamate synthase; 10, carbamoyl-phosphate synthase I (ammonia); 11, proline oxidase; 12, aspartate aminotransferase. Step 4 is a spontaneous, nonenzymatic reaction. Glutamyl-γ-semialdehyde is in chemical equilibrium with P5C. The chemical equilibrium favors P5C formation. Reactions 1–6 and 9–11 occur in mitochondria, reactions 7 and 8 take place in the cytosol, and reaction 12 can occur in both mitochondria and the cytosol (adapted from Wu G & Morris SM, Jr. (1998) Arginine metabolism: nitric oxide and beyond. Biochem J. 336, 1-17).

First, arginine can be hydrolyzed to urea and ornithine by arginase. Two forms of arginase are known, namely arginase I and arginase II, which are encoded by two distinct genes, and expressed in different tissues with a different subcellular localization [71]. Arginase I is a cytosolic enzyme which is primarily expressed in the periportal hepatocyte, and is thought to be mainly involved in the urea cycle. Minor expression is also observed in brains, small intestine and red blood cells of primates. Arginase II is a mitochondrial enzyme and is expressed in the kidney, prostate, small intestine, brain and endothelium [72]. This enzyme is thought to be involved in all other functions that arginase might have, such as the biosynthesis of polyamines, glutamate and proline, and the modulation of nitric oxide (NO) synthesis [73]. Ornithine production, which coincides with arginase activity, can be used for polyamine synthesis by ornithine decarboxylase.
Another arginine-metabolizing enzyme is NO synthase (NOS) which converts arginine to NO and citrulline. Citrulline can be recycled to arginine in a pathway known as the citrulline-NO or arginine-citrulline cycle [74]. Arginine is the only precursor for NO production, indicating that processes which regulate arginine availability, can play an important role in regulating NO synthesis [75]. Three isoforms of NOS are known, namely neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III) [76]. Noticeably, expression of eNOS and nNOS, the so-called constitutive NOS isoforms, can be induced under certain conditions, whereas iNOS may function constitutively [77]. NO production is quite low compared to overall arginine catabolism, and this is probably due to its role in processes such as signal transduction, cytotoxicity, vascular tone and inflammation [77].
Arginine is also used for creatine synthesis via the enzyme arginine:glycine amidinotransferase, an enzyme predominantly present in the renal tubules and pancreas. The enzyme transfers the guanidino group from arginine to glycine, with the formation of guanidinoacetate and ornithine. The kidneys are the main site for guanidinoacetate formation, whereas methylation of this molecule occurs mainly in the liver and the pancreas, yielding creatine which is released in the blood circulation [64]. Arginine decarboxylase activity, converting arginine to agmatine and CO_2, has been identified in brain, liver and kidney. Agmatine is hydrolyzed by the mitochondrial enzyme agmatinase with the formation of urea and putrescine, and hence this is an alternate route of polyamine synthesis [64]. Finally, arginine serves as a substrate for protein synthesis in the form of arginyl-tRNA. A schematic overview of arginine catabolism is shown in Figure 4.
Arginine is metabolized by two major pathways in the enterocytes, namely the conversion of arginine to ornithine via arginase, and the conversion of arginine to NO via NOS. In the small intestine of rat, arginase II mRNA and protein are abundant and the highest expression was observed in the jejunum. Expression

levels are observed from 3 weeks of age and levels increase up to 8 weeks of age [78]. Arginase activity results in ornithine production, and OAT and OCT are also expressed in the small intestine of rat. Immunohistochemical analysis of arginase II, OAT and OCT in the jejunum revealed their co-localization in absorptive epithelial cells of the jejunum, a finding which indicates the role of enterocytes in synthesizing polyamines, proline and citrulline [78]. The human small intestine also expresses arginase II [79]. NOS expression is observed in human enterocytes coming from duodenal tissue and both iNOS mRNA and nNOS mRNA isoforms are expressed, whereas eNOS expression is not observed. Protein expression was only detected for iNOS, and is constitutively expressed in enterocytes [80].

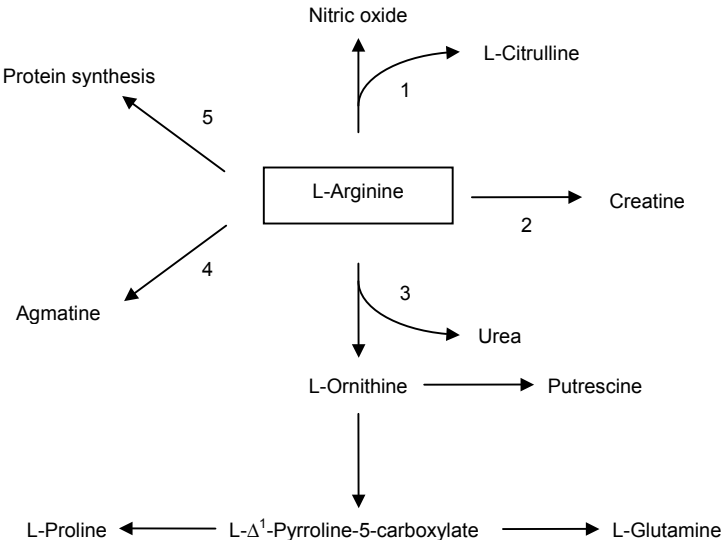

Figure 4. Metabolic fate of arginine in mammalian cells. The enzymes are: 1, nitric oxide synthase (NOS); 2, arginine:glycine amidinotransferase; 3, arginase; 4, arginine decarboxylase; 5, arginyl-tRNA synthase (adapted from Wu G & Morris SM, Jr. (1998) Arginine metabolism: nitric oxide and beyond. Biochem J. 336, 1-17).

As mentioned before, during periods of rapid growth and severe stress or injury, a state of arginine deficiency can occur. Arginine transport in intestinal cells was increased by LPS, probably reflecting the increased necessity under such conditions [69]. A recent study revealed that sepsis can be seen as an arginine-deficient condition, as plasma and muscle arginine levels were decreased [81]. Hyperdynamic endotoxemia in pigs induced a 6-fold decrease in the arginine efflux

from the portal drained viscera, while citrulline and ornithine fluxes remained unchanged [82]. In addition, a significant increase in NO synthesis was observed, which matches the increased arginine disposal. In the muscle of these rats an increased arginine production was observed, and was suggested to come from increased protein breakdown [82]. This arginine was used as a substrate for the induced NOS and arginase pathways and could be used for the synthesis of acute phase proteins [83]. In moderate inflammation, the balance between arginine anabolism and catabolism could be maintained, whereas in severe inflammation, arginine catabolism was higher than arginine anabolism [83]. As a result, decreased plasma arginine levels were observed in sepsis, probably caused by a diminished *de novo* arginine synthesis seen in septic patients [81, 84]. The arginine-deficient condition may be aggravated by a reduced food intake or diminished nutrient absorption in the gut which is often observed during critical illnesses [85]. Plasma arginine concentrations were also decreased in cancer patients [86], and preterm infants [87].

The effects of arginine on the intestine

Several studies suggest that arginine administration improves intestinal health, such as an enhanced mucosal repair after ischemia-reperfusion injury in rats. These effects were shown to involve accelerated morphological repair, enhanced cell proliferation and polyamine content [88, 89]. In addition, arginine has an important role in the GI mucosal defense by being the only precursor for NO synthesis [90]. An arginine-supplemented diet prior to a challenge increased the survival rate of mice after gut-derived sepsis, and the effect was suggested to be mediated by NO [91]. Furthermore, an arginine-enriched diet promoted ulcer healing, as indicated by decreased ulcer number and length in rats with indomethacin-induced ileitis. The underlying mechanism involved in the accelerated healing process was related to an increased cell proliferation [92]. Arginine becomes essential after massive bowel resection, because the main site of citrulline production is reduced considerably. The observed hypocitrullinemia was proportional to the severity of intestinal disease in patients with short bowel syndrome, and as a consequence, plasma arginine levels were decreased [93]. Arginine-treated rats submitted to small bowel resection had significantly greater villus height and crypt-cell mitoses in the remnant small intestine compared to control rats [94].
Hence, arginine has beneficial effects on the intestine, and this is thought to be mediated via its metabolites. In addition, an effect on gene expression could be involved.

Arginine as a regulator of gene expression

Arginine availability can influence the expression of specific genes, especially of those who are involved in arginine metabolism, such as ASS and ASL, in several nonhepatic cell lines [95]. In addition, iNOS expression in macrophages was markedly down-regulated by depletion of extracellular arginine [96]. Overexpression of arginase and a reduction of the capacity of arginine uptake had the same effect [97, 98]. A specific inhibition of protein translation as a consequence of arginine depletion and consecutive phosphorylation of eukaryotic translation initiation factor 2 subunit alpha (eIF2alpha) was shown in rat astrocytes [97]. The phosphorylation of eIF2alpha is part of an important pathway involved in amino acid regulation of gene and protein expression, which is called the 'general amino acid control' response. It is assumed that amino acid depletion causes an increase in cytosolic concentration of uncharged tRNAs that activate the kinase general control nonderepressible protein 2 (GCN2). GCN2 contains a regulatory domain homologous to histidyl-tRNA synthetase, which can bind to various uncharged tRNAs and acts as a sensor for amino acid depletion. Once activated, GCN2 phosphorylates eIF2alpha at serine 51 [99]. Phosphorylation results in reduced activity of the eIF2 complex, reduced rates of translation initiation, and reduced protein synthesis [100]. In contrast, translation and expression of activating transcription factor 4 (ATF4) is promoted. Atf4(-/-) cells are impaired for expressing genes involved in amino acid import, glutathione biosynthesis, and resistance to oxidative stress [101]. Expression of the zeta-chain of the T-cell antigen receptor is reduced when arginine levels were decreased [102]. Hence, these data suggest that arginine can regulate gene expression in a general and selective manner, and that the regulation is not restricted to genes involved in arginine metabolism.

THE CACO-2 CELL LINE AS AN *IN VITRO* MODEL OF THE HUMAN ENTEROCYTE

The Caco-2 cell line is derived from a human colorectal carcinoma and is the only enterocyte model capable of spontaneous differentiation, *i.e.* under standard culture conditions [103]. In differentiated state, the Caco-2 cell shares many characteristics with the small intestinal cell. A polarized monolayer with junctional complexes is formed, of which the apical side is covered with well-developed microvilli, with associated hydrolases [103-105].
A variety of intestinal processes have been studied by means of the Caco-2 cell model, and these include cell proliferation and differentiation, intestinal barrier function and transport of molecules under physiological and pathophysiological conditions [106-110].

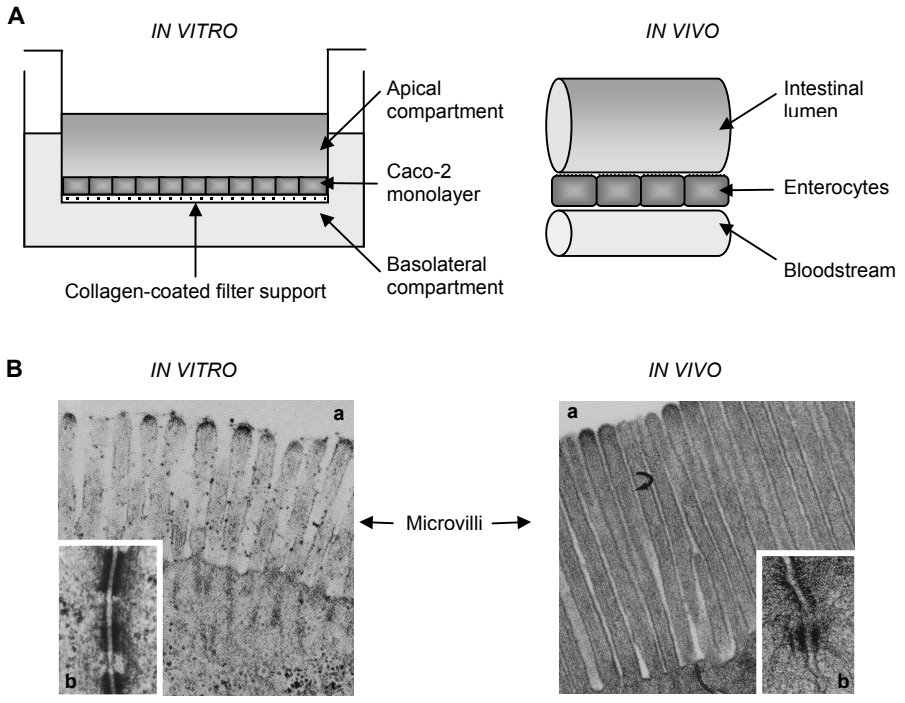

Figure 5. A. Comparison of the *in vitro* cell culture model with Caco-2 cells cultured on a collagen-coated membrane, and exposed to medium from both the apical and the basolateral compartment, and the *in vivo* situation where small intestinal cells are receiving nutrients from the luminal and the serosal side. **B.** Electron micrographs of the brush border (a) and desmosomes (b) of Caco-2 and the small intestinal cells. The brush borders are magnified 30000x and 27000x and the desmosomes 35000x and 45000x in the Caco-2 cells and the human enterocytes, respectively (adapted from Hidalgo IJ, Raub TJ & Borchardt RT (1989) Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. Gastroenterology. 96, 736-749 and Cross PC & Mercer KL (1993) Cell and Tissue Ultrastructure, A Functional Perspective. WH Freeman and Company, New York).

Caco-2 cells are often cultured on filter supports, which enables an effective separation of the apical or 'luminal' and the basolateral or 'systemic' compartment, once confluence is reached and cell junctions are formed. In this way, the model mimics the intestinal barrier *in vivo*, which is, under physiologic conditions, exposed to nutrients via the luminal side, and via the systemic circulation (Figure 5A). This cell culture approach has been applied often in nutrition research, and cellular mechanisms affected by deprivation of certain nutrients were revealed in this way [37, 38, 111, 112]. The amino acids arginine and glutamine are normally metabolized in these cells, since the main enzymes involved in their metabolism, like glutaminase, arginase, NOS are active in Caco-2 cells [109, 113-115]. The use of collagen-coated filter supports has an additional advantage, as the collagen matrix promotes cell attachment and growth leading to confluence of the Caco-2

monolayer more rapidly [116]. A detailed morphological characterization showed that, together with the length of time in culture, the epithelial cells displayed a more columnar shape, a better organized brush border with more and better shaped microvilli (Figure 5B) with associated enzymes such as alkaline phosphatase. The appearance of occluding tight junctions and desmosomes in this system are also similar compared to normal enterocytes [116].

PROTEOMICS IN NUTRITION RESEARCH

The proteome is defined as all the proteins that are present in a particular cell at a particular time, reflecting the expression of specific genes in the situation at that point. The term 'proteome' stands for 'the PROTEin complement expressed by a genOME' [117]. Proteomics can be defined as the study which compares proteomes under different conditions qualitatively and quantitatively to further unravel biological processes.

Nutrients or nutrient deficiencies can affect essentially every step in the flow of genetic information, from gene expression and mRNA stability to protein synthesis and protein degradation, thereby altering metabolic functions in the most complex ways. Alterations in mRNA and protein levels are critical parameters in controlling the flux of a nutrient or a metabolite through a biochemical pathway. Although mRNA levels of a certain gene can provide important information, the corresponding protein levels do not necessarily change in parallel [118]. In addition, several protein variants can exist from one gene, and proteins can be modified posttranslationally by phosphorylation, acetylation, glycosylation, cleavage etc. Therefore, the monitoring of proteins and hence the application of proteomics tools can be of great value in the field of molecular nutrition.

The development of advanced tools for protein profiling has gained interest over the past 20 years. Two dimensional gel electrophoresis (2-DE) coupled with protein spot analysis by mass spectrometry (MS), is still a commonly used technical approach in the study of proteomes [119]. 2-DE and various chromatography techniques have long been used as a tool for protein separation. However, only with the development, in the late 1980s, of soft ionization techniques, such as matrix-assisted laser desorption/ionization (MALDI) and electro-spray ionization (ESI), to evaporate peptides and proteins, their analysis has become possible using MS [119]. This has contributed considerably to the development of rapid-throughput proteome analysis.

Gel-based proteomics

Profiling of complex protein mixtures by 2-DE

The first step in the research of proteomes is the sample preparation, which includes disruption of cells or tissues and extraction of proteins with help of a lysis buffer [120]. The complex protein mixture is subjected to separation according to two physicochemical properties of a protein, which are the net charge at a certain pH and the molecular weight. In the first dimension, a protein mixture is separated by isoelectric focusing according to the isoelectric point of each individual protein. In the second dimension, proteins are separated according to their molecular weight by classical sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 6A). Proteins in the gel need to be visualized before semiquantitative analysis can be performed. Methods for visualization of proteins include silver staining, a complex procedure with a very high sensitivity but a rather small linear dynamic range, and fluorescent staining, which is somewhat less sensitive but has a better linear dynamic range leading to more reproducible results, especially for low-intensity protein spots [119]. Another advantage of the fluorescent dyes is their compatibility with modern downstream protein identification methods [121].
Next in the multi-step process of protein profiling comes the capturing of gel images with a (fluorescence) scanner and the analysis of these images with specialized software to seek for regulated protein spots, for example spots with a lower or higher abundance under a set condition (Figure 6B). These spots of interest that can provide new biological insights are excised out of the gel and submitted to the process of protein identification.

Protein identification by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

An excised gel plug, containing the protein of interest is digested enzymatically, usually with trypsin, which cleaves a protein after an arginine or a lysine. The tryptic digest, containing peptides originating from the protein, is embedded into an appropriate organic matrix on a metallic target plate. A laser beam is used for evaporation and ionization of peptides, which are accelerated in an electric field towards the mass analyzer. The generated ions will be dissociated in the vacuum of the mass analyzer based on their mass-to-charge ratio (m/z). The time of flight (TOF) between the acceleration process and their impact on the detector is measured very accurately. From the acceleration voltage and the length of the drift region, the m/z can be calculated, and therefore the mass. Ions from a tryptic digest with a different m/z analyzed by a MALDI-TOF mass spectrometer yield

different mass peaks with a certain intensity plotted into a mass spectrum providing a fingerprint of great specificity, also called a peptide mass fingerprint (PMF). This PMF can be compared with PMFs generated *in silico* from proteins in a database, which implies that the sequence of the protein must be known and present in that database (Figure 6C).

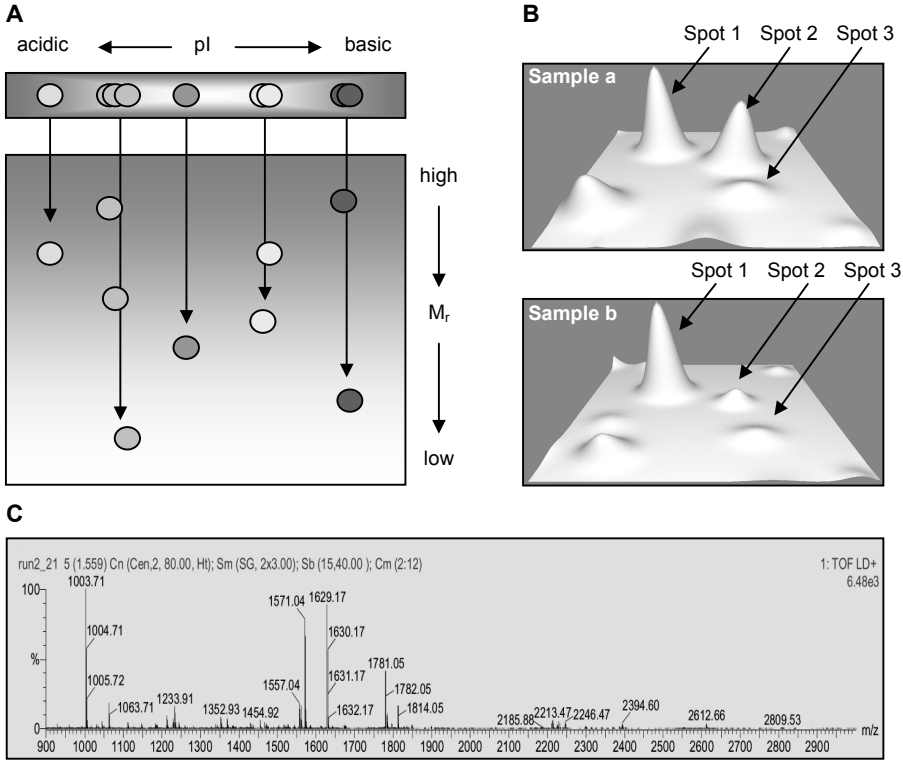

Figure 6. A. Separation of protein mixture according to the isoelectric points and molecular masses of individual proteins by 2-DE. **B.** Semiquantitative analysis of protein spot abundances with specialized software. Spot 2 is higher expressed in sample a than in sample b. This protein spot is cut from the 2-D gel, in gel digested with trypsin, and submitted to MALDI-TOF MS. **C.** MALDI-TOF mass spectrum of tryptic digest of differentially expressed protein spot. This PMF is used for database search to obtain the identity of the protein.

Array-based proteomics

Despite the high analytical power of 2-DE, the method is technically demanding, time-consuming and has a limited capacity in analyzing low-abundance proteins. Other emerging techniques, such as the antibody array technology, overcome some limitations of the 2-DE technique. Antibody arrays are composed of a panel of distinct monoclonal antibodies covalently immobilized on a glass slide. They can

be used to assess differences in expression of proteins between two biological samples. The method is less labor intensive and can detect also differences in proteins expressed at low abundance. A drawback is that only two samples can be compared, whereas the 2-DE method allows multiple comparisons. Furthermore, the number and choice of antibodies present on commercially available arrays is still limited.

OUTLINE OF THE THESIS

The study object of this thesis is the epithelial lining of the small intestine and the regulation of its protein profile under certain nutritional influences. The intestinal epithelium provides an important barrier to pathogens, and dysfunction of this barrier can be a threat to human health.

The amino acids glutamine and arginine have a potential therapeutic role in the intestine, and the molecular mechanisms by which these amino acids or their metabolites influence intestinal function remain largely unknown. Therefore, one aim of this thesis was to identify novel genes in the intestine whose expression is regulated at the protein level under glutamine- and arginine-deficient conditions.

In chapter 2, the effect of different glutamine concentrations delivered from the 'luminal' or 'serosal' side of the Caco-2 monolayer was assessed by 2-DE. In addition, incorporation of stable isotope-labeled glutamine into Caco-2 proteins was measured. It is known that human Caco-2 cells have a much higher glutamine synthesizing capacity compared to normal enterocytes. Therefore, the endogenous glutamine synthesis was blocked in the study described in chapter 3. In this manner, the effect of exogenous glutamine on the Caco-2 proteome could be assessed by 2-DE.

Chapter 4 focuses on the effects of arginine deficiency on the protein profile of proliferating Caco-2 cells, and the potential of arginine and citrulline supplementation to restore the observed effects. In parallel with the study described in chapter 3, differentiated Caco-2 cells were deprived of arginine after which the proteomes were analyzed.

An additional aim of this thesis was to identify the molecular mechanisms underlying the effects of malnutrition in the intestine. Accordingly, the effects of short-term and long-term fasting on the proteome of the mouse small intestine were investigated. The results of this study are described in chapter 5.

In chapter 6, a comparison was made between the protein profiles of commonly used intestinal cell lines, Caco-2 cells and HT-29 cells, and epithelial scrapings of the human intestine, in order to evaluate the usability of these *in vitro* models. In chapter 7, the major findings of all studies are reviewed in relation to human gut health, and implications for future research are discussed.

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CHAPTER 2

DIFFERENTIATION STAGE-DEPENDENT PREFERRED UPTAKE OF BASOLATERAL (SYSTEMIC) GLUTAMINE INTO CACO-2 CELLS RESULTS IN ITS ACCUMULATION IN PROTEINS WITH A ROLE IN CELL-CELL INTERACTION

Kaatje Lenaerts, Edwin Mariman, Freek Bouwman and Johan Renes

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ABSTRACT

Glutamine is an essential amino acid for enterocytes, especially in states of critical illness and injury. In several studies it is speculated that the beneficial effects of glutamine are dependent on the route of supply (luminal or systemic). The aim was to investigate the relevance of both routes of glutamine delivery to *in vitro* intestinal cells and to explore the molecular basis for proposed beneficial effects of glutamine (i) by determining the relative uptake of radiolabeled glutamine in Caco-2 cells, (ii) by assessing the effect of glutamine on the proteome of Caco-2 cells using a two-dimensional gel electrophoresis approach, and (iii) by examining glutamine incorporation into cellular proteins using a new mass spectrometry-based method with stable isotope-labeled glutamine. Results of this study show that exogenous glutamine is taken up by Caco-2 cells from both apical and basolateral side. Basolateral uptake consistently exceeds apical uptake and this phenomenon is more pronounced in 5-day-differentiated cells than in 15-day-differentiated cells. No effect of exogenous glutamine supply on the proteome was detected. However, we demonstrated that exogenous glutamine is incorporated into newly synthesized proteins and this occurred at a faster rate from basolateral glutamine, which is in line with the uptake rates. Interestingly, a large number of rapidly labeled proteins is involved in establishing cell-cell interactions. In this respect, our data may point to a molecular basis for observed beneficial effects of glutamine on intestinal cells and support results from studies with critically ill patients where parenteral glutamine supplementation is preferred above luminal supplementation.

INTRODUCTION

Glutamine has an important function in the small intestine with respect to maintaining the gut epithelial barrier in critically ill patients [1,2]. Several studies performed in different experimental settings reveal that it serves as an important metabolic fuel for enterocytes [3], and as a precursor for nucleotides, amino sugars, proteins and several other molecules like glutathione [4,5]. *In vitro* cell culture studies demonstrate that glutamine specifically protects intestinal epithelial cells against apoptosis [6,7], has trophic effects on the intestinal mucosa [8] and prevents tumor necrosis factor (TNF)-alpha induced bacterial translocation [9]. In experimental models of critical illness, glutamine was able to attenuate proinflammatory cytokine expression and to improve gut barrier function [1,10-12].

The intestinal cells obtain glutamine through exogenous and endogenous routes. The exogenous glutamine comes from uptake of the amino acid itself or of glutamine-containing peptides from the intestinal lumen via transporters in their apical brush border membranes [13], and from the bloodstream via their basolateral membranes [14]. The endogenous glutamine arises from conversion of glutamate and ammonia by glutamine synthetase [15]. However, in human and rat, intestinal glutamine synthetase activity is very low [16,17]. This suggests that enterocytes strongly depend on the external glutamine supply, either from the diet or from the blood circulation.

In many studies it is proposed that the beneficial effect of glutamine is dependent on the dose and route of supplementation. Data from a meta-analysis suggested that glutamine supplementation in critically ill patients may be associated with a decrease in complications and mortality rate, particularly when delivered parenterally at high dose [18]. Panigrahi *et al.* demonstrated that especially apical deprivation of glutamine in Caco-2 cells resulted in a significant rise of bacterial transcytosis [19]. Similar results were found in HT-29 cells, where apical delivery of glutamine decreased transepithelial permeability [20]. Le Bacquer *et al.* reported that, regardless of its route of delivery, glutamine is able to restore protein synthesis in cells submitted to apical fasting [21]. Another study showed that glutamine is utilized by the rat small intestine to a similar extent when given by luminal or systemic routes [22]. Hence, these studies indicate that both luminal and systemic routes can be used interchangeably to supply the enterocytes with glutamine. Altogether, these data do not allow a conclusion on the preferred side of glutamine supplementation.

Although the uptake rate of lumen-derived and blood-derived glutamine by the rat small intestine *ex vivo* and *in vivo* has been reported [22,23], the relative uptake from each glutamine source in *in vitro* cell culture systems is unknown. Another area that remains unexplored is the overall influence of glutamine on gene expression of

intestinal cells, which may reveal the underlying mechanism for the so-called 'health' effect of glutamine. In this respect, it is important to know whether glutamine taken up by the cells from the apical or basolateral side enters a common metabolic pool.

The purpose of this study was to investigate the relevance of the route of glutamine delivery to *in vitro* intestinal cells, and to explore a molecular basis for the proposed beneficial effects of glutamine (i) by determining the relative uptake of glutamine, (ii) by searching for changes in the intestinal proteome and (iii) by examining glutamine incorporation into cellular proteins. The Caco-2 cell line was used for this study. Although originally derived from a human colon adenocarcinoma, the cells undergo spontaneous enterocytic differentiation and share many characteristics with human small intestinal cells in their differentiated state. Caco-2 cells form a polarized monolayer with junctional complexes and a well-developed brush border with associated hydrolases [24-26]. This cell line is commonly used in a Transwell system, which enables an effective separation of the apical or 'luminal' and the basolateral or 'systemic' compartment, similar to the intestinal barrier in *in vivo* situations [27,28].

MATERIALS AND METHODS

Materials

The human colon carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagle's medium (DMEM) and most supplements were purchased from Invitrogen (Carlsbad, CA, USA). Fetal calf serum (FCS) was from Bodinco (Alkmaar, The Netherlands). SITE+3 Liquid Media Supplement, L-glutamine, 3-[(3-cholamidopropyl)dimethyl-amonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT) and Coomassie Brilliant Blue (CBB) were obtained from Sigma (St. Louis, MO, USA). Urea was from Bio-Rad Laboratories (Hercules, CA, USA). L-[^{3}H]glutamine (specific activity, 1.85 TBq/mmol) and immobilized pH gradient (IPG) buffer (pH 3-10, nonlinear) were from Amersham Biosciences (Little Chalfont, England), and L-[2,3,3,4,4-$^{2}H_5$]glutamine from Cambridge Isotope Laboratories (Andover, MA, USA).

Cell culture

Caco-2 cells (passages 5 to 19) were seeded at the density of $1.2x10^5$ cells/cm^2 onto 24 mm Transwell bicameral systems (Corning, Aston, MA, USA) with collagen-

coated membranes (0.4 μm pore size, 4.7 cm^2 surface area). Cells were grown in high glucose DMEM supplemented with 20% (v/v) FCS, 1% (v/v) nonessential amino acid solution, 100 units/mL penicillin and 100 μg/mL streptomycin. Monolayers were maintained in culture at 37 ºC in a humidified atmosphere of 5% CO_2/95% O_2 (v/v). Confluence of cells at approximately 6 days postseeding was determined by monitoring tight junction formation ending the paracellular diffusion of phenol red. At this point cells start their differentiation process. The final incubation periods were performed in experimental medium, *i.e.* DMEM containing 1% (v/v) SITE+3 Liquid Media Supplement as a substitute of FCS, and a defined amount of glutamine, as detailed below.

Measurement of L-glutamine uptake by Caco-2 cells

Glutamine uptake in Caco-2 cells was initiated by adding experimental medium containing 0.1, 2.0 or 8.0 mM L-glutamine, trace-labeled with 28.5 kBq/mL L-[^{3}H]glutamine to the apical or basolateral side of the Transwell system. The opposite side contained DMEM without glutamine. The uptake of L-glutamine was measured after 1 h incubation with experimental medium, when Caco-2 cells were differentiated for 1, 4, 6, 8, 12 and 15 days, respectively. In addition, a time course was made, in which uptake of 2.0 mM L-glutamine, trace-labeled with 28.5 kBq/mL L-[^{3}H]glutamine, from apical or basolateral side was measured from 5 min to 48 h by 5-day and 15-day-differentiated cells, respectively. After incubation, the monolayers were washed three times with ice-cold medium containing 100 mM of unlabeled L-glutamine. The cells were harvested by scraping membranes in 1 mL 0.1 mM NaOH. Cell-associated radioactivity was measured using a 1414 WinSpectral liquid scintillation counter (Wallac, Turku, Finland). Protein content of the radioactive samples was determined using a Bradford based protein assay (Bio-Rad Laboratories) [29].

Protein sample preparation from Caco-2 monolayers

Monolayers were washed three times with phosphate-buffered saline (PBS). Proteins were isolated by scraping membranes in ice-cold PBS, and centrifuging obtained cell suspensions at 350 x *g* for 5 min at 4°C. Cell pellets were dissolved in a cell lysis buffer containing 8 M urea, 2% (w/v) CHAPS, 65 mM DTT for one-dimensional gel electrophoresis 1-DE), supplemented with 0.5% (v/v) IPG buffer (pH 3-10, nonlinear) for two-dimensional electrophoresis (2-DE). This mixture was subjected to three cycles of freeze thawing, vortexed thoroughly and centrifuged at 20000 g for 30 min at 10ºC. Supernatant was collected and stored at

–80 ºC until further analysis. Protein concentration of the mixture was determined using a Bradford based protein assay.

Examination of glutamine effects on protein expression profiles from Caco-2 cells

Caco-2 cells (day 5 postconfluence) were exposed to experimental medium containing 0.1, 2.0 or 8.0 mM L-glutamine to the apical or basolateral side of the Transwell system for 24 h. The opposite side contained DMEM without glutamine. Protein extracts from the cells were obtained as described above and separated by 2-DE as described by Wang *et al.* [30]. Examination of differentially expressed proteins was performed by image analysis software (PDQuest 7.3) (Bio-Rad Laboratories) as described [30].

Determination of glutamine labeling of proteins of Caco-2 cells

Caco-2 cells (day 5 postconfluence) were exposed to experimental medium containing 4.0 mM stable isotope-labeled L-[2,3,3,4,4-$^2H_5$]glutamine for 0, 24, 48 and 72 h, apical or basolateral. The opposite side of the Transwell system contained DMEM without glutamine. Proteins were isolated from Caco-2 cells as described above and the accumulation of glutamine in proteins was measured by the method of Bouwman *et al.* [31]. Briefly, proteins were separated by 1-DE in which each lane represents another experimental condition. Protein samples obtained after 0 h and 72 h of labeling, apical and basolateral, were also separated by 2-DE. All gels were stained with CBB.

To assess labeling of the individual proteins of the 1-D gel, 36 clearly visible protein bands were arbitrarily excised from each lane of the gel from the entire M_r range. For identifying label-accumulating proteins from the 2-D gels, 120 protein spots were excised from each 2-D gel covering the pI range between 3 to 10 and the M_r range between 15 to 100 kDa. The excised protein bands and spots were subjected to tryptic in-gel digestion and peptide mass fingerprints were generated using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (Waters, Manchester, UK). ProteinLynx Global Server 2.0 (Waters) and the Mascot search engine (http://www.matrixscience.com) were used to search peptide mass lists from obtained spectra against the Swiss-Prot database (http://au.expasy.org/sprot/) for protein identification. One missed cleavage was allowed, carbamidomethylation was set as a fixed modification and oxidation of methionine as a variable modification. The peptide mass tolerance was set to 100 ppm and no restrictions were made for protein M_r and pI. A protein was regarded

as identified with a significant ProteinLynx or Mascot probability score ($p<0.05$) and at least five peptide mass hits or a sequence coverage of at least 30% of the complete protein sequence.
Glutamine-containing peptides from the obtained mass spectra were analyzed at high resolution and semiquantitative labeling measurements resulted in peak ratios as shown in Table 1 and 2. For each peptide, the peak ratio at 0 h labeling was subtracted from the peak ratios at 24, 48 and 72 h labeling. A peptide peak was regarded as labeled if the peak ratio was at least 33.3% and if it gradually increased over time.

RESULTS

Uptake of glutamine by differentiating Caco-2 cells

To determine whether the glutamine uptake is dependent on the differentiation stage of Caco-2 cells, monolayers were exposed to radiolabeled glutamine for 1 h at several time points after the formation of tight junctions (from day 1 to day 15 after reaching confluence). Three different concentrations of glutamine (0.1, 2.0 and 8.0 mM) were tested, administered from apical or basolateral side.
Higher glutamine concentrations in the medium resulted in higher glutamine uptake by the cells (Figure 1A). Uptake of apical- and basolateral-administered glutamine was significantly different at every time point, for each concentration used. Basolateral exposure of the monolayers to glutamine-containing medium for 1 h resulted in 15.3 ± 3.2 to 4.3 ± 0.7 times higher glutamine uptake compared to apical exposure. The difference between apical and basolateral glutamine uptake was smaller at the end of the differentiation period. This originated from the fact that basolateral L-[^{3}H]glutamine uptake decreased considerably during differentiation of the cells, especially from day 6 postconfluence. Comparing day 1 with day 15, we observed a 2.0 ± 0.6, 1.8 ± 0.5 and 1.4 ± 0.3 fold decrease, for, respectively 0.1, 2.0 and 8.0 mM basolateral glutamine, and only a 1.3 ± 0.2, 1.1 ± 0.2 and 1.3 ± 0.2 fold decrease for apical glutamine.

Time course of glutamine uptake in Caco-2 cells, at two stages of differentiation

To investigate the influence of exogenous glutamine on protein metabolism of Caco-2 cells, longer exposure times are required. To see whether exogenously added glutamine still contributed to the total glutamine pool in a side-dependent

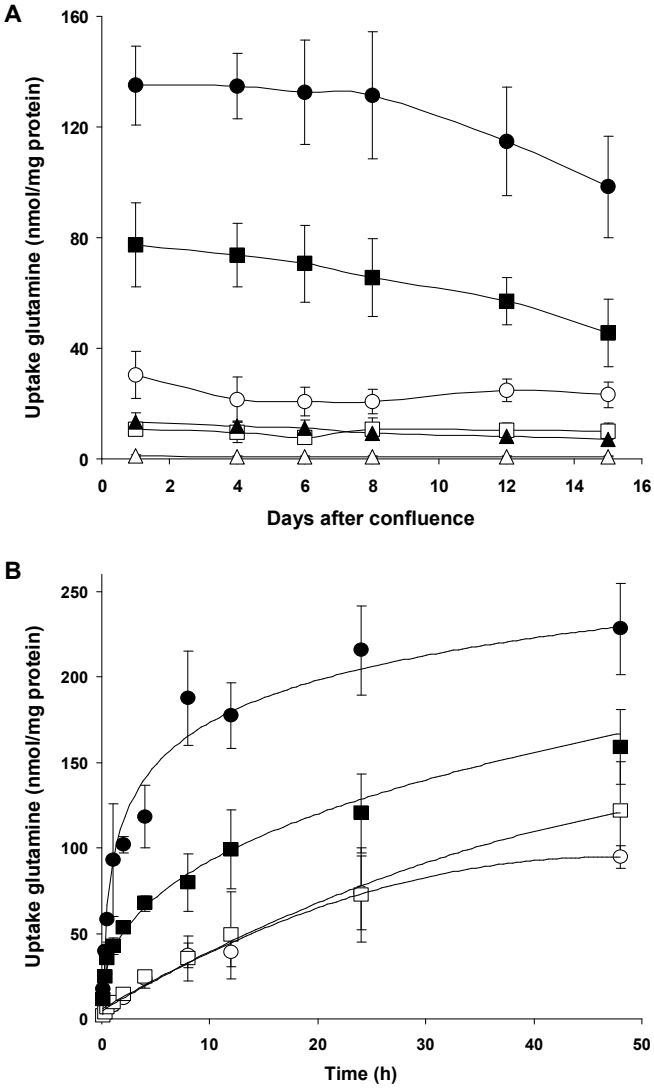

Figure 1. A. Glutamine uptake in Caco-2 monolayers across the apical (open symbols) and basolateral (closed symbols) membrane surface at various stages of differentiation (at day 1, 4, 6, 8, 12 and day 15 postconfluence). Uptake was measured after exposing cells to medium containing 0.1 mM (triangles), 2.0 mM (squares) and 8.0 mM (circles) glutamine, trace-labeled with 28.5 kBq/mL L-[^{3}H]glutamine for 1 h. Data represent mean ± SD for three monolayers. **B.** Time course of apical and basolateral glutamine uptake in Caco-2 monolayers. Apical (open symbols) and basolateral (closed symbols) uptake was measured after exposing cells to medium containing 2.0 mM glutamine, trace-labeled with 28.5 kBq/mL L-[^{3}H]glutamine, from apical or basolateral side for up to 48 h, at day 5 (circles) and day 15 postconfluence (squares). Data represent mean ± SD for three monolayers.

way after prolonged supplementation, cells were exposed to 2.0 mM glutamine for 5 min to 48 h. At day 5 (Figure 1B, circles), basolateral-administered glutamine led to a time-dependent increase of label in the cells with a maximum at 24 h, after which a steady state level was reached. Remarkably, an increase of radioactivity was observed at the apical compartment of the Transwell system when monolayers were exposed to radiolabeled glutamine from the basolateral side, and *vice versa* (data not shown). This was not due to leakage since paracellular diffusion of phenol red was not observed. Therefore, Caco-2 cells appeared not only to take up, but also to expel or secrete (metabolized) glutamine. With apical-administered glutamine, the accumulated label gradually increased till 48 h. At day 15 of differentiation (Figure 1B, squares) the absolute level of labeled glutamine in the cells again remained higher when administered from the basolateral side, but steady state levels were not yet reached.

Short exposure times (5 min to 30 min) did not result in a significantly different basolateral/apical uptake ratio compared to the ratio obtained at 1 h (data not shown). At 30 min the basolateral/apical uptake ratio was 9.1 ± 3.7 and 5.2 ± 0.3 for 5-day and 15-day-differentiated cells, respectively. At 24 h, the basolateral/ apical uptake ratio was 3.0 ± 0.6 and 1.7 ± 0.3 for 5-day and 15-day-differentiated cells, respectively. This indicates that the basolateral/apical uptake ratio depends on the differentiation state of Caco-2 cells. From these results, exogenous glutamine supply to 5-day-differentiated cells for 24 h was selected as the optimal condition for further studies.

Effects of glutamine availability on protein expression profiles of Caco-2 cells

In order to detect differences in protein expression related to glutamine addition to the Caco-2 cells, proteins were isolated from 5-day-differentiated cells exposed for 24 h to experimental medium containing 0.1, 2.0 and 8.0 mM glutamine from apical or basolateral side, and separated by 2-DE. Approximately 1600 spots were detected per gel within a pH range of 3 to 10, and a M_r range of 10 to 100 kDa. When comparing spot intensities after different glutamine treatment, none of them showed a significant up- or down-regulation (data not shown).

Accumulation of L-[$^2H_5$]glutamine in proteins of Caco-2 cells

We further investigated whether the supplied glutamine was incorporated into proteins and whether this was dependent on the delivery site. We examined this using our newly developed method [31] based on mass spectrometric detection of incorporated stable isotope-labeled amino acids into proteins. After an incubation

period of Caco-2 monolayers for 0, 24, 48 and 72 h with medium containing L-[$^2H_5$]glutamine from apical or basolateral side, proteins were isolated from the cells and separated in one dimension by SDS-PAGE (Figure 2).

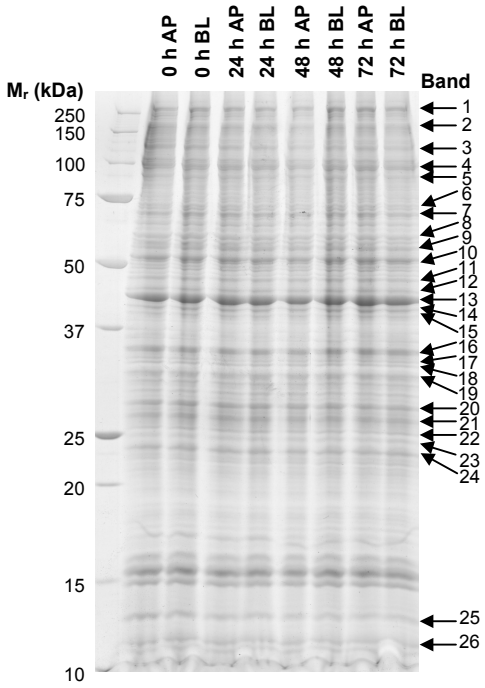

Figure 2. 1-D pattern of proteins extracted from Caco-2 cells after exposure to isotope-labeled glutamine for 0, 24, 48 and 72 h, apical (AP) and basolateral (BL). Protein bands were made visible by CBB staining. The 26 indicated protein bands were identified by MALDI-TOF MS and are depicted in Table 1.

MALDI-TOF MS analysis of 36 clearly visible protein bands covering the entire molecular mass range of the 1-D gel led to the identification of 33 distinct proteins in 26 bands by searching the Swiss-Prot database. This discrepancy is explained by the fact that one band in the gel can contain a mixture of several different proteins. Twelve out of those 33 proteins showed label incorporation (Table 1). In addition, protein samples of Caco-2 cells labeled with L-[$^2H_5$]glutamine for 0 and 72 h from apical or basolateral side were separated by 2-DE. An example of a 2-D gel is depicted in Figure 3. From each gel, 120 protein spots were subjected to MALDI-TOF MS analysis. This resulted in the identification of 80 distinct proteins represented by 114 spots in the gel, since some proteins were present as more than one spot due to protein processing or modification. In total, 20 proteins showed

label incorporation (Table 2), from which 8 proteins were also detected as labeled in the 1-DE experiment.

Table 1. List of identified proteins from bands of the 1-D gel. Thirty-three proteins out of 26 bands (see Figure 2) were identified by MALDI-TOF MS and semiquantitative analysis of glutamine-containing peptides and the corresponding isotopomer peaks at high resolution revealed significant labeling of 12 proteins, which are indicate in bold.

Band	Swiss-Prot accession number	Protein name	*m/z*	Peak ratio (x100%)			Peak ratio (x100%)		
				24 h AP	48 h AP	72 h AP	24 h BL	48 h BL	72 h BL
1	O15061	Desmulin	1608	7.7	12.3	13.7	11.4	21.0	18.8
2	Q00610	Clathrin heavy chain 1	NQ						
3	Q12864	**Cadherin-17**	1547	4.3	20.1	23.9	21.1	-	**73.2**
4	O43707	**Alpha-actinin 4**	1174	7.8	14.3	20.9	12.1	**33.4**	**75.1**
	P14625	Endoplasmin	1081	5.3	15.2	18.4	4.4	13.8	24.1
5	P08238	HSP 90-beta	2257	12.2	12.2	14.4	2.6	10.3	24.0
	P09327	Villin 1	NQ						
6	P38646	**Stress-70 protein, mit.**	1695	11.5	18.7	19.5	10.2	21.8	**33.7**
7	P31040	Succinate dehydrogenase [ubi-quinone] flavoprotein subunit, mit.	1268	0.0	2.0	11.0	3.6	3.8	14.0
8	P10809	60-kDa HSP, mit.	1919	5.6	8.6	23.2	2.1	4.7	10.2
9	P30101	**Protein disulfide-isomerase A3**	1515	9.5	10.0	21.2	14.1	29.3	**42.2**
	P07237	**Protein disulfide-isomerase**	1834	5.7	14.8	21.2	15.4	27.8	**47.9**
10	P05787	Keratin, type II cytoskeletal 8	1079	0.0	0.0	3.6	3.3	3.3	7.3
	P00367	Glutamate dehydrogenase 1, mit.	1738	5.0	6.9	9.0	1.6	2.6	8.2
11	P50454	**Collagen-binding protein 2**	1293	16.7	22.5	**34.9**	11.3	23.7	**58.4**
12	P04181	**Ornithine aminotransferase, mit.**	1811	-	21.3	16.5	19.5	**53.2**	**64.0**
13	P60709	Actin, cytoplasmic 1	1791	2.5	6.0	9.4	1.6	3.2	6.3
14	P08727	Keratin, type I cytoskeletal 19	1675	4.6	9.1	14.6	10.5	17.7	30.3
15	P00505	Aspartate aminotransferase, mit.	1449	0.0	2.3	2.5	11.0	18.0	21.6
16	P07355	**Annexin A2**	1111	1.9	5.0	10.1	14.1	26.1	**36.8**
	P22626	HnRNP A2/B1	1087	1.5	5.4	5.6	3.4	4.9	8.3
17	P09651	HnRNP A1	1049	15.4	3.0	5.2	0.0	0.0	10.2
	Q07955	Splicing factor, arginine/serine-rich 1	NQ						
18	P09651	HnRNP A1	1628	7.8	8.2	12.1	9.0	13.4	15.4
19	P09525	**Annexin A4**	1118	1.2	9.7	14.1	13.3	30.5	**49.0**
20	P17931	**Galectin-3**	1650	10.5	16.9	23.3	25.4	**38.8**	**57.9**
	P35232	Prohibitin	1396	4.0	5.9	9.6	5.0	7.1	11.8
21	P30084	Enoyl-CoA hydratase, mit.	1467	2.5	8.2	16.5	3.7	14.2	21.4
22	P60174	Triosephosphate isomerase	1458	0.1	2.0	6.9	3.3	5.1	10.4
23	P09211	**Glutathione S-transferase P**	1883	5.7	9.9	13.4	13.9	25.3	**37.3**
24	P62820	Ras-related protein Rab-1A	1316	3.3	8.4	14.7	8.2	19.0	32.7
	P51149	**Ras-related protein Rab-7**	1187	13.6	24.4	**36.2**	**36.0**	**76.3**	**97.5**
25	P61604	10-kDa HSP, mit.	1325	2.1	2.4	4.6	4.3	5.7	3.7
26	P62805	Histone H4	NQ						

AP, apical; BL, basolateral; NQ, no glutamine-containing peptides in spectrum peaks; HSP, heat shock protein; mit., mitochondrial; hnRNP, heterogeneous ribonucleoprotein

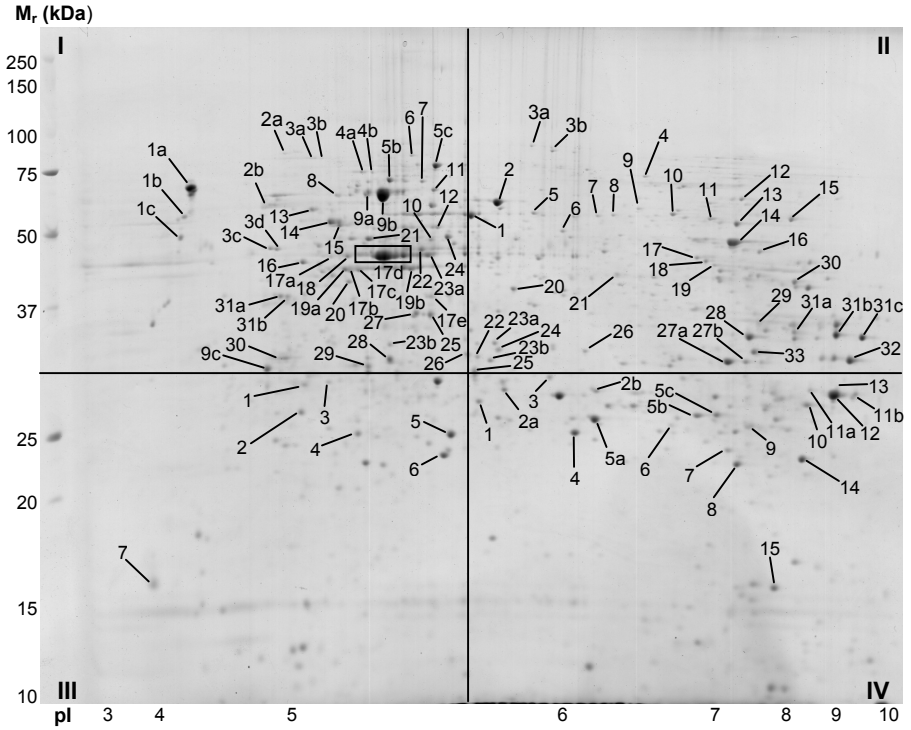

Figure 3. Example of a 2-D pattern of proteins extracted from Caco-2 cells after exposure to isotope-labeled glutamine for 0 and 72 h, apical and basolateral. Protein spots were made visible by CBB staining. The image is divided into four sections. The 114 indicated protein spots were identified by MALDI-TOF MS and are depicted in Table 2.

Table 2. List of identified proteins from the 2-D gel. Sections and protein numbers correspond with Figure 3. In total, 114 proteins were identified by MALDI-TOF MS and semiquantitative analysis of glutamine-containing peptides and the corresponding isotopomer peaks at high resolution revealed significant labeling of 20 distinct proteins, which are indicated in bold.

Spot	Swiss-Prot accession number	Protein name	*m/z*	Peak ratio (x100%) 72 h AP	Peak ratio (x100%) 72 h BL
Section I					
1a	P27797	Calreticulin	1476	11.5	14.7
1b	P27797	Calreticulin	1476	-	21.5
1c	P27797	Calreticulin	1476	18.9	17.1
2a	P14625	Endoplasmin	1081	20.1	23.8
2b	P14625	Endoplasmin[a)]	1081	18.4	24.5
3a	P11021	78-kDa glucose-regulated protein	1888	3.9	13.9
3b	P11021	78-kDa glucose-regulated protein	1888	17.8	20.6
3c	P11021	78-kDa glucose-regulated protein[b)]	NQ		
3d	P11021	78-kDa glucose-regulated protein[b)]	NQ		
4a	P20700	Lamin B1	1651	-	7.7
4b	P20700	Lamin B1	1651	3.4	5.8
5a	P38646	**Stress-70 protein, mit.**	1694	25.1	**43.3**

Spot	Swiss-Prot accession number	Protein name	*m/z*	Peak ratio (x100%) 72 h AP	Peak ratio (x100%) 72 h BL
5b	P38646	**Stress-70 protein, mit.**	1694	25.9	**45.8**
5c	P38646	**Stress-70 protein, mit.**	1694	15.1	**41.2**
6	P28331	**NADH-ubiquinone oxidoreductase 75-kDa subunit, mit.**	2071	**36.0**	**59.6**
7	P61978	**HnRNP K**	1518	**40.9**	-
8	O43707	**Alpha-actinin 4**[b]	1753	23.6	**47.5**
9a	P10809	60-kDa HSP, mit.	1919	9.3	18.6
9b	P10809	60-kDa HSP, mit.	1919	9.0	7.3
9c	P10809	60-kDa HSP, mit.[b]	1771	10.7	1.0
10	P15311	Ezrin[b]	1651	13.0	-
11	P48643	T-complex protein 1, epsilon subunit	1093	12.0	19.0
12	P05787	Keratin, type II cytoskeletal 8	1079	17.2	0.0
13	P68371	Tubulin beta-? chain	1130	12.3	19.1
14	P06576	ATP synthase beta chain, mit.	1601	10.0	7.0
15	Q15084	Protein disulfide-isomerase A6	1483	12.7	13.2
16	Q90473	**HSC 71-kDa protein**[b]	1081	**44.7**	**78.3**
17a	P60709	Actin, cytoplasmic 1	1790	5.3	8.7
17b	P60709	Actin, cytoplasmic 1	1790	11.4	4.8
17c	P60709	Actin, cytoplasmic 1	1790	16.7	7.0
17d	P60709	Actin, cytoplasmic 1	1790	7.6	9.5
17e	P60709	Actin, cytoplasmic 1[b]	1790	12.0	4.2
18	P06727	**Apolipoprotein A-IV**	1104	**100.3**	**656.1**
19a	P08727	Keratin, type I cytoskeletal 19	1674	19.8	32.9
19b	P08727	Keratin, type I cytoskeletal 19	1674	18.4	31.9
20	P07237	**Protein disulfide-isomerase**[a]	1833	20.9	**46.5**
21	P52597	HnRNP F	1935	13.3	23.3
22	P05783	Keratin, type I cytoskeletal 18	965	7.6	7.6
23a	P12277	Creatine kinase, B chain	1031	0.0	1.6
23b	P12277	Creatine kinase, B chain[a]	2518	10.0	13.8
24	P31930	Ubiquinol-cytochrome-c reductase complex core protein mit.	NQ		
25	P11177	Pyruvate dehydrogenase E1 component beta subunit, mit.	1801	14.8	18.7
26	P47756	F-actin capping protein beta subunit	1696	18.9	21.9
27	P07437	Tubulin beta-2 chain[a]	1130	10.8	14.3
28	P30101	**Protein disulfide-isomerase A3**[a]	1515	19.3	**51.4**
29	P07858	Cathepsin B[b]	1824	14.9	31.8
30	P12324	Tropomyosin alpha 3 chain	1243	25.9	10.3
31a	P06748	Nucleophosmin	1568	11.2	4.4
31b	P06748	Nucleophosmin	1568	32.2	22.5
32	O43852	**Calumenin**	1532	23.4	**36.5**
Section II					
1	P05787	Keratin, type II cytoskeletal 8	1079	9.0	6.6
2	P30101	**Protein disulfide-isomerase A3**	1515	19.6	**44.1**
3a	Q16891	Mit. inner membrane protein	1527	10.2	18.2
3b	Q16891	Mit. inner membrane protein	1527	13.1	14.9
4	P31040	**Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mit.**	1160	18.2	**59.6**
5	P05091	Aldehyde dehydrogenase, mit.	1789	15.1	7.2
6	P22307	Nonspecific lipid-transfer protein, mit.	1104	5.5	26.0
7	P30837	Aldehyde dehydrogenase X, mit.	1403	-	18.5
8	P78371	T-complex protein 1, beta subunit	1291	21.3	20.9
9	Q9UMS4	PRP19/PSO4 homolog	1614	15.4	26.3
10	P00352	Retinal dehydrogenase 1	1189	21.3	20.4

Spot	Swiss-Prot accession number	Protein name	*m/z*	Peak ratio (x100%) 72 h AP	Peak ratio (x100%) 72 h BL
11	P49419	Aldehyde dehydrogenase family 7 member A1	NQ		
12	P04040	Catalase	1812	12.5	32.7
13	P06733	Alpha enolase	1425	16.7	24.9
14	P00367	Glutamate dehydrogenase 1, mit.[b)]	1737	10.8	9.3
15	Q02252	Methylmalonate-semialdehyde dehydrogenase [acylating], mit.	NQ		
16	P07954	**Fumarate hydratase, mit.**	957	14.2	**38.1**
17	P49411	Elongation factor Tu, mit.	1483	18.6	18.3
18	O75874	**Isocitrate dehydrogenase [NADP] cyt.**	1009	14.1	**33.1**
19	P11310	Acyl-CoA dehydrogenase, medium-chain specific, mit.	1892	17.1	22.6
20	P50213	Isocitrate dehydrogenase [NAD] subunit alpha, mit.	1028	19.0	7.0
21	Q15084	Protein disulfide-isomerase A6	1191	12.8	14.5
22	P31937	3-hydroxyisobutyrate dehydrogenase, mit.	1567	17.2	-
23a	P09525	**Annexin A4**	1118	22.7	**50.9**
23b	P09525	**Annexin A4**	1118	18.1	**48.6**
24	P30101	Protein disulfide-isomerase A3[a)]	NQ		
25	P07339	**Cathepsin D[b)]**	1601	**42.0**	**143.1**
26	P49411	Elongation factor Tu, mit.[a)]	1483	10.4	-
27a	P13804	Electron transfer flavoprotein alpha-subunit, mit.	1812	8.5	20.6
27b	P13804	**Electron transfer flavoprotein alpha-subunit, mit.**	1812	31.3	**155.3**
28	P04406	Glyceraldehyde-3-phosphate dehydrogenase, liver	1613	12.8	13.9
29	P07355	**Annexin A2**	1111	11.3	**35.4**
30	P24752	Acetyl-CoA acetyltransferase, mit.	1544	10.7	13.9
31a	P22626	HnRNP A2/B1	1087	3.2	8.3
31b	P22626	HnRNP A2/B1	1087	16.2	11.0
31c	P22626	**HnRNP A2/B1**	1087	23.0	**37.1**
32	P21796	Voltage-dependent anion-selective channel protein 1	2103	14.4	17.4
33	P45880	Voltage-dependent anion-selective channel protein 2	2103	13.6	25.2
Section III					
1	P07237	Protein disulfide-isomerase[a)]	NQ		
2	P12277	Creatine kinase, B chain[a)]	NQ		
3	P11021	78-kDa glucose-regulated protein[a)]	1888	19.3	26.4
4	P07858	Cathepsin B[b)]	1824	8.3	2.5
5	P09211	**Glutathione S-transferase P**	1883	13.3	**43.4**
6	P32119	**Peroxiredoxin 2**	1211	19.7	**38.2**
7	P62158	Calmodulin	NQ		
Section IV					
1	Q13162	Peroxiredoxin 4	1225	15.5	26.0
2a	P30040	Endoplasmic reticulum protein ERp29	1247	5.2	5.5
2b	P30040	Endoplasmic reticulum protein ERp29	1247	26.5	27.8
3	P30101	**Protein disulfide-isomerase A3[b)]**	1515	22.0	**48.8**
4	P30048	Thioredoxin-dependent peroxide reductase, mit.	NQ		
5a	P60174	Triosephosphate isomerase	1458	14.7	11.2
5b	P60174	Triosephosphate isomerase	1458	13.0	15.2
5c	P60174	Triosephosphate isomerase	1458	9.4	13.9
6	P47985	Ubiquinol-cytochrome c reductase iron-sulfur subunit, mit.	1614	14.1	10.6
7	P25705	ATP synthase alpha chain, mit.[b)]	2367	17.0	16.4
8	P04179	Superoxide dismutase [Mn], mit.	NQ		
9	Q99714	3-Hydroxyacyl-CoA dehydrogenase type II	1621	11.7	-
10	P38117	Electron transfer flavoprotein beta-subunit	1339	17.6	11.6
11a	P22626	HnRNP A2/B1[b)]	NQ		
11b	P22626	HnRNP A2/B1[b)]	NQ		
12	P17931	**Galectin-3**	1694	21.8	**49.3**

Spot	Swiss-Prot accession number	Protein name	*m/z*	Peak ratio (x100%) 72 h AP	Peak ratio (x100%) 72 h BL
13	P10809	60-kDa HSP, mit.[a)]	1919	9.3	10.0
14	P06830	Peroxiredoxin 1	1211	21.5	30.0
15	P62937	Peptidyl-prolyl cis-trans isomerase A	1614	15.4	22.4

AP, apical; BL, basolateral; NQ, no glutamine-containing peptides in spectrum peaks; mit., mitochondrial; hnRNP, heterogeneous ribonucleoprotein; HSP, heat shock protein; HSC, heat shock cognate
a) N-terminal part of protein
b) C-terminal part of protein

As an example the spectra and coverage maps of actin and galectin-3, respectively band 13 and 20 in Table 1, are depicted in Figures 4A and 4B. Tryptic peptides that were matched with peaks in the spectrum are boxed in the amino acid sequence of the protein. A glutamine-containing spectrum peak of actin at m/z 1790 corresponds to the tryptic peptide SYELPDGQVITIGNER, and was analyzed at high resolution. No significant isotopomer peak (M+5) could be detected after labeling with [$^2H_5$]glutamine for up to 72 h, from apical or basolateral side (Figures 5A and 5B). Hence this protein did not incorporate labeled glutamine significantly during this time period. On the contrary, analysis of such a peak of galectin-3 at m/z 1650, which corresponds to the tryptic peptide VAVNDAHLLQYNHR, clearly shows the appearance of an isotopomer peak (M+5) after 24 h labeling (Figures 5C and 5D). According to our criteria, labeling was only significant after 48 h incubation with [$^2H_5$]glutamine at the basolateral side. The isotopomer peak appearing upon basolateral exposure to labeled glutamine for 72 h is 57.9% of the original mass peak, while the apical isotopomer peak is only 23.3% of the original peak. These data demonstrate incorporation of labeled glutamine into the protein galectin-3. Similar results were obtained for 11 other proteins of the 1-D gel (see Table 1), and for 20 proteins of the 2-D gel (see Table 2). This indicates that glutamine incorporates into a common pool of proteins independent from the site of application. The only difference is their rate of labeling which is for most of the proteins at least twice as high for basolateral-administered glutamine compared to apical-administered glutamine.

DISCUSSION

Essential in this study is that the gut epithelial lining utilizes glutamine from two sources, *i.e.* from the luminal and systemic side. By using an *in vitro* cell study approach, in which polarized human intestinal Caco-2 cells cultured on Transwell inserts are exposed to external glutamine from apical or basolateral side, we were able to investigate the influence of the polarity on cellular glutamine uptake and glutamine incorporation into proteins.

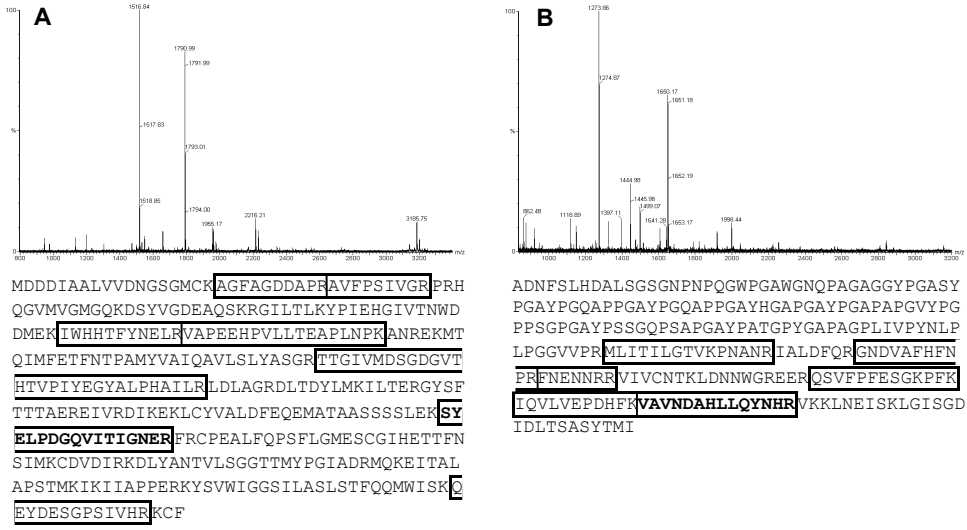

Figure 4. MALDI-TOF mass spectrum and coverage map of actin (A) and galectin-3 (B). Boxed peptides in the amino acid sequence of the protein show a clear match with peaks in the mass spectrum. **A.** The peptide SYELPDGQVITIGNER, indicated in bold in the sequence, contains a glutamine and corresponds to the spectrum peak with m/z value 1791. **B.** The peptide VAVNDAHLLQYNHR, indicated in bold in the sequence, contains a glutamine and corresponds to the spectrum peak with m/z value 1650.

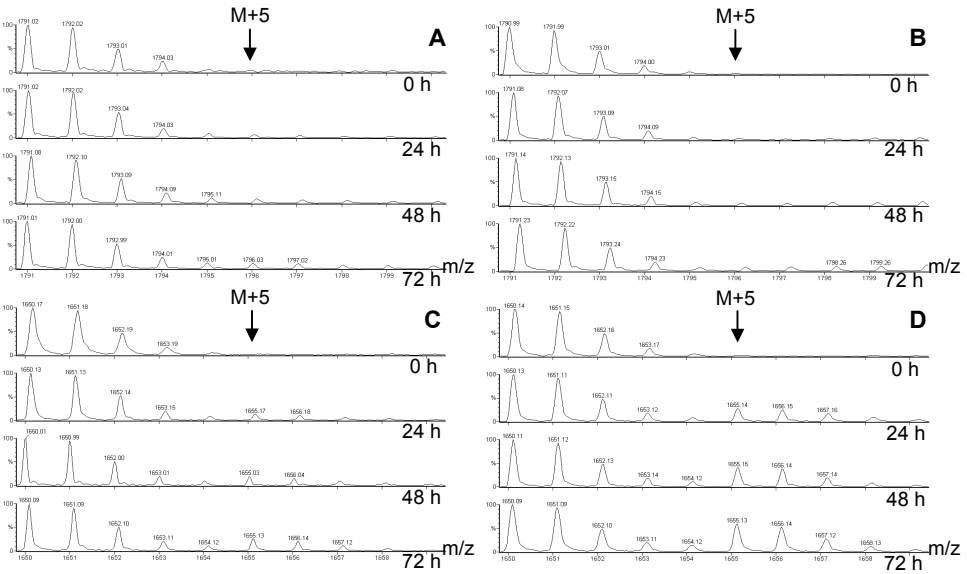

Figure 5. A, B. Peaks of mass spectrum of actin at high resolution, corresponding to m/z value 1791. No significant isotopomer peak (M+5) is present after labeling for up to 72 h with L-[2,3,3,4,4-$^{2}H_{5}$]glutamine, apical (panel A) and basolateral (panel B). **C, D.** Peaks of mass spectrum of galectin-3 at high resolution, corresponding to m/z value 1650, and the upcoming isotopomer peak (M+5) due to incorporation of L-[2,3,3,4,4-$^{2}H_{5}$]glutamine in the peptide after 24, 48 and 72 h of labeling, apical (panel C) and basolateral (panel D).

We demonstrated that, compared to the apical side, the overall glutamine uptake from the basolateral side is consistently higher. It is known that uptake of glutamine across the apical (brush border) membrane of Caco-2 cells is mainly dependent on three mechanisms, (i) Na^+-dependent and (ii) Na^+-independent saturable transport processes as well as (iii) passive diffusion, which even exceeds Na^+-independent uptake at high concentrations of glutamine (above 3.0 mM) [32-34]. The Na^+-dependent uptake of glutamine occurs mainly via the Na^+-dependent neutral amino acid transporter B^0 (ATB^0), which is also expressed in Caco-2 cells [35] and was found to mediate the majority of total glutamine uptake across the apical membrane. Na^+-independent glutamine uptake in Caco-2 cells occurs largely through system L [33]. Although it is suggested that systemic (basolateral) glutamine plays an important role in enterocyte homeostasis and function [36], also in intestinal injury [37], few data are available on the uptake mechanisms of glutamine by the basolateral membrane of Caco-2 cells. As mentioned above, system L plays a role in glutamine uptake across the brush border membrane of Caco-2 cells and it is suggested that especially LAT-1, the first isoform of system L, is responsible for that [38]. A second isoform of this system, known as LAT-2, is prominently expressed in the basolateral membranes of epithelial cells in the villi of the mouse intestine [39]. A study performed in Caco2-BBE cells also showed a basolateral localization of LAT-2 [40]. Since the Caco2-BBE cell line is a clone isolated from the cell line Caco-2 [41], it is most likely that the LAT-2 protein has a similar distribution pattern in the cells used in this study. In addition, experiments with rodent and human LAT isoforms revealed that glutamine is more efficiently transported by LAT-2 than by LAT-1 [34]. Together, these data provide an explanation for the observed difference between apical and basolateral glutamine uptake in our experiments. Since passive diffusion plays also a considerable role in cellular glutamine uptake, another explanation for this difference may be the ratio of basolateral to apical surface area which is 3 : 1 in Caco-2 cells early in differentiation [42].

When cells become more differentiated, we observed a decrease in glutamine uptake across the basolateral membrane. This decrease may parallel changes in membrane composition, like a decrease of passive diffusion and a reduction of transporter protein expression or activity that coincides with Caco-2 cell differentiation. For example, it is suggested that the differentiation process in Caco-2 cells is associated with a decrease in system B and system L activity [43,44]. This could also influence glutamine transport via these systems. Together with the length of time in culture, cell height and the number and length of microvilli increase and cell width decreases [45]. This leads to different ratios of basolateral to apical membrane surface area at different time points in differentiation, which might underlie the declining basolateral/apical glutamine uptake ratio.

By using a 2-DE approach, we searched for differences in protein expression profiles of Caco-2 cells subjected to diverse glutamine treatment. No protein spots could be recognized with a significant differential expression pattern. This observation can be interpreted in several ways. Using this method, a substantial number of proteins occurs below the detection level, meaning that proteins, which do show a glutamine-dependent expression, could have been missed. However, from the fact that none out of 1600 examined protein spots showed any significant change, this seems unlikely. Another explanation may be the overall slow turnover rate of proteins in Caco-2 cells. Alternatively, our findings can be explained by the relative high endogenous glutamine synthesis capacity of Caco-2 cells compared to human small intestinal cells [46],[16]. This may limit the influence of exogenous glutamine on the Caco-2 proteome, demonstrating a shortcoming of the *in vitro* model system. Therefore, it cannot be excluded that exogenous glutamine does change the proteome of human intestinal cells *in vivo*.

We found exogenous glutamine incorporated into proteins of Caco-2 cells. Some proteins (24 out of 113) are labeled more rapidly than others, and the labeling rate is for most of the proteins at least twice as high when L-[$^2H_5$]glutamine was delivered from the basolateral side compared to the apical side. This phenomenon is in close agreement with the uptake experiments, where basolateral exposure to glutamine leads to higher exogenous glutamine concentrations in the Caco-2 cells, and thus resulting in considerable competition between externally administered glutamine and endogenously synthesized glutamine for protein synthesis. Despite the sidedness in uptake rate, our labeling results indicate that similar proteins are labeled when glutamine is supplied from either side. This suggests that apical and basolateral glutamine enter a common pool and are used for similar purposes. Thus, the hypothesis that the effects of glutamine are dependent on the route of supplementation [19,20], is not supported by our labeling results.

The labeling method that we used has proven its ability to reveal important information about essential processes in cultured cells [31]. In the present study the most rapidly labeled proteins (Table 1 and 2) can roughly be divided into four functional groups. The first group of proteins (annexin A2, annexin A4, cadherin-17, galectin-3 and alpha-actinin 4) is involved in membrane stabilization, cell-cell adhesion and cell-matrix adhesion, and thus seems important for establishing the barrier integrity of the 5-day-differentiated Caco-2 monolayer. The second group concerns proteins which play a role in protein folding and processing (protein disulfide-isomerase, protein disulfide-isomerase A3, collagen-binding protein 2, mitochondrial stress-70 protein and heat shock cognate 71-kDa protein). The third group of proteins is involved in the regulation of the redox status in cells and the fourth group in glutamine metabolism.

Annexin A2 and A4 belong to a family of soluble cytoplasmic proteins that can bind to the membrane surface in response to elevations in intracellular calcium [47]. Annexin A2 is an F-actin binding protein and participates in the formation of membrane-cytoskeleton connections [47]. A recent study has revealed also morphological and functional evidence for a role of annexin A2 in tight junction assembly in MDCK II monolayers [48]. The other family member, annexin A4 is closely associated with the apical membrane in secretory and absorptive epithelia. It is reported that annexin A4 interactions with membranes did reduce membrane permeability by reducing the fluidity of the bound leaflet [49]. Another protein, which is also important for cell-cell adhesion is cadherin-17 or liver-intestine cadherin (LI-cadherin). LI-cadherin appears to be a third Ca^{2+}-dependent cell adhesive system in the intestinal mucosa, next to co-expressed E-cadherin and to desmosomal cadherins. LI-cadherin acts as a functional Ca^{2+}-dependent homophilic cell-cell adhesion molecule without any interaction with cytoplasmic components [50]. It is most likely responsible for flexible intercellular adhesive contacts outside the junctional complexes [51]. In addition, galectin-3 is suggested to be involved in cell-cell and cell-matrix interactions. It is an intracellular and extracellular lectin, which interacts with intracellular glycoproteins, cell surface proteins and extracellular matrix proteins. Overexpression of galectin-3 in human breast carcinoma cell lines exerted an enhanced adhesion to laminin [52]. A recent study showed that galectin-3 probably interacts with LI-cadherin by its carbohydrate recognition domain, on the cell surface of pancreatic carcinoma cells [53]. Alpha-actinin 4, like annexin A2, is an F-actin cross-linking protein which seems to regulate the actin cytoskeleton and increases cellular motility [54]. At least one member of the alpha-actinin protein family, alpha-actinin 1, has shown to be involved in cadherin-mediated cell-cell adhesion via alpha-catenins in adherens junctions of epithelial cells [55]. The fact that these proteins show a rapid labeling with glutamine suggests a functional link between them and may provide a molecular basis for the improved gut barrier function observed after glutamine supplementation [56].

The importance for developing Caco-2 cells of producing proteins involved in cell-cell adhesion may be reflected in the second group of labeled proteins. For instance, collagen-binding protein 2, also known as colligin-2, is a collagen-binding glycoprotein localized in the ER. It is suggested that colligin-2 functions as a collagen-specific molecular chaperone [57] assisting extracellular matrix remodeling during changing cell-cell interactions. Another example is protein disulfide-isomerase which is found to be a component of prolyl 4-hydroxylase, an enzyme involved in the synthesis of collagen [58].

The third group consists of proteins with a role in the redox regulation in cells. Glutathione S-transferase P (GSTP1-1) is involved in the conjugation of reduced

glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles, and thus acts as a cytoprotective agent. This protein is highly expressed in various carcinoma's, including colon carcinoma, acting as a protection against apoptosis [59]. Cytosolic NADP-dependent isocitrate dehydrogenase has a protective role against oxidative damage being a source of NADPH [60], while peroxiredoxin 2 functions as an antioxidant enzyme through its peroxidase activity [61].

Several proteins with a role in the metabolism of glutamine are labeled (group 4). Ornithine aminotransferase is a key enzyme necessary for synthesis of arginine from glutamine in the small intestine of neonatal and postweaning pigs [62]. The fact that this enzyme has a quite high turnover in the Caco-2 cells may indicate that a substantial amount of glutamine is used for conversion to ornithine, were it can enter several metabolic routes. Two proteins from the tricarboxylic acid cycle, succinate dehydrogenase flavoprotein subunit and fumarate hydratase, can play a role in the oxidative metabolism of glutamine via alpha-ketoglutarate to yield energy or to provide precursors for synthesis of compounds derived from tricarboxylic acid cycle intermediates [63]. GSTP1-1 also belongs to this group of proteins.

Eight other proteins were found to be relatively rapidly labeled. Rab-7 regulates endocytic membrane traffic and is an essential participant in the autophagic pathway, which is necessary to sequester and target cytoplasmic components to the lytic compartment for degradation and recycling [64]. Cathepsin D is a lysosomal protease. Two heterogeneous nuclear ribonucleoproteins, K and A2/B1, also show label incorporation. These proteins have the capacity to bind DNA and RNA sequence elements and thereby regulate gene expression at various levels [65]. Apoliprotein A-IV and calumenin are known to be secreted, the former is primarily synthesized by the intestine, and also by differentiated Caco-2 cells [46]. Finally, NADH-ubiquinone oxidoreductase 75-kDa subunit and electron transfer flavoprotein alpha-subunit are components of the mitochondrial respiratory chain.

In conclusion, our experiments have provided clear evidence that exogenous glutamine is taken up by Caco-2 cells, from both apical and basolateral side. Glutamine uptake across the basolateral membrane consistently exceeds uptake across the apical membrane of the cells and this phenomenon is more pronounced in partially differentiated cells (at day 5 postconfluence) than in completely differentiated cells (at day 15 postconfluence). No effects of exogenous glutamine supply on the proteome were detected. However, we demonstrated incorporation into proteins with a role in cell-cell interactions, redox status and glutamine metabolism. This may provide an explanation for improved gut barrier function after glutamine supplementation. Our data indicate that systemic supplementation

is preferred above luminal glutamine supply, which is in line with studies in critically ill patients [1].

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CHAPTER 3

GLUTAMINE REGULATES THE EXPRESSION OF PROTEINS WITH A POTENTIAL HEALTH-PROMOTING EFFECT IN HUMAN INTESTINAL CACO-2 CELLS

Kaatje Lenaerts, Edwin Mariman, Freek Bouwman and Johan Renes

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ABSTRACT

Glutamine is an essential amino acid for the enterocytes with respect to maintaining the gut mucosal integrity and function. This study was conducted to explore a molecular basis for the beneficial effects of glutamine on intestinal cells by searching for glutamine-dependent changes in the proteome. Caco-2 cells were exposed to different concentrations of L-glutamine with or without L-methionine sulfoximine, an inhibitor of the glutamine synthetase activity. Two-dimensional gel electrophoresis combined with matrix-assisted laser desorption/ionization-time of flight-mass spectrometry was used to identify proteins whose expression is changed by glutamine. To assess the relative protein synthesis rate, incorporation of L-[$^2H_5$]glutamine into individual proteins was monitored. The expression levels of fourteen proteins changed significantly with the glutamine availability. Examples of differentially expressed proteins with potential health-promoting effects on the intestine are plasma retinol-binding protein, ornithine aminotransferase, apolipoprotein A-I, mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase and acyl-CoA synthetase 5. Expression of these proteins was not changed by arginine deprivation. The differential change in the expression levels of the proteins was not correlated with their rate of synthesis, excluding an effect of glutamine depletion on general protein synthesis. Together, this study shows a gene-specific effect of glutamine on intestinal cells.

INTRODUCTION

Glutamine is the most abundant free amino acid in the human body and plays a key role in the metabolism of rapidly dividing cells, including enterocytes. The small intestine consumes large amounts of glutamine either as an energy source [1] or as a metabolic precursor for other amino acids, peptides and proteins, nucleotides and glutathione [2-4]. In addition, glutamine protects intestinal cells against apoptosis [5,6], enhances the intestinal blood flow [7], acts as a trophic factor for the intestinal mucosa [8], stimulates the synthesis of polyamines [9] and prevents TNF-alpha induced bacterial translocation [10]. In experimental models of critical illness, glutamine is able to attenuate proinflammatory cytokine expression and to improve gut barrier function [11-14]. Recently, we found in Caco-2 cells that glutamine rapidly incorporates in proteins mainly involved in cell-cell interactions, redox status and glutamine metabolism [15].

The glutamine pool in intestinal cells originates, besides from uptake of nutrients out of the lumen and/or the systemic circulation, from *de novo* synthesis by glutamine synthetase. The glutamine synthetase activity in human intestines is very low [16], suggesting that enterocytes depend on the external glutamine supply for normal functioning. Under certain catabolic conditions like injury or infection, the circulating amount of this amino acid decreases, indicating that the ability of endogenous glutamine production to meet demands during a variety of illnesses is insufficient [17]. It has been proposed that gut mucosal turnover and barrier function are compromised during stress states, partly due to relative glutamine deficiency [18]. This has led to the reclassification of glutamine from a nonessential to a conditionally essential amino acid [14]. Since the physiological importance of this amino acid for promoting and maintaining cell function is now widely accepted, strong interest has focused on glutamine as a nutraceutical to enhance bowel function and integrity. However, molecular mechanisms and pathways underlying the health-promoting potential of glutamine in the intestine remain unclear.

In this study, we applied a combination of two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to investigate changes in the protein profiles of intestinal cells corresponding to the availability of cellular glutamine. The observed changes may well lead to new insights and a better understanding of the effects of glutamine on the intestine. As a model for the small intestine we used the human Caco-2 cell line. These cells are originally derived from a colon adenocarcinoma, but undergo spontaneous differentiation towards a small intestinal enterocyte-like phenotype. Caco-2 cells, grown on filter supports, form a polarized monolayer with junctional complexes and a well-developed brush border with hydrolases [19-21]. The utility of this cell culture approach to examine cellular mechanisms affected by

deprivation of nutrients was already demonstrated before [10,22]. Collagen-coated filter supports were used, since it was shown that the collagen matrix promotes cell attachment and growth leading to confluence of the Caco-2 monolayer more rapidly [23]. A detailed morphological characterization showed that together with the length of time in culture, the epithelial cells displayed a more columnar shape, a better organized brush border with more and better shaped microvilli with associated enzymes like alkaline phosphatase. The appearance of occluding tight junctions and desmosomes in this system are also similar compared to normal enterocytes [23]. Therefore, this model was chosen to examine the effects of glutamine on the intestinal proteome.

MATERIALS AND METHODS

Materials

The human colon carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagles medium (DMEM) and most supplements for cell culture were purchased from Invitrogen (Carlsbad, CA, USA). Fetal calf serum (FCS) was from Bodinco (Alkmaar, The Netherlands). SITE+3 Liquid Media Supplement, L-glutamine, L-methionine sulfoximine (MSO), 3-[(3-cholamidopropyl)dimethyl-amonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT) and Coomassie Brilliant Blue (CBB) were obtained from Sigma (St. Louis, MO, USA). Urea and SYPRO Ruby Protein Stain were from Bio-Rad Laboratories (Hercules, CA, USA). Immobilized pH gradient (IPG) strips and buffer (pH 3-10, nonlinear) were from Amersham Biosciences (Little Chalfont, England), and L-[2,3,3,4,4-$^2H_5$]glutamine from Cambridge Isotope Laboratories (Andover, MA, USA).

Measurement of Caco-2 cell growth

To assess the effect of glutamine on cell growth, Caco-2 cells (passage 12 to 22) were seeded at a low density (2800 cells/cm^2) in T25 flasks (Corning, Aston, MA, USA) in DMEM containing 4.0 mM glutamine, supplemented with 20% (v/v) FCS, 1% (v/v) nonessential amino acid solution, 100 units/mL penicillin and 100 μg/mL streptomycin. Cells were maintained in culture at 37 ºC in a humidified atmosphere of 5% CO_2/95% O_2. One day after seeding, cells were washed with phosphate-buffered saline (PBS) and subsequently divided into four groups. The fist group received experimental medium, *i.e.* glutamine-free DMEM with 1% (v/v) nonessential amino acid solution, 100 units/mL penicillin, 100 μg/mL

streptomycin and 1% (v/v) SITE+3 Liquid Media Supplement as a substitute of FCS. The second group received the same medium supplemented with 8.0 mM glutamine. Each group was then divided in a group receiving no MSO and a group receiving 10 mM MSO. To determine the cell number, the Caco-2 cells were rinsed with PBS after 0, 1, 2, 3, 4 and 5 days exposure to the experimental medium, trypsinized and counted using a Bürker-Türk counting chamber.

Preparation of Caco-2 monolayers for 2-DE

In order to study the effect of glutamine on the proteome of intestinal cells, Caco-2 cells (passages 17 to 20) were seeded at the density of $1.2x10^5$ cells/cm^2 onto 24 mm Transwell bicameral systems (Corning) with collagen-coated membranes (0.4 μm pore size, 4.7 cm^2 surface area). Cells were grown in DMEM supplemented with 20% (v/v) FCS, 1% (v/v) nonessential amino acid solution, 100 units/mL penicillin and 100 μg/mL streptomycin. Confluence of cells at approximately 6 days postseeding was determined by monitoring tight junction formation ending the paracellular diffusion of phenol red. At this point cells start their differentiation process. Cells were used for experiments after 5 days of differentiation. The final incubation periods were performed in experimental medium (described above) and a defined amount of glutamine, as detailed below.

Experimental conditions for 2-DE

Protein profiling

To determine the effects of glutamine on the proteome of Caco-2 cells (day 5 postconfluence), cells were exposed to experimental medium containing 0.0, 0.1 or 8.0 mM L-glutamine, either with or without 10.0 mM MSO at the apical side of the bicameral system for 24 h. The opposite side contained culture medium without glutamine or MSO. Protein extracts from the cells were obtained and separated by 2-DE as detailed below.

Label accumulation in proteins

To measure the accumulation rate of stable isotope-labeled glutamine in proteins, Caco-2 cells (day 5 postconfluence) were exposed to experimental medium containing 4.0 mM L-[$^2H_5$]glutamine for 0, 24, 48 and 72 h from the apical side [15]. Protein extracts from the cells were obtained and separated by 2-DE as detailed below.

Protein sample preparation from Caco-2 monolayers

Monolayers were washed three times with PBS. Proteins were isolated by scraping membranes in ice-cold PBS, and centrifuging the obtained cell suspensions at 350 x *g* for 5 min at 4°C. Cell pellets were dissolved in a cell lysis buffer containing 8 M urea, 2% (w/v) CHAPS, 65 mM DTT and 0.5% (v/v) IPG buffer (pH 3-10, nonlinear) for 2-DE. This mixture was subjected to three cycles of freeze-thawing, vortexed thoroughly and centrifuged at 20000 g for 30 min at 10°C. Supernatant was collected and stored at –80°C until further analysis. Protein concentration of the mixture was determined using a Bradford based protein assay (Bio-Rad Laboratories) [24].

2-DE

The first dimension separation of proteins was performed on an IPGphor isoelectric focusing (IEF) system (Amersham Biosciences) at 20°C using precast IPG strips (pH 3-10, 24 cm, nonlinear). 100 μg of total protein, diluted with the same lysis buffer used for sample preparation to a total volume of 450 μl, was loaded onto the IPG strips. After 12 h of rehydration at 30 V, IEF was performed according to the following protocol: 1 h at 500 V, 1 h at 1000 V, 2 h gradient from 1000 V to 8000 V, 52 kVh at 8000 V. After IEF separation, the IPG strips were equilibrated for 2 x 15 min with a buffer containing 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol and 2% (w/v) SDS. DTT (1% w/v) was added in the first step, and iodoacetamide (2.5% w/v) in the second step. After equilibration of the strips, they were placed onto 12.5% SDS-polyacrylamide gels and run for 6 h at a constant voltage of 200 V for separation in the second dimension. Gels were run simultaneously using a Protean Dodeca Cell electrophoresis chamber (Bio-Rad Laboratories) to reduce technical variability. For protein profiling, four replicates (from two independent experiments) were made for each group of Caco-2 cells subjected to either 0.0, 0.1 or 8.0 mM of glutamine, with or without MSO. These gels were stained with SYPRO Ruby Protein Stain according to the manufacturer's protocol. The proteins were visualized by gel scanning with the Molecular Imager FX (Bio-Rad Laboratories). Additional preparative gels were made with a higher protein load and stained with CBB to increase the success rate of protein identification by MALDI-TOF MS. 2-D gels for measuring label incorporation in Caco-2 proteins were also stained with CBB.

Image analysis

Examination of differentially expressed proteins was performed using the specialized image analysis software PDQuest 7.3 (Bio-Rad Laboratories). Data were normalized with respect to the total density of the gel image. Gels from samples with the same treatment from independent experiments formed one replicate group with average spot intensities. A spot was regarded as significantly differentially expressed between groups if the average spot intensity of these groups differed more than 1.4-fold and if $p<0.05$ (obtained from Student's t-test). Spots, which showed a significant change in expression levels between conditions, were excised from the gels with an automated Spot Cutter (Bio-Rad Laboratories).

MS and protein identification

Excised protein spots were subjected to tryptic in-gel digestion and MALDI-TOF MS (Waters, Manchester, UK) generating peptide mass fingerprints with MassLynx 4.0.5 (Waters) for each peptide mixture as described by Bouwman *et al.* [25]. ProteinLynx Global Server 2.0 (Waters) and the Mascot search engine (http://www.matrixscience.com) were used to search the peptide mass lists from obtained spectra against the Swiss-Prot database 48.0 (http://au.expasy.org/sprot/). One missed cleavage was allowed, carbamidomethylation was set as a fixed modification and oxidation of methionine as a variable modification. The peptide mass tolerance was set to 100 ppm and no restrictions were made for protein mass and p*I*. Mascot probability scores were calculated using 30 mass peaks of the peptide mass list with the highest signal intensity, trypsin peaks were excluded. A protein was regarded as identified with a significant Mascot probability score, *i.e.* protein scores greater than 60 ($p<0.05$) and at least four peptides, from which different forms of the same peptide were excluded, assigned to the protein. Semiquantitative measurements of label incorporation was performed as described [15,25].

RESULTS

Effect of glutamine on Caco-2 cell growth

To investigate whether the proliferation of Caco-2 cells is dependent on glutamine levels in the medium, cell growth was studied under four different conditions. Cells were exposed to experimental medium containing either no glutamine or 8.0 mM glutamine. Both these cultures were subsequently divided in a group receiving no

MSO and a group receiving 10.0 mM MSO, to inhibit *de novo* glutamine synthesis. The FCS in the culture medium was substituted by SITE+3 Liquid Media Supplement to exclude possible effects of glutamine present in the serum. When monitoring Caco-2 cell growth for a period of 5 days under the different conditions, it became obvious that cells maintained in glutamine-free medium were able to proliferate efficiently (Figure 1). However, a small, but significant decline in cell growth was observed compared to growth in glutamine-supplemented medium. In contrast, cell growth was completely impaired in glutamine-free medium supplemented with MSO. This inhibitory effect on Caco-2 cell proliferation could be partly reversed by adding 8.0 mM of glutamine to this medium (Figure 1). To summarize, the removal of glutamine from the medium for 5 days resulted in a decrease in cell number of 17%. The removal of glutamine together with the addition of MSO resulted in a decreased cell growth of 92%. In the presence of glutamine this decrease was only 53%.

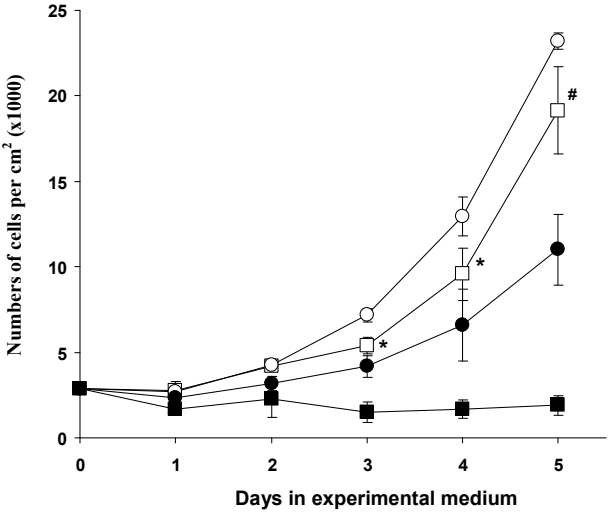


Figure 1. Effect of glutamine and MSO on Caco-2 cell proliferation. The cell number was monitored after exposing Caco-2 cells to experimental medium with 8.0 mM glutamine (circles) or without glutamine (squares), in the absence (open symbols) or the presence (closed symbols) of MSO for 1, 2, 3, 4 and 5 days. Data represent mean ± SD of three independent experiments. * $p<0.05$ and # $p=0.05$, if compared to cells treated with glutamine and without MSO (obtained from Student's t-test).

Effect of glutamine on protein expression profiles of Caco-2 cells

In order to detect glutamine-dependent changes in the protein expression of intestinal cells, 5-day-differentiated Caco-2 cells were exposed to experimental

medium containing 0.0, 0.1 and 8.0 mM glutamine with or without MSO, from the apical side for 24 h. Subsequently, proteins were isolated from the cells and separated by 2-DE. By staining gels with fluorescent SYPRO Ruby protein stain, approximately 1500 spots were detected per gel within a pH range of 3 to 10 and a molecular mass range of 10 to 100 kDa. The image analysis software PDQuest was used to discover statistically significant changes in protein expression (of at least 1.4-fold) between the different conditions. An example of a 2-D gel, in which the differentially expressed spots are indicated with an arrow, is shown in Figure 2. A list of the identified differentially expressed proteins together with the changes in expression patterns due to different glutamine treatment are displayed in Table 1.

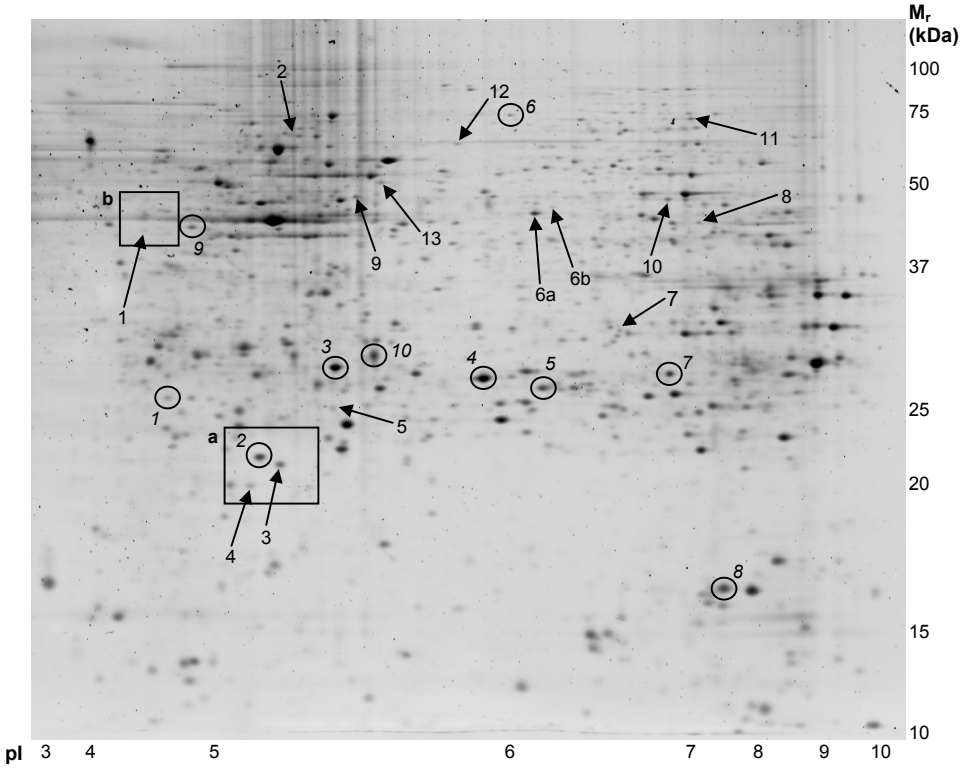
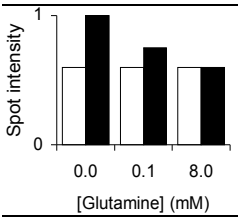
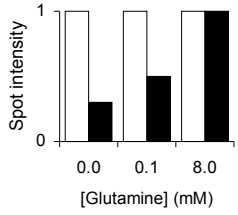
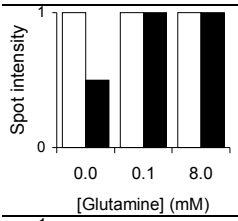
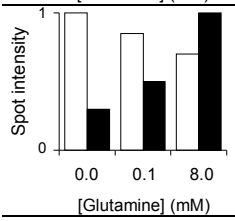


Figure 2. Example of a 2-D pattern of proteins extracted from 5-day-differentiated Caco-2 cells. The gel was stained with SYPRO Ruby Protein Stain. Expression levels of protein spots indicated with an arrow were changed between the different conditions (0.0, 0.1 and 8.0 mM glutamine, with or without MSO) and their identity is depicted in Table 1. The incorporation of stable isotope-labeled glutamine was assessed in differentially expressed and not-differentially expressed proteins (encircled spots) in a separate experiment (data shown in Table 2). The sections indicated with a and b are enlarged in Figure 3.

Table 1. Expression patterns and identity of significantly changed protein spots (see Figure 2, arrows). In the first column spot intensities of proteins from Caco-2 cells with or without MSO treatment, represented by black bars and white bars, respectively, are shown. The last column indicates the fold change in protein expression between Caco-2 cells treated with 0.0 mM glutamine and cells treated with 0.0 mM glutamine plus MSO. * $p<0.05$, ** $p<0.01$, obtained from Student's t-test.

Pattern	Spot	Swiss-Prot accession number	Protein name	Fold change
	1	Q15293	Reticulocalbin 1	2.05*
	2	P61978	Heterogeneous nuclear ribonucleoprotein K	1.43*
	3	P02753	Plasma retinol-binding protein	-4.29**
	4	Q13185	Chromobox protein homolog 3	-1.47**
	5	P02647	Apolipoprotein A-I	-2.80*
	6a	P04181	Ornithine aminotransferase, mit.	-1.90*
	6b	P04181	Ornithine aminotransferase, mit.	-2.90*
	7	Q9ULC5	Long-chain-fatty-acid-CoA ligase 5	-2.52*
	8	P11310	Acyl-CoA dehydrogenase, medium-chain specific, mit.	-1.89*
	9	P35900	Keratin, type I cytoskeletal 20	-2.07*
	10	P54868	HMG-CoA synthase, mit.	-2.04**
	11	Q16822	Phosphoenolpyruvate carboxykinase, mit. [GTP]	-2.04*
	12	P17987	T-complex protein 1, alpha subunit	-1.56*
	13	P02679	Fibrinogen gamma chain	-2.47**

Mit., mitochondrial

When comparing the profiles of Caco-2 cells treated with different concentrations of only glutamine, a significant change was observed for one protein spot (spot 13, Figure 2, Table 1). The expression level of this protein was decreased with high glutamine concentrations in the medium. More changes were found when comparing the profiles of the MSO-treated Caco-2 cells with the corresponding profiles of cells not treated with MSO, especially when glutamine was absent from the medium. Under the glutamine-depleted condition (0.0 mM glutamine and 10.0

mM MSO in experimental medium) two proteins were up-regulated and twelve proteins were down-regulated. By adding glutamine to this medium, the observed changes became smaller and even disappeared depending on the concentration of glutamine present in the medium (Table 1). Two small sections (Figure 2, a and b) with typical examples of differentially expressed protein spots are shown in Figures 3A and 3B.

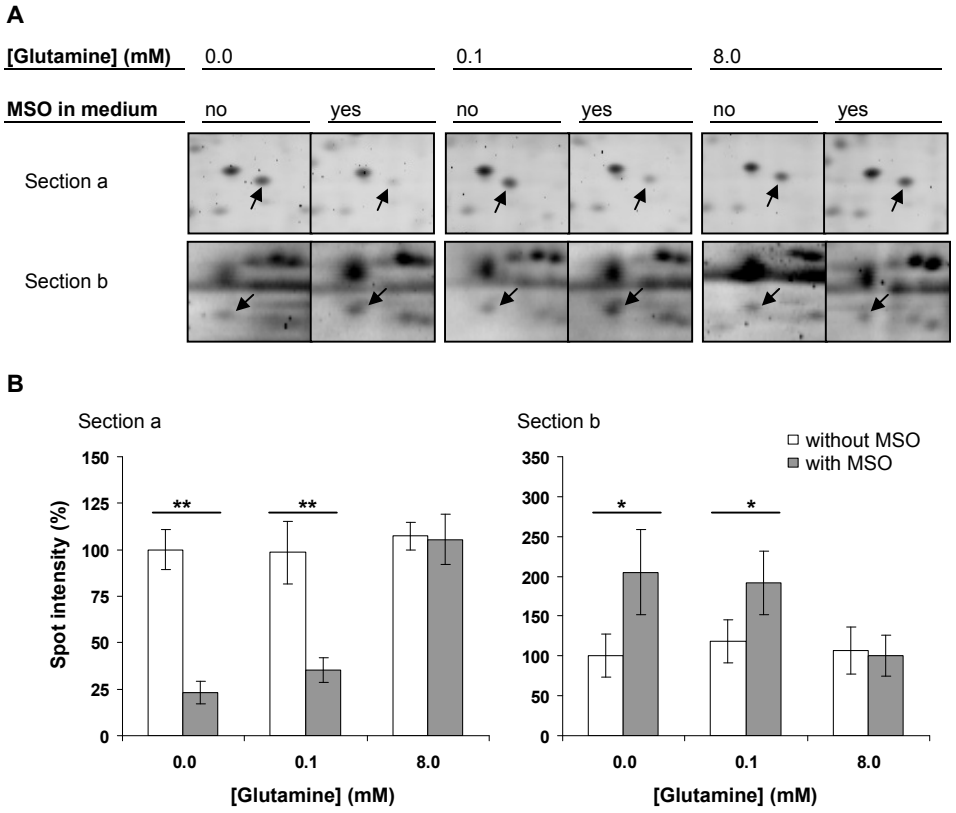


Figure 3. A. Examples of differentially expressed protein spots (section a and section b in Figure 2). Spot 3 in section a (plasma RBP) shows a decrease in intensity in the glutamine-deprived state. When glutamine is supplied to the medium at high concentration (8.0 mM) the spot intensity returned to normal levels. Spot 1 in section b (reticulocalbin 1) shows an increase in intensity caused by glutamine deprivation, which disappears when 8.0 mM glutamine is added to the medium. **B.** Graphical presentation of the expression patterns of spots depicted in A. The average spot intensity of the condition 0.0 mM without MSO is set to 100%. Data represent mean ± SD of four replicate gels from two independent experiments. * $p<0.05$ and ** $p<0.01$, obtained from Student's t-test.

To investigate whether the observed effects on the proteome of Caco-2 cells were specific for glutamine, the expression levels of those fourteen protein spots were analyzed in 2-D gels of 5-day-differentiated Caco-2 cells subjected to arginine-free

culture conditions, as its removal from the culture medium also prevented Caco-2 cell growth [26]. The proteins were not significantly differentially expressed in Caco-2 cells maintained in arginine-free versus arginine-supplemented medium for 24 h. One example of a protein spot under the different culture conditions is shown in detail in Figures 4A and 4B. This indicated protein spot was identified as plasma retinol-binding protein (RBP) and contains 201 amino acids, from which 4.0% is glutamine and 7.5% is arginine. In contrast to the glutamine conditions, the average spot intensities of this protein are not significantly different between the applied conditions of 0.0 mM arginine and 2.0 mM arginine in the medium.

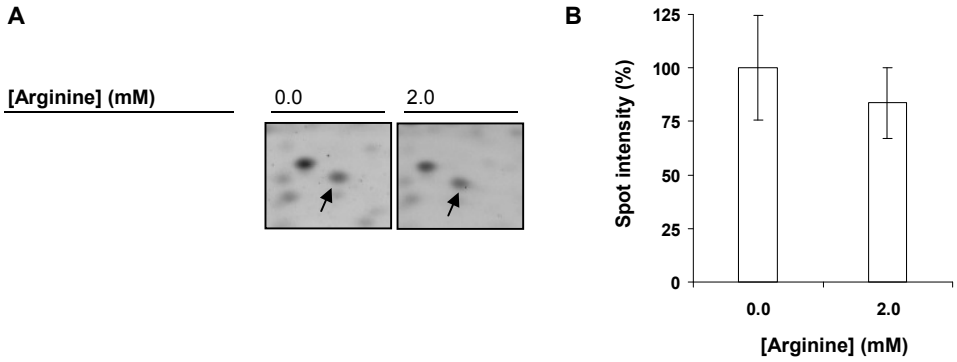


Figure 4. A. Spot 3 in section a (plasma RBP) of Figure 2 in arginine-depleted (0.0 mM) and arginine-supplemented (2.0 mM) conditions. **B.** Graphical presentation of the expression patterns of spot depicted in A. The average spot intensity of the condition 0.0 mM arginine is set to 100%, and this intensity is not significantly different between both experimental groups. Data represent mean ± SD of four replicate gels from two independent experiments.

Semiquantitative determination of glutamine accumulation as a measure for protein synthesis

Since glutamine has a role as protein monomeric unit, we hypothesized that a lack of this amino acid could lead to a decrease of the corresponding charged tRNA pool, which subsequently causes a general decrease in protein synthesis. This phenomenon would be more rapidly visible in proteins with a high synthesis rate and a high glutamine content. To test this hypothesis, we determined the relative synthesis rate of individual proteins by incubating 5-day-differentiated Caco-2 cells with medium containing stable isotope-labeled L-[$^2H_5$]glutamine for 0, 24, 48 and 72 h before the proteins were isolated and separated by 2-DE. Scanned images of the gels were matched to the images of the 2-DE experiment described in the previous section. Protein spots that showed a change in expression levels due to a different glutamine treatment, were now excised from the 'labeled' gels at each time point (*i.e.* 0, 24, 48 and 72 h). As a control, eight protein spots that did not

show any change in expression levels after different glutamine treatment (encircled spots in Figure 2), were also excised from each 'labeled' gel. To generate mass spectra and to obtain the protein identifications, all spots were subjected to MALDI-TOF MS (see data Table 2). Two protein identifications with a low Mascot score were confirmed using LC-MS/MS [27]. MALDI-TOF mass spectra were analyzed at high resolution and semiquantitative measurement of label accumulation was assessed, resulting in the enlisted peak ratios. The percentage of glutamine and arginine molecules present in the amino acid sequence of each protein was also calculated. The data demonstrate that the differential change in protein expression is not correlated with the molecular mass or the glutamine content of that protein and, interestingly, also not with the rate of protein synthesis. Therefore, the effects of glutamine depletion are apparently caused by a gene-specific molecular mechanism.

For example, premature ovarian failure 1B (spot *6*) shows a relatively high incorporation rate of stable isotope-labeled glutamine, which equals that of ornithine aminotransferase (OAT) (spot 6a), suggesting that both proteins are newly synthesized at a similar rate. Although the protein sequence of premature ovarian failure 1B is longer and has a higher percentage of glutamine than OAT, its expression level remains constant in the glutamine-depleted state in contrast to OAT. More proteins, like heat shock cognate 71-kDa protein and cathepsin D, showed a high rate of label incorporation [15] and are not significantly differentially expressed under glutamine-depleted conditions. Thus, the incorporation rate of glutamine does not determine the change in protein expression levels.

DISCUSSION

Optimal growth and differentiation of intestinal cells in culture requires sufficient glutamine levels in the medium [28]. In our experiments we observed that cellular growth was modestly reduced when glutamine was removed from the medium. These results are in agreement with a study of Le Bacquer *et al.* [29], where it was shown that Caco-2 cells were able to maintain normal intracellular glutamine concentrations when grown in glutamine-free medium for up to 20 days. However, in that study a small amount of glutamine (0.1 mM) was present in the medium, due to the FCS added to the culture medium. We excluded the possible effects of serum-derived glutamine in the current study by using the serum replacer SITE+3 Liquid Media Supplement. Our data clearly show that Caco-2 cells maintain their proliferation properties under glutamine-free medium conditions, which indicates an endogenous glutamine synthesis capacity sufficient to proceed with important physiological processes under glutamine-deprived conditions.

Table 2. List of identified differentially expressed and not-differentially expressed proteins, indicated with arrows and circles, respectively, in Figure 2. Semiquantitative analysis of glutamine-containing peptides and the corresponding isotopomer peaks at high resolution resulted in peak ratios as depicted.

Protein ID						*m/z*[a]	Peak ratio (x100%)			Protein sequence		
Spot	**Swiss-Prot accession number**	**Protein name**	**Mas-cot score**	**Seq. cov. (%)**	**MP**		**24 h**	**48 h**	**72 h**	**M_r (kDa)**	**Gln (%)**	**Arg (%)**
Differentially expressed proteins												
1	Q15293	Reticulocalbin 1	35[b]	18	5	969	31.8	34.1	45.2	39	3.0	6.9
2	P61978	HnRNP K	74	19	8	1579	7.9	25.4	22.9	51	4.5	7.8
3	P02753	Plasma RBP	65	20	4	/	/	/	/	23	4.0	7.5
4	Q13185	Chromobox protein homolog 3	31[c]	20	4	1524	38.4	56.8	72.0	21	2.7	4.4
5	P02647	Apolipoprotein A-I	124	44	11	1283	138.6	118.7	95.9	31	7.1	6.4
6a	P04181	OAT, mit.	192	42	13	1560	18.7	37.7	46.9	49	2.1	4.8
6b	P04181	OAT, mit.	82	24	8	/	/	/	/	49	2.1	4.8
7	Q9ULC5	Long-chain-fatty acid-CoA ligase 5	92	19	10	1565	13.1	13.5	15.6	76	4.1	3.7
8	P11310	Acyl-CoA dehydrogenase, medium-chain specific, mit.	101	26	9	1465	23.0	30.7	34.3	47	4.8	6.2
9	P35900	Keratin, type I cytoskeletal 20	177	15	18	1164	15.5	20.6	33.1	48	7.8	6.8
10	P54868	HMG-CoA synthase, mit.	70	19	8	1052	9.5	15.5	24.5	57	4.7	5.5
11	Q16822	Phosphoenolpyruvate carboxykinase, mit. [GTP]	160	26	16	952	0.0	8.8	7.7	71	3.6	7.2
12	P17987	T-complex protein 1, alpha subunit	112	23	11	1411	5.4	9.8	15.3	60	3.1	4.9
13	P02679	Fibrinogen gamma chain	210	50	16	1682	72.2	84.5	/	52	5.3	2.6
Not-differentially expressed proteins												
1	P50990	T-complex protein 1, theta subunit[d]	100	21	10	1158	3.7	8.5	10.9	59	2.7	3.3
2	O75947	ATP synthase D chain, mit.	77	48	6	1624	3.0	12.1	9.7	18	3.1	1.9
3	P35232	Prohibitin	80	24	6	1396	5.7	8.9	12.8	30	5.5	7.0
4	P30084	Enoyl-CoA hydratase, mit.	113	31	7	1466	7.0	11.5	17.8	31	4.1	4.1
5	O95571	ETHE1 protein, mit.	86	40	8	1796	4.4	8.6	11.6	28	4.3	7.5
6	Q5H9E9	Premature ovarian failure, 1B	109[e]	22	11	1193	19.5	30.1	43.6	68	9.7	4.8
7	P18669	Phosphoglycerate mutase 1	137	49	8	/	/	/	/	29	3.2	6.7
8	P62937	Peptidyl-prolyl cis-trans isomerase A	84	32	6	1598	8.1	12.1	15.2	18	1.8	3.7
9	P11142	HSC 71-kDa protein[d]	74	14	7	1081	/	/	44.7	71	4.0	4.3
10	P07339	Cathepsin D[d]	76	20	6	1601	/	/	42.0	45	4.1	2.9

Seq. cov., sequence coverage; MP, matched peptides; gln, glutamine; arg, arginine; hnRNP, heterogeneous ribonucleoprotein; mit., mitochondrial; HSC, heat shock cognate

a) *M/z* of glutamine-containing peptide peak of MALDI-TOF mass spectrum

b) Data confirmed by LC-MS/MS, matched peptides: T**FDQLTPDESK**, **AADLNGDLTATR**, **YIFDNVAK** and **ISWEEYK** with mass error -0.06, 0.05, 0.56, 0.44 respectively, charge +1 and Mascot score 89

c) Data confirmed by LC-MS/MS, matched peptides: **DSDEADLVLAK**, **WKDSDEADLVLAK** and **LTWHSCPEDEAQ** with mass error -0.01, -0.33 and -0.31 respectively, charge +1 and Mascot score 154

d) C-terminal part of protein

e) Searched against NCBInr Database 20050907

This is supported by the fact that addition of 10 mM MSO, an irreversible inhibitor of glutamine synthetase [30] and thereby of *de novo* glutamine synthesis [29], to the glutamine-free culture medium resulted in a complete inhibition of cell growth. This effect was counteracted by the presence of exogenous glutamine and prompted us to use MSO in the present study to bring the cells in a severe glutamine-depleted state.

MSO was found to down-regulate total protein synthesis in Caco-2 cells [29]. On the other hand glutamine deprivation (with MSO) enhanced the production of the pro-inflammatory cytokine IL-8 by Caco-2 cells after LPS stimulation [31,32]. These findings suggest that glutamine may either up- or down-regulate the synthesis of proteins in a general and a gene-specific manner. The present study was conducted to discover such proteins whose expression is altered in a glutamine-dependent gene-specific manner and thereby provide molecular insight in the cellular processes which are responsible for the beneficial effects of glutamine on the intestine.

By using a wide-screen proteomics approach, combining 2-DE with MALDI-TOF MS analysis, we observed glutamine-mediated regulation of protein expression in intestinal cells. Two out of approximately 750 properly matched proteins showed an increase in expression levels, whereas twelve proteins showed a decrease when MSO was added to glutamine-free experimental medium. Addition of glutamine to this medium normalized their expression levels. This indicates that the effects on the proteome level were due to the inhibition of glutamine synthetase and not to any other effect of MSO. Data of our study confirm also that the differential change in expression level is not correlated with the glutamine content or the relative synthesis rate of the proteins. Moreover, proteins whose expression was altered in a glutamine-dependent manner, were not changed when cells were starved for arginine. These findings together show that the changes in protein expression are not due to a lack of glutaminyl-tRNA for normal protein synthesis or a common effect of single amino acid deprivation, and thus a more specific, yet undefined, regulation is involved.

Regulation of gene expression by individual amino acids was already described for several genes, for example *CHOP*, a CCAAT/enhancer-binding protein [C/EBP]-related gene [33], and asparagine synthetase [34]. The promotor regions of these genes contain similar amino acid response element (AARE) consensus sequences, essential for the transcriptional regulation by amino acids [35]. In contrast, neither AAREs nor nutrient-sensing response elements could be identified on the proximal promotor of the argininosuccinate synthetase gene whose expression was found to be regulated by glutamine in Caco-2 cells via modification of the Sp1 transcription factor [36]. Hence, cells possess more ways by which protein expression can be regulated in response to amino acid deprivation.

Since the intestinal glutamine synthetase activity is shown to be very low in humans [16], MSO-treated Caco-2 cells may resemble the exogenous glutamine-dependency of the *in vivo* intestinal situation more closely. In addition, in some clinical circumstances like injury, sepsis, inflammation or extremely low birth weight infants there is excess of glutamine utilization by body tissues and the demand for glutamine cannot be fully met by *de novo* synthesis and normal dietary intake, which often is impossible under those conditions [3]. This results in a glutamine-deficient state in the intestine. The differentially expressed proteins observed here provide interesting leads to explain the beneficial effects of glutamine supplementation during such catabolic states, in particular plasma RBP, OAT, apolipoprotein AI (apoA-I), mitochondrial 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase and long-chain fatty-acid-CoA ligase 5.

Epithelial cells of the small intestine are known to require vitamin A, derived from retinyl esters in the diet, for maintenance of normal differentiated function and epithelial integrity [37]. Plasma RBP is a retinol-specific transport protein present in plasma and primarily synthesized in the liver. More sites of RBP synthesis have been reported, which include kidney, adipose, lacrimal gland, retinal pigment epithelium, testes, and brain [38]. The stomach contains RBP mRNA, but it is not determined whether the protein is synthesized and secreted [39]. Interestingly, we isolated the protein from Caco-2 cells and data of a study performed in rats suggest that the brush border of the intestinal mucosa contains a receptor that recognizes retinol-RBP complexes [40]. This may be an indication for RBP to be secreted in the gut lumen and this secretion may be metabolically significant to fulfill the vitamin A requirement of the intestine. In this respect, the glutamine-dependent regulated expression of this protein may contribute to the beneficial effects of glutamine on the small intestine.

Glutamine from the diet is metabolized extensively in the mucosa of the small intestine and glutamine-derived carbon is found mainly in CO_2, but also in other molecules like ornithine [41,42]. OAT is a key enzyme, present predominantly in the small intestine, and is involved in the conversion of glutamine to ornithine. The expression of this protein is down-regulated in glutamine-deprived Caco-2 cells resulting probably in a decreased amount of ornithine in the intestinal cells. Ornithine is an immediate precursor for synthesis of polyamines, molecules known for their involvement in cell proliferation, cell differentiation and repair of intestinal cells [43]. In a porcine jejunal enterocyte cell line, glutamine could stimulate ornithine decarboxylase activity, the rate-limiting enzyme in polyamine biosynthesis, in a dose- and time-dependent manner [9]. Recent findings of a study in *in vitro* intestinal epithelial cells suggest that polyamines are necessary for maintaining the normal structure of the tight junctions and maintenance of the intestinal epithelial barrier [44]. Ornithine is also a precursor for arginine. The

small intestine plays an important role in the endogenous synthesis of arginine, because pyrroline-5-carboxylate synthase (one step ahead of OAT in the formation of ornithine from glutamine) is almost exclusively located in the intestinal mucosa [45,46]. Arginine is the physiologic precursor of NO, which plays an important role in regulating intestinal integrity [47]. Therefore, the supply of glutamine to the intestine could promote the health and functioning in the intestine in two ways via ornithine.

Another health-promoting protein found to be regulated by glutamine is apoA-I, primarily synthesized in the small intestine and the liver [48,49] and a major protein component of the high-density lipoprotein (HDL) particles. HDLs are mainly involved in the removal of cholesterol from extrahepatic tissues, and exhibit antiatherogenic properties [50]. However, other functions not linked to their role in cholesterol transport are described, also in the intestine. Reconstituted HDL particles are protective in experimental colitis in rats, probably by its inhibitory effect on TNF-alpha and interleukin-1 beta formation in the intestine [51]. In this view, sufficient intestinal apoA-I production may have multiple beneficial effects. Research has already focused on the regulation of hepatic and intestinal apoA-I expression in response to dietary stimuli, including amino acids [52-54], and fasting [55]. To our knowledge, this study is the first to report that intestinal apoA-I protein expression is reduced under glutamine-depleted conditions.

Mitochondrial HMG-CoA synthase is a control enzyme in ketogenesis and catalyzes the condensation of acetoacetyl-CoA and acetyl-CoA to produce HMG-CoA in addition to free CoA. The HMG-CoA is then converted, through the actions of HMG-CoA lyase and D-3-hydroxybutyrate dehydrogenase, into the ketone bodies acetoacetate and ß-hydroxybutyrate, which are used as a source of oxidative fuels in several nonhepatic tissues [56], including the small intestine [1]. A study in isolated rat jejunal cells showed that equimolar concentrations of acetoacetate or 3-hydroxybutyrate have a similar stimulatory effect on protein synthesis as glutamine, and the effect most likely represents the provision of energy [57]. Sufficient glutamine levels in the intestine seem to maintain the level of HMG-CoA synthase and thereby the formation of ketone bodies.

The mRNA of long-chain fatty-acid-CoA ligase 5, also known as acyl-CoA synthetase 5, is predominantly present in the small intestine, mainly in the villous epithelium [58]. Recently, evidence was provided that the acyl-CoA synthetase 5 status reflects the state of the villous architecture and epithelial homeostasis in the human small intestine [58]. Therefore, reduced levels of acyl-CoA synthetase 5 in Caco-2 cells submitted to glutamine-depletion may point to a disturbed villous morphology.

The expression level of mitochondrial phosphoenolpyruvate carboxykinase (PEPCK), a major control gene of the gluconeogenesis catalyzing the conversion

of oxaloacetate to phosphoenolpyruvate, is decreased in glutamine-depleted conditions. The gene transcript is strongly expressed in liver and kidney, but also in small intestine of humans [59]. Glutamine is the main gluconeogenic substrate in this tissue [60]. However, it must be noted that especially the role of cytosolic PEPCK is studied, and not of the mitochondrial form that is shown to be constitutively expressed and not inducible by glucagon [61]. The reduced PEPCK expression in Caco-2 cells in glutamine-deprived conditions may come from the reduced amount of available substrate and could lead to a decreased supply of energy to the cells.

The function of other proteins we found to be specifically regulated by glutamine in Caco-2 cells in relation to intestinal functioning is currently unknown. These proteins are acyl-CoA dehydrogenase (medium-chain specific), reticulocalbin 1, heterogeneous nuclear ribonucleoprotein K, chromobox protein homolog 3, keratin, T-complex protein 1 (alpha subunit) and fibrinogen gamma chain. Additional research is needed to find out the exact mechanisms involved in the glutamine-dependent regulation of these and other identified proteins and their relevance for the *in vivo* situation.

In conclusion, the use of the present proteomics approach allowed us to explore the glutamine-enterocyte interactions under strictly controlled conditions. Data of this study clearly demonstrated that glutamine regulates the expression of proteins in the Caco-2 cells in a gene-specific manner. Proteins with potential health-promoting effects on the intestine are plasma RBP, OAT, apoA-I, mitochondrial HMG-CoA synthase and acyl-CoA synthetase 5.

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CHAPTER 4

ARGININE DEFICIENCY IN PRECONFLUENT INTESTINAL CACO-2 CELLS MODULATES EXPRESSION OF PROTEINS INVOLVED IN PROLIFERATION, APOPTOSIS, AND HEAT SHOCK RESPONSE

Kaatje Lenaerts, Johan Renes, Freek Bouwman, Jean-Paul Noben, Johan Robben, Egbert Smit and Edwin Mariman

Proteomics, in press

ABSTRACT

Arginine is classified as a conditionally essential amino acid required exogenously during catabolic disease states and periods of rapid growth, both characterized by increased arginine utilization. Arginine plays an important role in the intestine, where it is extensively metabolized, and enhances its immune-supportive function and mucosal repair. Cell proliferation is important for the latter process. This study aimed for a better molecular insight in the response to arginine deprivation/supplementation of preconfluent and 5-day-confluent, differentiated Caco-2 intestinal cells. The potential of citrulline to counteract the effects of arginine deprivation was investigated in preconfluent cells. Two-dimensional gel electrophoresis combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, and the antibody microarray technology were applied. Evidence is provided that arginine deficiency modulates the protein expression profiles of preconfluent Caco-2 cells differently than of postconfluent differentiated cells. In preconfluent cells, certain proteins changed in direct response to arginine deficiency, whereas other proteins did not, but instead responded during the recovery phase after an arginine/citrulline resupplementation. The protein changes suggest that arginine deprivation decreases cell proliferation and heat shock protein expression, and enhances the cell's susceptibility to apoptosis. These processes are critical for proper cell function, and hence a state of arginine deficiency can be detrimental for intestinal cells which proliferate actively *in vivo*.

INTRODUCTION

Arginine is an important molecule for the human body and is involved in many metabolic processes. This amino acid is considered to be nonessential to healthy humans and adult mammals, since the body can synthesize sufficient amounts of arginine. Some conditions, however, are characterized by increased arginine utilization, for instance, periods of rapid growth and catabolic disease states. A condition of arginine deficiency may occur when the requirements of this amino acid are not fulfilled [1, 2]. This has led to classifying arginine as a conditionally essential amino acid.

Arginine in the human body comes from endogenous synthesis (intestinal-renal axis: conversion of ornithine to citrulline in the intestine and citrulline to arginine in the kidney), diet, and protein breakdown [3]. The amino acid can be metabolized via several enzymes thereby producing essential molecules for normal cell growth and function, like nitric oxide (NO) via NO synthase (NOS), creatine via arginine:glycine amidinotransferase, agmatine via arginine decarboxylase, arginyl-tRNA for protein synthesis via arginyl-tRNA synthetase, and ornithine via arginase [4]. During first-pass intestinal metabolism, approximately 40% of the arginine absorbed from the intestinal lumen is converted into the intestinal mucosa due to arginase activity [5].

Arginine or its metabolites can act as signaling molecules and it has been described that arginine availability can influence the expression of specific genes, especially of those which are involved in arginine metabolism, like argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). When arginine is present in excess in the culture medium, cellular levels of those enzymes are repressed [6]. Expression of inducible NOS (iNOS) is reduced when extracellular arginine availability is limited [7]. The regulation of protein expression by arginine availability is, however, not restricted to genes involved in arginine metabolism. For instance, expression of the zeta-chain of the T-cell antigen receptor is reduced when arginine levels are decreased [8].

The influence of arginine has been studied mostly in relation to immunity and inflammation including its immune-supportive function in the intestine [9]. Arginine exerts beneficial effects on the intestinal mucosal repair after ischemic damage in rats [10]. Furthermore, arginine transport in intestinal epithelial cells is shown to be increased by lipopolysaccharide, probably reflecting its necessity under such conditions [11]. Many more studies regarding the function of arginine in the intestine have been performed and various beneficial effects have been ascribed to arginine [12]. However, the importance of this amino acid *per se* for the intestinal epithelial cells is yet unclear and molecular effects of arginine deficiency on intestinal cells remain largely unknown.

The present study aimed to determine molecular adaptations in intestinal cells induced by arginine deficiency. An additional issue addressed in this study is the potential of arginine, and its precursor citrulline to normalize changes observed after arginine deficiency. A comparative proteomics approach, including two-dimensional gel electrophoresis (2-DE) combined with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), and the antibody (Ab) microarray technology, was applied.
Human colonic carcinoma Caco-2 cells were used as a model for the small intestine, as they undergo spontaneous *in vitro* epithelial differentiation [13]. Caco-2 cells show arginase activity under normal culture conditions when arginine is present in the medium [14]. Like normal intestinal cells, Caco-2 cells express neuronal NOS (nNOS), a so-called constitutive NOS isoform, and iNOS [15, 16]. In addition, this cell model was previously applied in studies examining cellular mechanisms affected by deprivation of nutrients [17, 18]. We have used the cells in proliferating as well as in differentiating state, because it was shown that the arginine uptake in Caco-2 cells was considerably reduced with the number of days postseeding [19]. As arginine uptake is higher in preconfluent cells, and arginine is shown to be beneficial in intestinal repair, a process in which intestinal cell proliferation is very important, we hypothesized that the effects of arginine deficiency are more profound in the preconfluent, proliferating cell population.

MATERIALS AND METHODS

Materials

The human colon carcinoma cell line Caco-2 was obtained from the American Type Culture Collection (Rockville, MD, USA). Dulbecco's modified Eagles medium (DMEM) and supplements for cell culture were purchased from Invitrogen (Carlsbad, CA, USA), except fetal calf serum (FCS) which was from Bodinco (Alkmaar, The Netherlands) and SITE+3 Liquid Media Supplement which was obtained from Sigma (St. Louis, MO, USA). Urea, SYPRO Ruby Protein Stain and all the reagents for SDS-PAGE and blotting were from Bio-Rad Laboratories (Hercules, CA, USA). Immobilized pH gradient strips and buffer (pH 3-10, nonlinear), Cy3 and Cy5 fluorescent dyes, PD-10 Columns and Hybond ECL nitrocellulose membrane were from Amersham Biosciences (Little Chalfont, England). The Ab microarray 500 was obtained from Clontech BD (Mountain View, CA, USA). Cellular apoptosis susceptibility protein (CAS) and HSF (heat shock transcription factor)-4 Ab were obtained from Pharmingen (BD

Biosciences, San Diego, CA, USA). Monoclonal anti-beta actin and all other chemicals were obtained from Sigma.

Measurement of Caco-2 cell growth

Caco-2 cells were seeded at a low density (4000 cells/cm^2) in T25 flasks (Corning, Aston, MA, USA) in DMEM, supplemented with 20% (v/v) FCS, 1% (v/v) nonessential amino acid solution, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in culture at 37 ºC in a humidified atmosphere of 5% CO_2/95% O_2. Two days after seeding, cells were washed with phosphate-buffered saline (PBS) and divided into five groups. All groups received experimental medium, *i.e.* arginine-free DMEM with 1% (v/v) nonessential amino acid solution, 100 units/mL penicillin, 100 μg/mL streptomycin and 1% (v/v) SITE+3 Liquid Media Supplement as a substitute of FCS. One group received experimental medium supplemented with 0.4 mM arginine for 8 days. The other groups were deprived of arginine for 1, 2, 3 or 5 days after which arginine-supplemented medium was added for 8 days. To determine the cell number, the Caco-2 cells were rinsed with PBS, trypsinized, and counted using a Bürker-Türk counting chamber (time points of counting are indicated with an arrow in Figure 1A).

Preparation of differentiated Caco-2 monolayers for 2-DE and Ab microarray

Caco-2 cells were seeded at the density of 1.2x10^5 cells/cm^2 onto 24 mm Transwell bicameral systems (Corning) with collagen-coated membranes (0.4 μm pore size, 4.7 cm^2 surface area). Cells were grown in DMEM containing supplements as described above. After reaching confluence at approximately 6 days postseeding, cells start their differentiation process. Cells were used for experiments after 5 days of differentiation.

Experimental conditions

2-DE

1. Two days after seeding (at a density of 5000 cells/cm^2), Caco-2 cells were exposed to experimental medium containing 0.0 or 0.4 mM arginine for 48 h, after which proteins were isolated (described below). Cells from arginine-free cultures were exposed to medium supplemented with 0.4 mM arginine or 0.4 mM citrulline for 6 days, followed by protein isolation. Cells from the arginine-supplemented

cultures were exposed for another 6 days to medium with 0.4 mM arginine, followed by protein isolation (see Figure 2A). The latter cells reached confluence at the end of the experiment, whereas the cells that were exposed to arginine-free medium for 48 h, after which arginine or citrulline was added for 6 days, did not (comparably to the cell growth curve shown in Figure 1B). The concentration of arginine chosen for this experiment is identical to the concentration which is present in standard DMEM. Equimolar concentrations of citrulline were used to analyze the normalizing effect of citrulline in case of arginine deprivation.

2. Higher concentrations of arginine have shown to exert additional beneficial effects in intestinal cells [20]. Therefore, a wider concentration range was chosen for this experiment. Differentiated Caco-2 monolayers (described above), were hence exposed to experimental medium with 2.0, 0.2 or 0.0 mM arginine at the apical side of a bicameral system for 24 h.

Ab microarray

Differentiated Caco-2 monolayers were exposed to experimental medium containing 2.0 or 0.0 mM arginine at the apical side of a bicameral system for 24 h.

Protein sample preparation, 2-DE, image analysis, and protein identification

Protein sample preparation of Caco-2 cells and the 2-DE were performed exactly as described in Lenaerts *et al.* [17]. At least four replicate gels from two independent experiments were made per condition. Examination of differentially expressed proteins was performed using image analysis software PDQuest 7.3 (Bio-Rad Laboratories). Data were normalized with respect to the valid spot quantity per gel. Gels from samples with the same treatment from independent experiments formed one replicate group with average spot intensities. A spot was regarded as differentially expressed between groups if the average spot intensity of these groups differed at least 1.4-fold and if $p<0.05$ (Student's t-test). In addition, the influence of log-transformation of spot intensities was tested, since it converts the distribution of intensities from a non-normal distribution to a normal distribution when image analysis is done in PDQuest [21]. The outcome of both experiment changed only marginally, and initially significant *p*-values of spots that were ≥ 0.05 after log-transformation were indicated with superscript e) and d) in Table 1 and 2, respectively. Differentially expressed spots were excised from the gels with a Spot Cutter (Bio-Rad Laboratories).
Excised protein spots were subjected to tryptic in-gel digestion and MALDI-TOF MS (Waters, Manchester, UK) generating peptide mass fingerprints with MassLynx

4.0.5 (Waters) as described by Bouwman *et al.* [22]. Protein identification was performed as described in ref. [17]. Taxonomy was set to *Homo sapiens* and Mascot probability scores (http://www.matrixscience.com) were calculated using 20 mass peaks or less with highest signal intensity, and trypsin peaks were excluded. Protein identifications with a score greater than 60 and at least four matched peptides, from which different forms of the same peptide were excluded, were significant ($p<0.05$). Some proteins were identified using LC-MS/MS as described [23]. Detailed identification characteristics of protein spots are shown in the supplemental material.

Ab microarray and Western blotting

The Clontech Ab microarray consisted of 500 Ab spotted in duplicate upon a glass slide. Caco-2 monolayers were washed three times with PBS. Cells were harvested by scraping the Transwell membranes in ice-cold PBS, and centrifuging the obtained cell suspensions at 350 x *g* for 5 min at 4°C. Protein extraction, protein labeling with Cy3 and Cy5 and Ab microarray incubation were performed exactly as described by the manufacturer's protocol. Two slides were used for reverse color labeling. The slides were scanned by a ScanArray laser scanner at two wavelengths to obtain fluorescence intensities for both dyes, which were calculated by ImaGene software (BioDiscovery, Los Angeles, CA). The ratios of Cy5 to Cy3 were imported in a Microarray Analysis Workbook (developed specifically for the Ab microarray) and internally normalized ratios were calculated per Ab. A ratio <0.77 or >1.3 indicates that the protein is differentially expressed between the experimental conditions.

For confirmation of Ab microarray results, total protein (10 μg per condition) was separated by SDS-PAGE on a 10% (w/v) polyacrylamide gel at 150 V and transferred to nitrocellulose membrane for 1 h at 100 V. Blocking and Ab incubation steps of the membrane were performed in TBST (10 mM Tris-HCl, 100 mM NaCl, 0.1% Tween-20) supplemented with 5% nonfat dry milk. The membrane was incubated overnight with antimouse CAS (1:500), HSF-4 (1:700) and beta-actin (1:15000) monoclonal Ab at 4°C. After washing with TBST, the membrane was incubated with a horseradish peroxidase-conjugated secondary Ab (Dako, Glostrup, Denmark). Signals were detected by SuperSignal West Pico Chemiluminescent Substrate and CL-XPose clear blue X-ray film (Perbio Science, Etten-Leur, The Netherlands).

RESULTS

Caco-2 cell growth is dependent on arginine availability

Incubation of Caco-2 cells in arginine-free medium resulted in complete inhibition of cell proliferation, whereas cell growth was initiated again by adding arginine to the culture medium. Pretreatment of Caco-2 cells with arginine-free medium for 1 day followed by an 8-day period of arginine supplementation showed a cell growth reduction of 59% compared to control cells (Figure 1). Hence, cells are able to recover their proliferative activity in arginine-supplemented medium after an arginine-deprived period. However, the longer the deprivation period, the more difficult the proliferative activity restores (Figure 1B). Adding citrulline to the medium instead of arginine also restored cell growth, although it was less effective as arginine (data not shown).

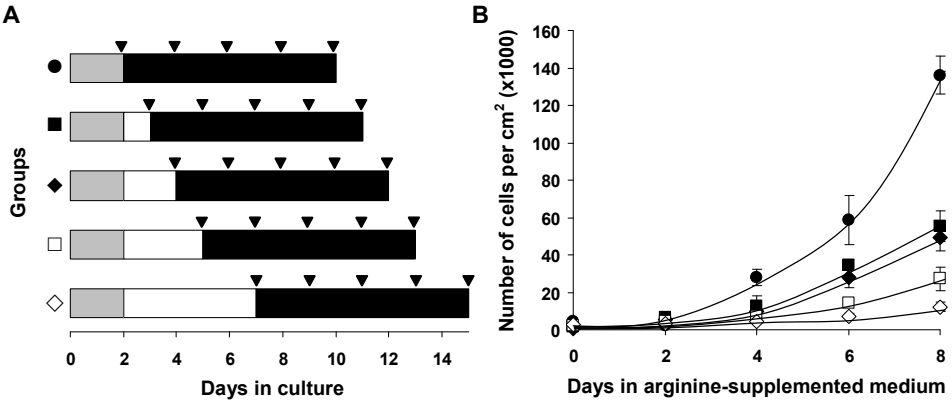


Figure 1. A. Schematic representation of the experiment to assess the effect of arginine on Caco-2 cell proliferation. Gray bars indicate the 2-day period of the cells in culture before the arginine intervention. White and black bars indicate the period in arginine-free and arginine-supplemented medium, respectively. Arrows indicate the time points at which the cell number was measured. Each group is represented by a symbol used in section B. **B.** The cell number in arginine-supplemented medium was monitored after exposing Caco-2 cells to experimental medium without arginine for 1, 2, 3 or 5 days. Data represent mean ± SD of three independent experiments.

Effect of arginine deprivation and subsequent exposure to arginine or citrulline on the protein profiles of preconfluent Caco-2 cells

Since previous results showed that Caco-2 cells were able to restart proliferation in the presence of arginine after 48 h of arginine deprivation (comparably with 24 h of arginine deprivation), this condition was selected for protein profiling. The results were compared with a condition where cells were cultured with normal arginine supply for 48 h. Protein profiles of both conditions were also evaluated

after 6 days of arginine supplementation, since cells regained their normal proliferative activity by then. The effect of citrulline supplementation after 48 h of arginine deprivation was evaluated as well. Figure 2A schematically represents the experimental set-up, whereas Figure 2B gives an overview of the 80 differentially expressed protein spots, clustered according to their change in expression pattern. We were able to identify almost 80% of these proteins, which are displayed in Table 1.

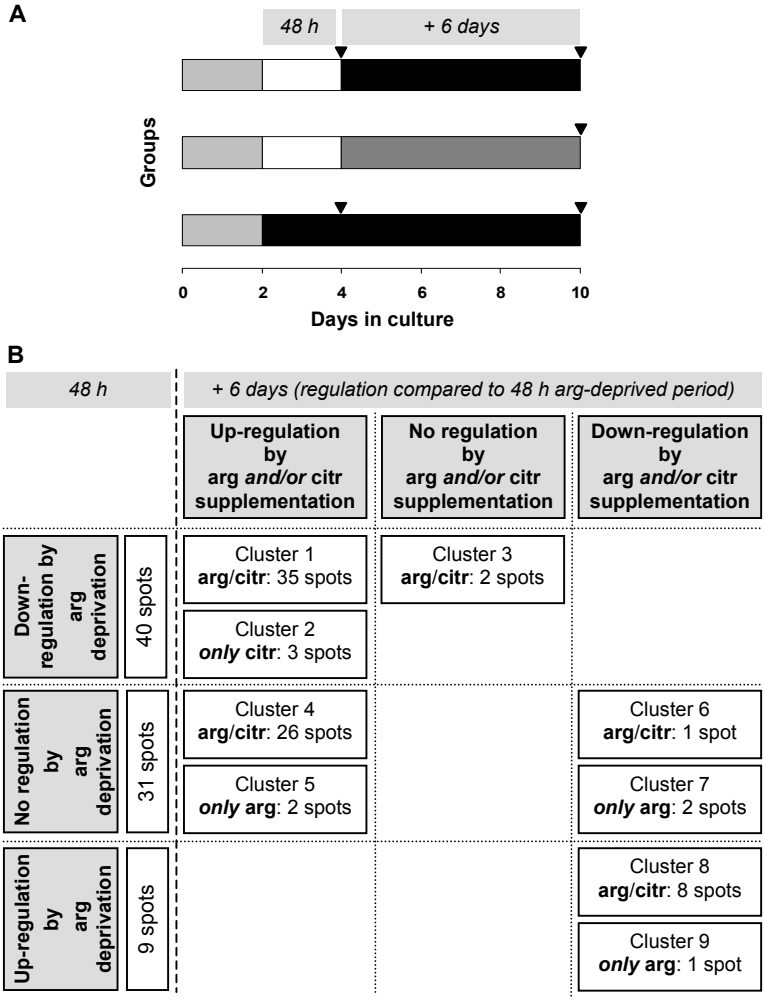


Figure 2. A. Schematic representation of the different conditions to assess the effect of arginine on the proteome of preconfluent Caco-2 cells. Light gray bars indicate the 2-day period of the cells in culture before the arginine intervention. White, black, and dark gray bars indicate the period in arginine-free medium, arginine-supplemented, and citrulline-supplemented medium, respectively. Arrows indicate the time points at which cell protein was isolated to perform 2-DE. **B.** Overview of changes in protein spot expression. Spots exhibiting similar expression patterns are clustered, and their identity is depicted in Table 1.

Table 1. Spot numbers and identity of changed protein spots in preconfluent Caco-2 cells (corresponding to Figure 3). Fold changes (FC) and *p*-values are calculated for differences in average spot intensities induced by arginine deprivation for 48 h, and differences induced by subsequent arginine and citrulline supplementation for 6 days, respectively. Spots are clustered according to the change in expression profile (Figure 2B). The last column indicates the percentage of arginine residues (R) present in the amino acid (AA) sequence.

Spot	Swiss-Prot accession number	Protein name	48 h deprivation arginine		6 days supplementation arginine		6 days supplementation citrulline		R/AA (%)
			FC	*p*	FC	*p*	FC	*p*	
Cluster 1									
4	P27797	Calreticulin[a)]	-3.0	0.010	2.6	0.008	2.7	0.003	1.9
5	P27797	Calreticulin[a)]	-1.6	0.028	2.0	0.021	2.2	0.000	1.9
14	P27797	Calreticulin[a,b)]	-1.9	0.030	2.0	0.004	2.1	0.003	1.9
50	P27797	Calreticulin[a,c)]	-2.5	0.036	2.7	0.002	2.1	0.011	1.9
7	P11021	78-kDa glucose-regulated protein[a,b)]	-3.0	0.000	3.7	0.004	4.6	0.010	4.3
11	P11142	HSC 71-kDa protein[b)]	-2.2	0.001	2.0	0.006	2.6	0.000	4.3
43	P11142	HSC 71-kDa protein[c)]	-3.3	0.017	3.7	0.001	3.3	0.024	4.3
47	P11142	HSC 71-kDa protein[c)]	-3.1	0.019	5.8	0.000	5.8	0.004	4.3
12	P08107	Heat shock 70-kDa protein 1[b)]	-2.0	0.028	2.7	0.017	3.0	0.015	4.8
3	P14625	Endoplasmin[c)]	-3.0	0.000	2.8	0.020	4.5	0.000	4.7
16	P08238	HSP 90-beta[b)]	-2.3	0.001	2.3	0.026	2.4	0.002	4.4
17	P08238	HSP 90-beta[b)]	-2.1	0.008	1.8	0.040	1.9	0.029	4.4
18	Q07955	Splicing factor arginine/serine-rich 1	-2.6	0.007	3.0	0.000	2.7	0.024	17.4
19	P12277	Creatine kinase B-type[d)]	-1.8	0.012	2.9	0.002	3.2	0.000	5.5
41	P07355	Annexin A2	-1.7	0.036[e)]	2.1	0.004	2.6	0.001	6.2
25	O95336	6-phosphogluconolactonase	-1.8	0.015	3.3	0.002	2.5	0.001	5.4
26	P30101	Protein disulfide-isomerase A3[a,c)]	-2.4	0.000	3.0	0.000	2.8	0.000	4.6
40	P30101	Protein disulfide-isomerase A3[a,b)]	-1.5	0.008	2.3	0.002	2.1	0.001	4.6
30	P00558	Phosphoglycerate kinase 1	-1.8	0.000	2.4	0.016	1.7	0.003	2.6
38	P14618	Pyruvate kinase M1/M2[c)]	-1.5	0.019	1.7	0.003	1.8	0.002	5.9
42	P04075	Fructose-bisphosphate aldolase A	-2.3	0.011	2.4	0.001	2.8	0.010	4.1
45	P22626	HnRNP A2/B1[a)]	-1.8	0.011	2.9	0.003	2.4	0.016	7.1
59	P09211	Glutathione S-transferase P[a)]	-1.6	0.006	2.0	0.002	2.7	0.008	3.8
60	Q13185	Chromobox protein homolog 3	-2.0	0.030	2.4	0.016	2.2	0.028	4.4
56	Q13185	Chromobox protein homolog 3	-2.1	0.000	2.3	0.000	1.9	0.000	4.4
54	P33316	dUTPase	-3.1	0.010	2.2	0.005	2.3	0.017	7.3
55	P55795	HnRNP H'[c)]	-2.0	0.000	3.0	0.000	2.7	0.000	6.9
51	P29043	47-kDa HSP[b)]	-1.9	0.010	2.9	0.000	2.4	0.002	4.6
Cluster 2									
20	Q07955	SF2/ASF	-1.7	0.012	1.5	0.132	2.3	0.037	17.4
34	P49368	T-complex protein 1 gamma	-1.9	0.018	1.3	0.303	1.6	0.044[e)]	6.4
Cluster 3									
10	Q9BQE3	Tubulin alpha-6 chain	-1.8	0.024	1.9	0.160	1.7	0.240	4.7
53	Q9BV57	1,2-dihydroxy-3-keto-5-methylthiopentene dioxygenase	-2.1	0.017	1.9	0.057	1.8	0.115	8.4
Cluster 4									
2	P49321	Nuclear autoantigenic sperm protein	-2.0	0.065	2.2	0.018	2.1	0.012	2.3
8	Q9UNZ2	NSFL1 cofactor p47	-1.0	0.763	2.0	0.003	2.0	0.043	7.0
9	O43707	Alpha-actinin-4[b)]	-1.3	0.309	2.2	0.015	2.9	0.025	5.6
15	P05198	eIF2alpha	-1.4	0.055	2.4	0.010	2.6	0.014	8.0
21	P12277	Creatine kinase B-type[c,d)]	-1.4	0.095	2.9	0.007	2.3	0.001	5.5
27	P12277	Creatine kinase B-type[d)]	-2.5	0.087	6.1	0.000	4.8	0.006	5.5
29	P12277	Creatine kinase B-type[d)]	-1.3	0.066	1.7	0.001	1.7	0.000	5.5
22	P30101	Protein disulfide-isomerase A3[a,c)]	-1.3	0.042	2.3	0.010	2.5	0.001	4.6

Spot	Swiss-Prot accession number	Protein name	48 h deprivation		6 days supplementation				R/AA (%)
			arginine		arginine		citrulline		
			FC	*p*	FC	*p*	FC	*p*	
23	Q9BT58	HSDL2 protein	-1.3	0.114	2.2	0.023	2.1	0.012	2.0
24	P07355	Annexin A2	-2.1	0.113	2.4	0.029[e)]	2.9	0.048[e)]	6.2
31	P06733	Alpha-enolase[a)]	-1.4	0.121	1.7	0.017	1.8	0.011	3.9
37	P06733	Alpha-enolase[a)]	-1.5	0.162	1.7	0.042[e)]	1.9	0.016	3.9
58	P50990	T-complex protein 1 theta[b)]	-1.7	0.050	2.3	0.000	1.9	0.001	3.3
57	P07858	Cathepsin B[a,d)]	1.4	0.312	3.0	0.000	2.1	0.024	4.1
49	P55795	HnRNP H'	-1.3	0.472	2.1	0.031[e)]	2.0	0.045[e)]	6.9
52	P29373	Cellular retinoic acid-binding protein 2[d)]	-1.1	0.531	2.8	0.047	2.7	0.035	5.1
48	P09651	HnRNP A1[c)]	-2.0	0.099	3.3	0.001	3.6	0.006	7.0
61	/	Tubulin alpha chain	-1.5	0.151	2.2	0.038	2.0	0.006	/
Cluster 5									
28	P07437	Tubulin beta-2 chain[a,d)]	-1.2	0.196	3.0	0.015	3.0	0.120	5.0
44	P22626	HnRNP A2/B1[a,d)]	-1.4	0.110	1.8	0.008	2.1	0.084	7.1
Cluster 7									
33	P06733	Alpha-enolase[a,d)]	1.0	0.942	-2.0	0.023	-1.5	0.127	3.9
Cluster 8									
1	P14314	Glucosidase 2 beta subunit	2.2	0.032	-2.1	0.010	-1.6	0.037	3.8
6	Q15293	Reticulocalbin-1	2.0	0.031	-1.7	0.041	-1.8	0.034	7.0
32	O00330	Pyruvate dehydrogenase protein X component, mit.	2.1	0.002	-2.3	0.001	-1.6	0.032	5.4
35	Q16822	Phosphoenolpyruvate carboxykinase [GTP], mit.	1.6	0.015	-2.3	0.003	-1.8	0.018	7.2
36	Q99541	Adipophilin[d)]	3.3	0.007	-3.8	0.006	-2.3	0.018	3.0
39	Q14376	UDP-glucose 4-epimerase	2.2	0.015	-2.1	0.030	-2.3	0.023	4.9
46	P60174	Triosephosphate isomerase[a)]	1.8	0.007	-2.4	0.001	-2.1	0.003	3.2
62	P62807	Histone H2B.a/g/h/k/l	3.7	0.002	-2.6	0.004	-2.4	0.015	6.4
Cluster 9									
13	Q01105	Protein SET	2.0	0.038	-2.4	0.019	-1.4	0.189	3.5

HSC, heat shock cognate; HSP, heat shock protein; hnRNP, heterogeneous nuclear ribonucleoprotein; mit., mitochondrial

a) more spots of protein present in 2-D pattern that are not regulated by arginine

b) C-terminal peptide

c) N-terminal peptide

d) spot influenced by proliferation and/or differentiation

e) $p \geq 0.05$ after log-transformation of spot intensities

Spot numbers of identified differentially expressed proteins correspond to those indicated in Figure 3, a typical 2-D pattern of preconfluent Caco-2 cells. Fold changes and *p*-values were calculated to quantify the effect of arginine withdrawal and subsequent arginine and citrulline supplementation on protein expression in Caco-2 cells. Results show that most changes induced by arginine deprivation were restored to control levels when cells were afterwards supplemented with arginine or citrulline (see Figure 2B and Table 1). Figure 4 shows an example of two spots with such a reciprocal expression pattern.

The expression of 31 protein spots was not different between arginine-deprived and arginine-supplemented conditions (48 h); however, these spots showed a

difference in expression level when cells were subsequently exposed to arginine or citrulline for 6 days. Of those spots, 20 showed this change exclusively when they were deprived of arginine during the first 48 h of the treatment, whereas 11 spots changed irrespective of the presence or absence of arginine during the first 48 h (9 of these 11 spots were identified and indicated by superscript d in Table 1).

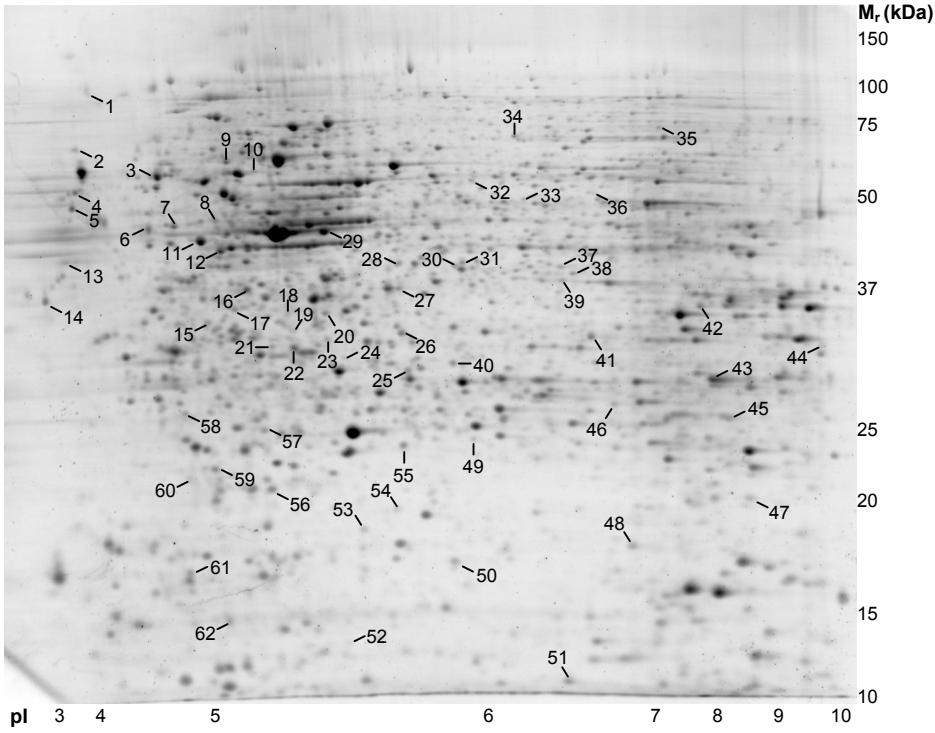


Figure 3. Example of a typical 2-D pattern of proteins extracted from preconfluent Caco-2 cells. About 1500 spots are visible within a pH range of 3-10 and a molecular mass range of 10-100 kDa. Expression levels of indicated protein spots were changed between the different conditions and their identity is depicted in Table 1.

Some of the spots that did not change by different arginine treatments were identified by matching current 2-D patterns with our published 2-D protein map of Caco-2 cells [17, 24]. When multiple spots of one protein were present in the gel (due to protein procession or modification), and did not show the same response, they were not discussed because their expression pattern is too complex (marked in Table 1 with superscript a). The percentage of arginine molecules present in the amino acid sequence of the proteins was also calculated, but was not correlated with the change in protein expression under arginine-deprived conditions.

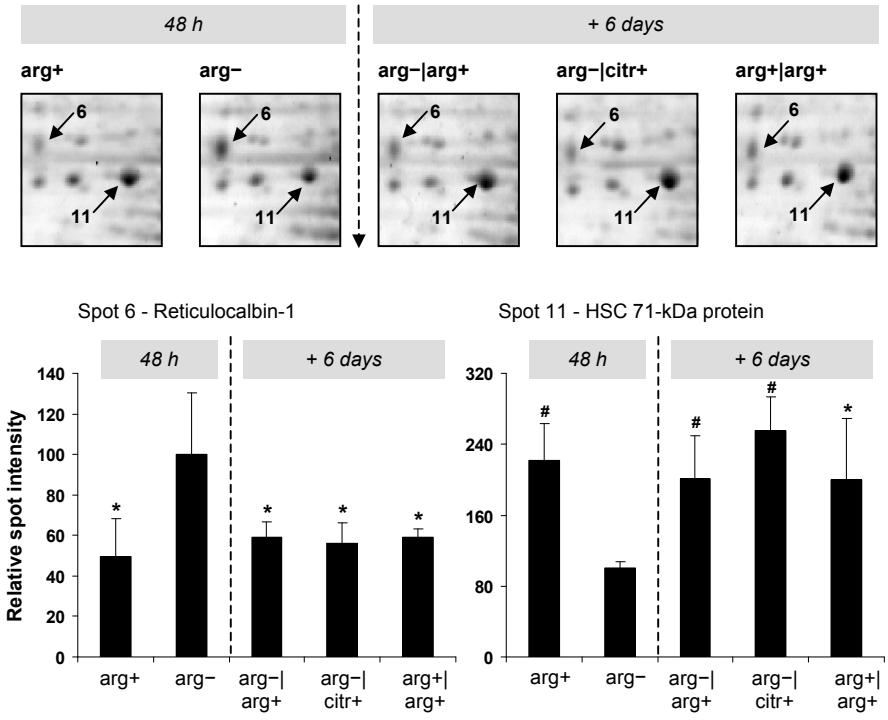


Figure 4. Two differentially expressed protein spots under different conditions (spot 6 and 11 in Figure 3). Arg− and arg+ indicate the 48-h-arginine-deprived and 48-h-arginine-supplemented conditions, respectively. When arg− cells are subsequently supplemented with arginine or citrulline for 6 days, this is indicated as arg−|arg+ and arg−|citr+, respectively. When arg+ cells are subsequently supplemented with arginine for 6 days, this is indicated as arg+|arg+. The spot intensity of arg+ is set to 100. Values are means + SD of four replicate gels of two independent experiments, * and # indicate significant differences compared to arg− condition with $p<0.05$ and $p<0.01$ (Student's t-test), respectively.

Effect of arginine on the protein profile of postconfluent Caco-2 cells

2-DE

To investigate if arginine-depletion responsive proteins in undifferentiated Caco-2 cells showed the same behavior in differentiated Caco-2 cells, another 2-DE experiment was performed in which 5-day-differentiated Caco-2 cells were exposed to decreasing arginine concentrations (2.0, 0.2 and 0.0 mM arginine from the apical side) for 24 h. Comparison of 2-D profiles resulted in 24 differentially expressed protein spots. A list of the identified proteins, 17 in total, together with the change in expression patterns is displayed in Table 2. A representative 2-D gel image, in which the changed spots are indicated, is shown in Figure 5.

Most of the protein spots were detected in profiles of both preconfluent and postconfluent Caco-2 cells, and matching of these profiles made it possible to assess differences and similarities in the response to arginine deprivation in preconfluent and postconfluent Caco-2 cells. Only three protein spots showed a similar response, namely spot 14, 22 and 48 in Table 1, which correspond to spot 1, 7 and 13 in Table 2, respectively.

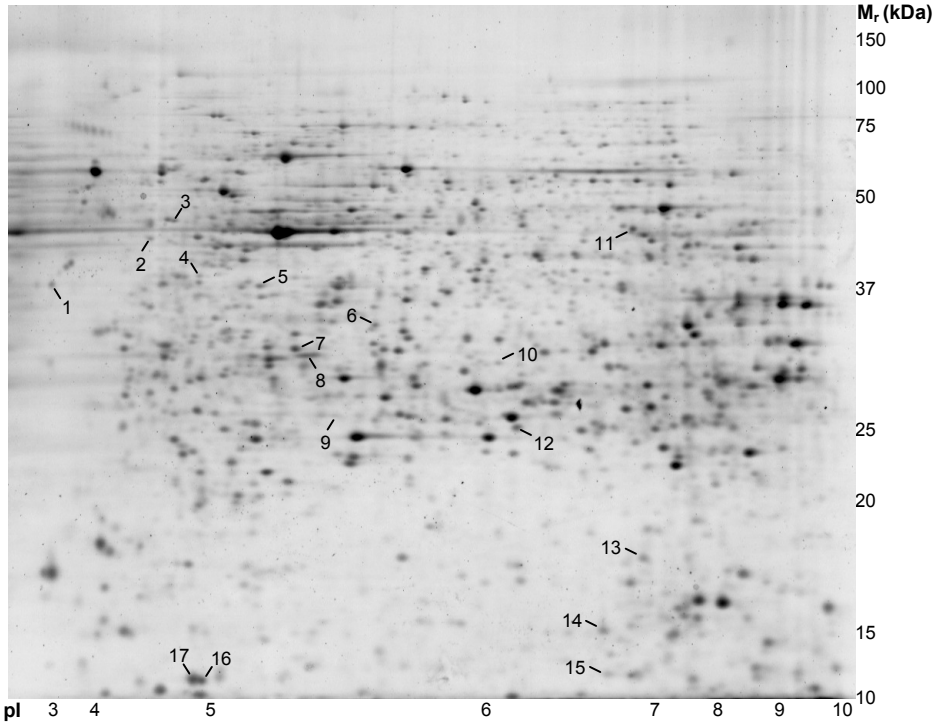
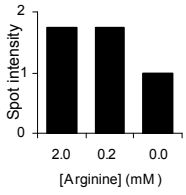
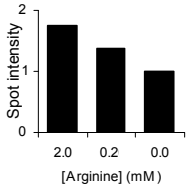
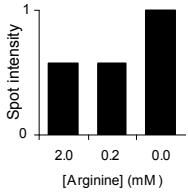
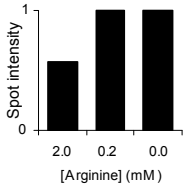
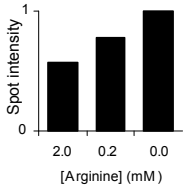


Figure 5. Example of a 2-D pattern of proteins extracted from 5-day-differentiated Caco-2 cells. Expression levels of indicated protein spots were changed between the different conditions (2.0, 0.2 and 0.0 mM arginine) and their identity is shown in Table 2.

Ab microarray

Since 2-DE is limited in the detection of low-abundance proteins, we applied an additional method for protein profiling. By using Ab microarrays, we were able to detect expression differences of two proteins in 5-day-differentiated Caco-2 cells exposed to a medium containing 2.0 or 0.0 mM arginine for 24 h. Two proteins, CAS and HSF-4A, were found to be up-regulated under arginine-deprived conditions, and these results were confirmed by Western blotting (Figure 6). In preconfluent Caco-2 cells exposed to medium containing 0.0 or 0.4 mM arginine for 48 h no significant change in HSF-4A levels could be observed (not shown).

Table 2. Spot numbers and identity of changed protein spots in differentiated Caco-2 cells, which correspond to those indicated in Figure 5. Fold change (FC) and *p*-value (Student's t-test) are calculated for comparisons of average spot intensities from three conditions (2.0, 0.2 and 0.0 mM arg). Spots are clustered according to the change in their expression profile.

Pattern	Spot	Swiss-Prot accession number	Protein name	0.0/2.0		0.0/0.2		0.2/2.0	
				FC	*p*	FC	*p*	FC	*p*
	1	P27797	Calreticulin[a,b)]	-1.5	0.037	-1.5	0.004	1.0	0.929
	9	P02647	Apolipoprotein A-I	-1.6	0.001	-1.5	0.012	1.0	0.743
	13	P09651	HnRNP A1[c)]	-1.4	0.037	-1.4	0.001	1.0	0.893
	3	P11021	78-kDa glucose-regulated protein[a,b)]	-2.2	0.014	-1.7	0.061	-1.3	0.359
	4	Q92597	Protein NDRG1	-2.4	0.001	-1.8	0.009	-1.3	0.152
	7	P30101	Protein disulfide-isomerase A3[a,c)]	-1.8	0.021	-1.4	0.075	-1.3	0.221
	16	P20674	Cytochrome c oxidase polypeptide Va, mit.	-2.0	0.001	-1.4	0.113	-1.4	0.061
	2	Q15293	Reticulocalbin-1	1.6	0.000	1.5	0.002	1.1	0.587
	6	P50224	Monoamine-sulfating phenol sulfotransferase	1.5	0.010	1.8	0.003	-1.2	0.313
	11	O75874	Isocitrate dehydrogenase [NADP] cyt.	1.4	0.016	1.5	0.015	-1.1	0.695
	15	P49773	Histidine triad nucleotide-binding protein 1	1.7	0.018	1.5	0.010[d)]	1.1	0.548
	8	P31943	HnRNP H[b)]	2.1	0.042[d)]	1.2	0.519	1.8	0.086
	12	P30048	Thioredoxin-dependent peroxide reductase, mit.	1.9	0.028	1.0	0.885	1.8	0.067
	17	P25705	ATP synthase alpha chain, mit.[a,c)]	1.3	0.042	-1.1	0.151	1.5	0.010
	14	Q969H8	Interleukin-25	1.5	0.010	-1.1	0.386	1.7	0.005
	5	P55735	SEC13-related protein	1.6	0.027[d)]	1.2	0.127	1.3	0.179
	10	P78417	Glutathione transferase omega-1	1.7	0.007	1.3	0.021	1.3	0.170

HnRNP: heterogeneous nuclear ribonucleoprotein; mit.: mitochondrial; cyt.: cytoplasmic

a) more spots of protein present in 2-D pattern that are not regulated by arginine

b) N-terminal peptide

c) C-terminal peptide

d) $p \geq 0.05$ after log-transformation of spot intensities

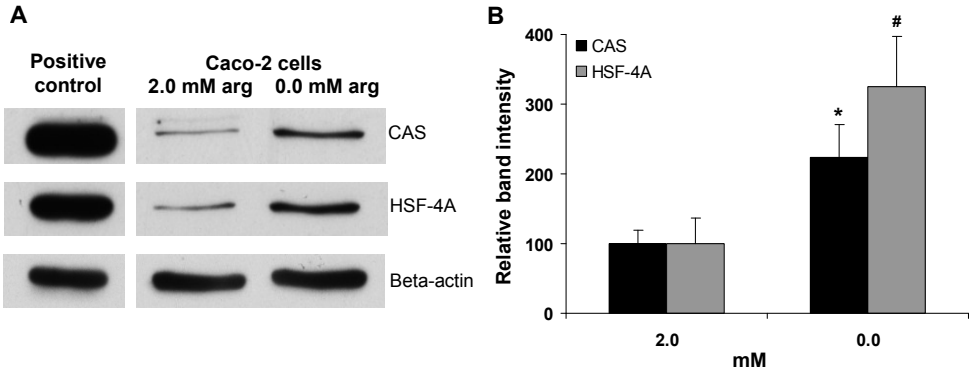


Figure 6. A. A representative Western blot visualizing CAS (100 kDa) and HSF-4A (55 kDa) levels in differentiated Caco-2 cells exposed to DMEM with(out) arginine. Beta-actin was used as an internal standard (42 kDa) **B.** Graphical representation of relative band intensities expressed as mean + SD of triplicate measures. * and # indicate significant differences compared to arginine-supplemented condition with $p<0.05$ and $p<0.01$ (Student's t-test), respectively.

DISCUSSION

Since cell proliferation is required for proper functioning and health of the intestine, we studied the effects of arginine, particularly in preconfluent cells, which are still in a proliferative state. The aim was to obtain a better insight in the response of the Caco-2 cell proteome to arginine deprivation/supplementation. We found that proteins involved in cell proliferation and DNA replication, protein synthesis and processing, apoptosis and the heat shock response are regulated by the arginine availability to these cells.

Changed proteins with a role in cell proliferation

Arginine deprivation causes an increase in protein SET expression levels. This protein is widely expressed in human tissues and has multiple functions. Overexpressing SET in two cell types induced inhibition of cell cycle progression at G_2/M transition, and was associated with inhibition of cyclin B-CDK1 activity [25]. Induction of exogenous SET at a 1:1 ratio to the endogenous protein resulted in suppression of cell proliferation, possibly by inhibiting G_1/S transition [26]. The reduced proliferation capacity of arginine-deprived Caco-2 cells might result from increased SET levels.

Arginine can modulate deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase) expression, another protein related to cell proliferation. The enzyme is involved in the formation of dUMP from dUTP, thereby limiting dUTP levels in

the cell to prevent uracil incorporation during DNA synthesis. A nuclear and a mitochondrial form are known of human dUTPase [27], and expression of the nuclear isoform was induced in human lung fibroblasts in response to the onset of DNA replication, while the mitochondrial isoform was constitutively expressed [28]. Arginine withdrawal decreases nuclear dUTPase levels in Caco-2 cells and this effect can be restored by subsequent arginine supplementation, which appears to stimulate the DNA replication process.

During mitosis, the Golgi system breaks down and reassembles within each daughter cell. NSFL1 cofactor p47 (also known as p97 cofactor p47) is shown to be involved in the reassembly of the Golgi apparatus and the endoplasmatic reticulum (ER) at the end of mitosis [29]. This protein is not differentially expressed in 48-h-arginine-deprived Caco-2 cells compared to 48-h-arginine-supplemented cells; however, arginine exposure after an arginine-deprivation period is able to induce its expression. Under this condition, cells start proliferating and thus require NSFL1 cofactor p47 for Golgi and ER assembly. Nuclear autoantigenic sperm protein (NASP) shows a similar expression pattern. It was shown recently that without sufficient NASP in the cells, they are unable to replicate their DNA and progress through the cell cycle [30].

Changed proteins with a role in the synthesis and processing of proteins

Eukaryotic translation initiation factor 2 subunit alpha (eIF2alpha) is up-regulated in Caco-2 cells exposed to arginine after 48 h of arginine deprivation. Eukaryotic translation initiation factor eIF2, composed of an alpha, a beta and a gamma chain, and its 'exchange factor' eIF2B play a key role in the regulation of protein synthesis. Several stress-responsive kinases can phosphorylate eIF2alpha. Phosphorylated eIF2 acts as an inhibitor of the guanine nucleotide exchange factor eIF2B and inhibits translation initiation [31]. Here, eIF2alpha (unphosphorylated) is up-regulated indicating that translation initiation is promoted.

Two ER proteins displaying increased expression levels under arginine-deficient condition are glucosidase II beta subunit and reticulocalbin-1. Glucosidase II has an important function in the folding and maturation of glycoproteins and contains an alpha and a beta subunit [32]. Amino acid deprivation was able to induce expression the alpha subunit of glucosidase II [33]. We showed here that arginine deprivation induces expression of the beta subunit in preconfluent Caco-2 cells. Reticulocalbin-1 is a Ca^{2+}-binding protein localized in the lumen of the ER and expressed in various cell types which suggests a role in protein synthesis, modification, and intracellular transport but, at present, its function remains unknown [34]. A 44-kDa and a 46-kDa form are known, and the 46-kDa protein is probably produced by N-glycosylation (high-mannose type) [34]. Arginine

deprivation increases the expression of the 46-kDa protein in preconfluent Caco-2 cells, while it increases expression of the 44-kDa protein in differentiated Caco-2 cells. It remains to be elucidated what the exact role of reticulocalbin (glycosylated and unglycosylated form) is and why its expression is increased upon arginine deprivation. Noteworthy is our finding that glutamine deprivation in differentiated Caco-2 cells is also able to increase the 44-kDa form of reticulocalbin [17].
Together, the above data strongly suggest that arginine is essential for intestinal cell proliferation, protein synthesis and processing. Thus, a state of arginine deficiency can be detrimental for intestinal cells which are highly proliferative *in vivo*.

Changed proteins with a role in apoptosis

Histone H2B protein is found to be higher expressed in Caco-2 cells subjected to arginine deprivation. In cell lysates of lymphoblasts, this protein was up-regulated after induced apoptosis [35]. H2B was also found to be phosphorylated upon apoptotic stimuli in different mammalian cell types [36]. We propose that arginine deficiency causes an increase in the phosphorylated form of H2B, since the corresponding protein spot is localized in the acidic region of the 2-D gel instead of the basic region, a characteristic shift during protein phosphorylation.
Two protein spots identified as splicing factor arginine/serine-rich 1, also known as splicing factor 2 or alternate splicing factor (SF2/ASF), show reduced expression levels when growing Caco-2 cells are exposed to arginine-free medium. SF2/ASF has an important role in constitutive and alternative splicing of pre-mRNAs. Depleting a chicken B-cell line of SF2/ASF caused cell cycle arrest followed by cell death in a manner that displays many characteristics of apoptosis [37]. Although not observed in general, withdrawal of arginine from the medium could lead to a specific down-regulation of proteins which are very rich in arginine residues, including SF2/ASF which consists for 17.4% of arginine molecules. However, also other factors can modulate its expression, for instance, tumor necrosis factor-alpha was able to decrease SF2/ASF expression in a mouse muscle cell line [38].

Changed heat shock proteins

Diverse proteins belonging to the heat shock protein (HSP) family, like 47-kDa HSP, heat shock 70-kDa protein 1, heat shock cognate 71-kDa protein, HSP 90-beta and endoplasmin show decreased expression levels in arginine-deficient preconfluent Caco-2 cells. Arginine supplementation following this deprivation period reestablishes their expression levels. Thus, arginine modulates protein

expression in favor of increased HSP levels, needed for proper protein folding and function. Such processes are critical for normal cellular proliferation and differentiation, and consequently sufficient arginine levels may have a protective effect on the intestine.

Effect of citrulline

It was shown that arginine supplementation counteracts most changes observed after an arginine deficiency period in intestinal cells, and it is interesting to know if citrulline has the same effect. This could be of clinical importance since citrulline is thought to be a better nutrient to administer during arginine deficiency, since this amino acid is not a substrate for arginase, and is not taken up by the liver [39, 40]. We observed that citrulline can be used as a substitute for arginine since citrulline stimulates cell proliferation after arginine deficiency, and normalizes the expression of most changed proteins (90%) after an arginine-deprivation period. It appears that the intestinal cells can use citrulline effectively and convert it to arginine (by ASS and ASL) during periods of arginine deprivation, to sustain its cell proliferative capacity and maintain normal protein levels. ASS and ASL were shown to be expressed at higher levels in several cell lines exposed to growth-limiting concentrations of arginine [6].

Postconfluent Caco-2 cells

The arginine deprivation experiment in differentiated Caco-2 cells revealed that fewer proteins are changed in expression. This may be not only ascribed to the shorter period of arginine deprivation (24 h instead of 48 h) but, also to the cellular need for arginine, which seems to be reduced in differentiated Caco-2 cells [19]. In addition, we observed that arginine deprivation affected proteins involved in cell proliferation and DNA replication, and this is not an active process in postconfluent cells. This was reflected by the very small overlap which was observed in the response to arginine deficiency.

By using the Ab microarray, we found CAS protein expression to be regulated by arginine depletion in 5-day-differentiated Caco-2 cells, and this protein is a nuclear transcription factor that plays a role in apoptosis [41]. Reduction of CAS expression by antisense cDNA decreases the sensitivity for tumor necrosis factor- and toxin-mediated apoptosis in cells [42]. In this manner, arginine-deprived cells could be more sensitive to apoptosis. This would mean that sufficient arginine levels may prevent apoptosis in mature intestinal cells. Its expression in the intestine *in vivo* is not yet documented, but CAS is expressed in many adult tissues

and the gene shows a high interspecies homology indicating that it might have an essential function [43].
Concerning the heat shock response in differentiated Caco-2 cells, we observed increased HSF-4A expression upon arginine deprivation. It is the splice variant of the HSF-4 gene which can repress transcription of HSPs by binding heat shock response elements within the promoter regions of HSP-coding genes [44]. This was however not apparent from the 2-DE and the Ab microarray experiment, in which several HSPs are represented. Preconfluent Caco-2 cells exposed to 48 h of arginine deprivation showed no increase of HSF-4A, whereas they did show reduced HSP expression. Possibly, arginine deprivation up-regulates HSF-4A expression only transiently, whereas changes in HSP levels may occur at a later stage requiring a longer period after HSF-4A induction. However, other mechanisms cannot be excluded.
Two protein profiling methods were applied in this study, namely the 2-DE and the Ab microarray technique, and both have their advantages and disadvantages. For example, the latter method is less labor intensive and can detect differences in low-abundance proteins. A drawback is that only two biological samples can be compared, whereas the 2-DE method allows multiple comparisons. Furthermore, the number and choice of Ab present on the arrays is still limited. Current data show that both techniques are complementary in generating new biological insights about the effects of nutrients on the proteome of the intestinal cells. However, a direct comparison of the two techniques in this study is difficult since different protein preparations were used.

CONCLUSION

Arginine is suggested to have a role in the intestinal repair mechanism, a process in which intestinal epithelial cell proliferation is an essential event. Furthermore, enterocytes proliferate actively under physiological conditions. Understanding the role of arginine in proliferating intestinal cells is therefore highly relevant. The present study shows that arginine deprivation modulates the protein expression profiles of preconfluent, proliferating intestinal Caco-2 cells differently than those of postconfluent, differentiated cells. The protein changes in preconfluent Caco-2 cells suggest that arginine deficiency decreases cell proliferation and HSP expression, and enhances the susceptibility to apoptosis, and the observed changes can be mostly counteracted by subsequent supplementation of arginine or citrulline. The regulated processes are critical for proper cell function, and hence a state of arginine deficiency, which occurs during catabolic disease states, seems damaging for proliferating intestinal cells.

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CHAPTER 5

STARVATION INDUCES PHASE-SPECIFIC CHANGES IN THE PROTEOME OF MOUSE SMALL INTESTINE

Kaatje Lenaerts, Milka Sokolović, Freek Bouwman, Wouter Lamers, Edwin Mariman and Johan Renes

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ABSTRACT

Food deprivation results in metabolic, structural, and functional changes in the small intestine that influences gut mucosal integrity, epithelial cell proliferation, mucin synthesis, and other processes. The underlying mechanisms are still unclear, which lead to the study of molecular effects of short-term and long-term starvation in the intestine of mice. A comparative proteomics approach, combining two-dimensional gel electrophoresis with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, was used to identify intestinal proteins whose expression is changed under different starvation conditions (0, 12, 24, and 72 h). In total, the expression levels of 80 protein spots changed significantly between the different groups. The results demonstrate that after 12 h of starvation mainly proteins involved in glycolysis and energy metabolism show decreased expression levels. Starvation for 24 h results in a down-regulation of proteins involved in protein synthesis and amino acid metabolism. Simultaneously, proteins with a protective role, e.g., reg I and II, glutathione peroxidase 3, and carbonic anhydrase 3, are clearly up-regulated. The last starvation phase (72 h) is characterized by increased ezrin expression, which may enhance villus morphogenesis critical for survival. Together, these results provide novel insights in the intestinal starvation response and may contribute to improved nutritional support during conditions characterized by malnutrition.

INTRODUCTION

Alterations in nutritional status have an impact on metabolic pathways in the human body. Malnutrition and starvation generate a number of adaptive biochemical, physiological, and molecular responses that lead to a reduction in body weight and visceral organ mass, immune dysfunction, inhibition of reproduction, etc. According to the main energy sources available and the associated loss in body weight, the starvation response in mammals is characterized by consecutive phases. Phase I is the postabsorptive phase characterized by glycogenolysis. During phase II, energy requirements are mostly derived from fat oxidation leading to depletion of lipid stores. Phase III is characterized by increased protein utilization as a substitute fuel [1].

The small intestine, the primary organ for nutrient digestion and absorption, undergoes dramatic structural and functional changes when deprived of food. Adaptive mechanisms following starvation influence gut mucosal integrity leading to increased mucosal permeability [2]. Other processes such as epithelial cell proliferation and mucin synthesis are inhibited, and apoptosis is induced, however this seems to be dependent on the length of the starvation period [3-5]. Food intake regulates gastrointestinal hormones that are important for growth and repair, including gastrin, epidermal growth factor, and insulin-like growth factor-I, which interact with gut mucosal receptors to stimulate regeneration and function of enterocytes [6].

Currently, the molecular mechanisms underlying the effects of food deprivation in the intestine are unclear. Hence, we used a proteomics approach to determine starvation-induced adaptations at the molecular level, and to discriminate between the effects of short-term (12 h) and long-term starvation (24 and 72 h) on the mouse intestine. In mice, glycogen stores are maximally reduced after 12 h of starvation, which should correspond to a shift from phase I to II [7]. The transition between phase II and III occurs probably after 48 h of fasting [1]. A further understanding of molecular adaptive responses to food restriction in the intestine may lead to an improved nutritional support in conditions characterized by starvation and malnutrition, such as critical illness, surgery, sepsis, cancer, anorexia nervosa and malabsorption syndromes.

MATERIALS AND METHODS

Materials

Zirkonia-Silica beads (2.5 mm) were from BioSpec Products (Bartlesville, OK, USA). Dithiothreitol (DTT), 3-[(3-cholamidopropyl)dimethyl-amonio]-1-propane-

sulfonate (CHAPS), goat antirabbit Ig-alkaline phosphatase, and levamisole (Tetramisolehydrochloride) were from Sigma (St. Louis, MO, USA). NBT/BCIP solution was from Roche Applied Science (Penzberg, Germany). Urea and SYPRO Ruby Protein Stain were from Bio-Rad Laboratories (Hercules, CA, USA). Immobilized pH gradient (IPG) strips (pH 3–11, nonlinear), IPG buffer, and a 2-D Clean-Up kit were from Amersham Biosciences (Little Chalfont, England).

Animals and tissue collection

Male FVB mice, obtained from Charles River (Maastricht, The Netherlands), were housed per two in cages under controlled environmental conditions (12-h light period, temperature of 20–22°C, 50 to 60% humidity). They had free access to food and water until six weeks of age. At that time point, six mice per group were fasted for 0, 12, 24, or 72 h. The 72-h-starved animals were housed in metabolic cages and were kept warm with an infrared lamp. Animals were killed by cervical dislocation. The small intestine was removed immediately and made free of mesentery, pancreas and fat. Proximal and distal parts of the small intestine were opened longitudinally, washed with phosphate-buffered saline (PBS), and blotted dry. Tissues were snap frozen in liquid nitrogen and stored at –80°C.
The study was approved by the Animal Experiments Committee from the Academic Medical Center (Amsterdam, The Netherlands) and was performed in accordance with the Dutch guidelines for the use of experimental animals.

Protein sample preparation

Equal quantities of proximal and distal parts of the intestine were pooled per mouse. Tissue samples were homogenized in lysis buffer containing 6 M urea, 2 M thiourea, 30 mM DTT, 4% (w/v) CHAPS with a Biospec Mini-beadbeater and centrifuged at 20000 g for 30 min at 10°C. A 2-D Clean-Up kit was used to remove nonprotein contaminants of supernatants. Protein pellets were dissolved in lysis buffer containing 0.5% (v/v) IPG buffer (pH 3–10, nonlinear). After centrifuging protein samples at 8000 g for 10 min at 10°C, supernatants were stored at –80°C until further analysis. Protein concentration was determined using a Bradford-based protein assay (Bio-Rad Laboratories) [8].

Two-dimensional gel electrophoresis (2-DE)

The 2-DE procedure was performed as described [9]. Briefly, 100 μg of total protein was separated by isoelectric focusing using IPG strips (24 cm, pH 3–11,

nonlinear) according to the following protocol: 12 h at 30 V, 1 h at 500 V, 1 h at 1000 V, 3 h gradient from 1000 to 8000 V, 30 kVh at 8000 V. Strips were equilibrated and placed onto 12.5% SDS-polyacrylamide gels for protein separation in the second dimension. Gels were stained with SYPRO Ruby Protein Stain, and proteins were visualized by scanning gels with the Molecular Imager FX (Bio-Rad Laboratories). To reduce technical variability, 12 IPG strips and 12 gels were run simultaneously using an IPGphor isoelectric focusing system (Amersham Biosciences) and a Protean Dodeca Cell electrophoresis chamber (Bio-Rad Laboratories), respectively. The six biological replicates per condition were evenly distributed between the two runs.

Image analysis

Examination of differentially expressed proteins was performed using PDQuest 7.3 (Bio-Rad Laboratories). Gels from samples with the same treatment formed one replicate group with average normalized spot intensities. A spot was regarded as significantly differentially expressed between groups (0, 12, 24, and 72 h starvation) if the average spot intensity differed 2-fold or more and if $p<0.05$ (Student's t-test) for at least one comparison. Changed spots were excised from the gels with a Spot Cutter (Bio-Rad Laboratories).

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and protein identification

Excised protein spots were subjected to tryptic in-gel digestion and MALDI-TOF MS (Waters, Manchester, UK), generating peptide mass fingerprints with MassLynx 4.0.5 (Waters) as described [10]. Protein identification was performed as described [9]. Taxonomy was set to Mus musculus and Mascot probability scores (http://www.matrixscience.com) were calculated using 30 mass peaks or less with the highest signal intensity; trypsin and keratin peaks were excluded. Protein identifications with a score greater than 54 and at least four matched peptides were considered significant ($p<0.05$).

Data analysis

GenMAPP and MAPPFinder (version 2.0) (Gladstone Institute, San Fransisco, CA, USA) were used to analyze data of identified changed protein spots in response to starvation and to explore biological processes altered at the protein level under these conditions [11, 12].

Immunohistochemistry

Intestinal samples, fixed overnight in 4% formaldehyde, were embedded in paraffin, sectioned at 6 μm, and stained immunohistochemically according to the following procedure. The slides were boiled in 10 mM Na–citrate (pH 6.0) for 10 min to retrieve epitopes and inactivate endogenous alkaline phosphatase and blocked for 30 min at room temperature in Teng-T (10 mM TrisHCl with pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.25% (w/v) gelatin and 0.05 (v/v) Tween-20) containing 10% normal goat serum (NGS). Subsequently, the slides were incubated overnight with the polyclonal anti-ornithine aminotransferase (OAT) antibody (kindly provided by dr. T. Matsuzawa[13]) diluted in Teng-T/10%NGS. Sections were thoroughly washed in PBS and incubated with goat antirabbit Ig-alkaline phosphatase diluted 1:40 in Teng-T/10%NGS. Antibody-bound alkaline phosphatase activity was visualized by incubation in NBT/BCIP solution 1:50 in 100 mM TrisHCl, pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 1 mM levamisole for 30 min.

RESULTS

Starvation-induced effects on the proteome of mice intestine

Male FVB mice were deprived of food for 0, 12, 24, or 72 h, and changes in the protein profiles were determined by a 2-DE approach. Approximately 1500 spots were detected per gel within a pH range of 3–11 and a molecular mass range of 10–100 kDa. Differentially expressed protein spots (73) (≥2-fold change, $p<0.05$) were found when 2-D patterns of the control group (0 h starvation) were compared to 2-D patterns of the experimental groups (12, 24 or 72 h starvation). Figure 1 shows the number of changed protein spots per time point. Compared to the fed condition, 36, 58, and 27 protein spots were changed at 12, 24, and 72 h of fasting, respectively. Comparing the three experimental groups with each other resulted in 7 additional changed protein spots.

Differentially expressed proteins (62 of the 80) were identified with MALDI-TOF MS corresponding to 46 unique protein entries, as some proteins were present as more than one spot due to protein processing or modification. Figure 2 shows a representative 2-D gel indicating the identified protein spots. A list of the same proteins together with their change in expression during starvation is displayed in Table 1.

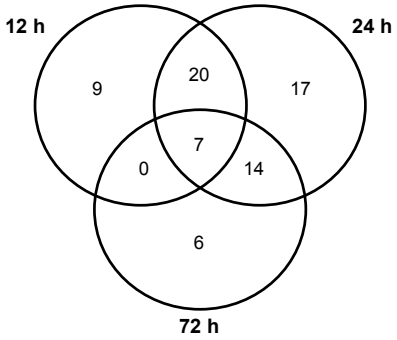

Figure 1. Venn diagram of 73 differentially expressed protein spots (12, 24, or 72 h fasting compared to control group). The intersections indicate the spots that are changed at more than one time point.

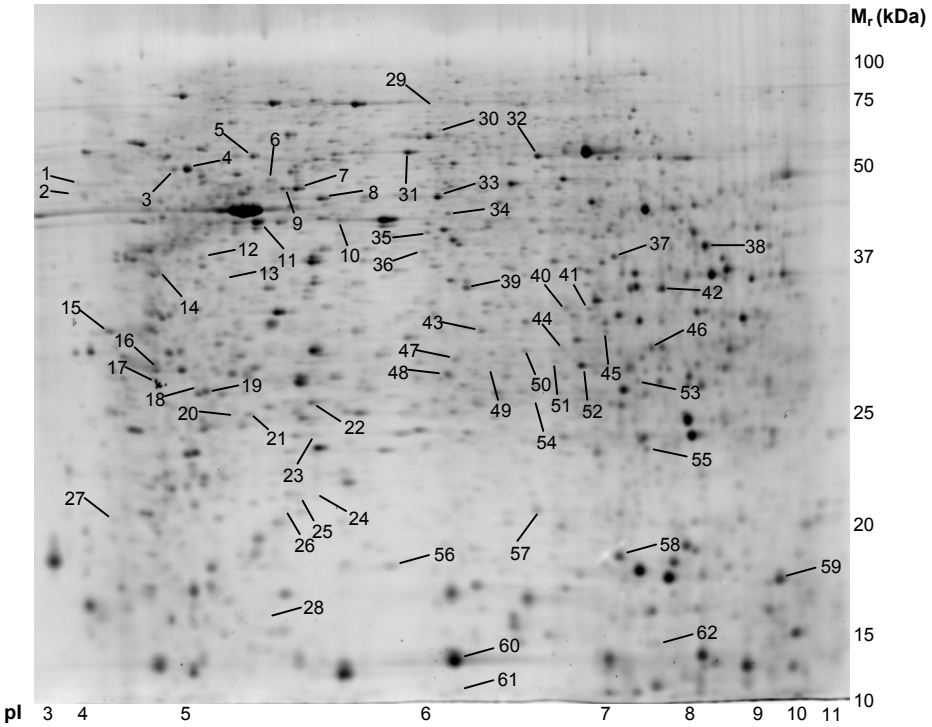

Figure 2. Example of a 2-D pattern of proteins extracted from a mouse small intestine (at 12 h of fasting). The gel was stained with SYPRO Ruby Protein Stain. Expression levels of numbered protein spots were changed between the different conditions (0, 12, 24, and 72 h of starvation), and their identity is depicted in Table 1.

Table 1. Spot numbers, corresponding to Figure 2, and identity of changed protein spots during starvation. The expression fold change (FC) and the t-test p-value were calculated for each comparison with control (0 h of starvation). Values are indicated in bold when the spot intensity of the groups differed 2-fold ore more and $p<0.05$.

Spot	Swiss-Prot accession number	Protein ID	0 h vs 12 h FC	*p*	0 h vs 24 h FC	*p*	0 h vs 72 h FC	*p*
Glycolysis/gluconeogenesis								
44	Q91Y97	Fructose-bisphosphate aldolase B	**-2.5**	**0.000**	-1.8	0.014	-1.4	0.071
45	Q91Y97	Fructose-bisphosphate aldolase B	**-2.2**	**0.009**	-1.6	0.051	-1.2	0.527
38	Q91Y97	Fructose-bisphosphate aldolase B	**-2.0**	**0.007**	-1.7	0.011	1.2	0.546
41	P16858	Glyceraldehyde-3-phosphate dehydrogenase	**-2.5**	**0.001**	**-2.6**	**0.001**	**-2.6**	**0.002**
52	Q9DBJ1	Phosphoglycerate mutase 1	-1.7	0.028	**-2.2**	**0.004**	-1.6	0.135
46	P06151	L-lactate dehydrogenase A chain	-1.6	0.046	**-2.1**	**0.006**	-1.6	0.023
39	P14152	Malate dehydrogenase, cyt.	**-2.0**	**0.019**	**-2.1**	**0.001**	-1.4	0.156
24	P17751	Triosephosphate isomerase	**2.0**	**0.010**	**2.5**	**0.001**	1.9	0.017
Energy metabolism								
7	Q9CZ13	Ubiquinol-cytochrome-c reductase protein I, mit.	**-2.2**	**0.022**	-1.3	0.398	-1.3	0.379
4	P56480	ATP synthase beta chain, mit.	**-2.7**	**0.030**	-2.1	0.064	-1.0	0.961
17	P56480	ATP synthase beta chain, mit.[a)]	**2.0**	**0.007**	**3.4**	**0.005**	**2.4**	**0.001**
8	Q04447	Creatine kinase B-type	**-2.8**	**0.032**	-2.1	0.105	-1.2	0.640
58	Q01768	Nucleoside diphosphate kinase B	-1.6	0.009	**-2.0**	**0.001**	-1.6	0.055
Alcohol-metabolizing enzymes								
37	Q9JII6	Alcohol dehydrogenase [NADP+]	**-2.2**	**0.001**	**-2.7**	**0.001**	-1.6	0.119
32	gi|56270548	Aldehyde dehydrogenase 1 family, B1	**-2.9**	**0.009**	-1.9	0.056	-1.4	0.285
Protein folding and processing								
27	P09103	Protein disulfide-isomerase[a)]	**2.2**	**0.023**	**2.8**	**0.001**	2.1	0.089
61	P17742	Peptidyl-prolyl cis-trans isomerase A[b)]	**2.9**	**0.025**	**3.3**	**0.012**	2.0	0.211
1	P14211	Calreticulin	**2.9**	**0.001**	**2.3**	**0.028**	1.1	0.825
2	P14211	Calreticulin	1.3	0.218	-1.3	0.258	**-2.1**	**0.006**
30	P11983	T-complex protein 1 subunit alpha B	1.4	0.082	**2.8**	**0.002**	**2.7**	**0.009**
49	P63017	Heat shock cognate 71-kDa protein[b)]	1.6	0.139	1.9	0.004	**2.3**	**0.002**
Protein synthesis								
15	O70251	Elongation factor-1-beta	-1.3	0.298	**-2.1**	**0.006**	-1.3	0.377
36	P58252	Elongation factor-2[a)]	-1.5	0.044	**-2.2**	**0.001**	**-2.0**	**0.003**
Amino acid metabolism								
33	P29758	Ornithine aminotransferase, mit.	-1.9	0.008	**-2.0**	**0.002**	**-2.0**	**0.003**
16	P29758	Ornithine aminotransferase mit.[a)]	-1.2	0.406	**-2.3**	**0.002**	**-3.3**	**0.000**
34	O08691	Arginase-2, mit.	-1.8	0.030	-1.6	0.051	1.2	0.478
Extracellular space proteins								
23	P46412	GPx-3	1.9	0.087	**2.8**	**0.036**	**2.9**	**0.001**
62	P43137	Lithostathine 1/reg I	1.1	0.695	**5.0**	**0.002**	**2.5**	**0.030**
56	Q08731	Lithostathine 2/reg II	1.7	0.126	**4.4**	**0.000**	**3.6**	**0.049**
51	P00688	Pancreatic alpha-amylase[b)]	1.9	0.052	**2.6**	**0.003**	2.7	0.108
40	P00688	Pancreatic alpha-amylase[b)]	1.4	0.229	1.9	0.023	**2.1**	**0.007**
59	O88312	Anterior gradient protein 2 homolog/ gob-4 protein	**-2.0**	**0.020**	**-2.1**	**0.008**	-1.5	0.111
42	O88310	Intelectin-1a	-1.6	0.050	**-2.2**	**0.005**	**-2.1**	**0.010**
18	P07724	Serum albumin[a)]	**4.4**	**0.005**	**6.5**	**0.004**	**5.9**	**0.000**
13	P07724	Serum albumin[a)]	**3.2**	**0.001**	**3.4**	**0.009**	2.5	0.141
22	P07724	Serum albumin[b)]	1.7	0.017	1.3	0.270	-1.6	0.058
Cytoskeleton-related proteins								
5	P31001	Desmin	-1.5	0.258	**-3.2**	**0.024**	-1.3	0.522

Spot	Swiss-Prot accession number	Protein ID	0 h vs 12 h FC	*p*	0 h vs 24 h FC	*p*	0 h vs 72 h FC	*p*
12	Q62468	Villin-1[a)]	-1.9	0.003	**-2.3**	**0.000**	-1.3	0.198
25	Q62468	Villin-1 (fragment)	**2.1**	**0.003**	**2.7**	**0.009**	2.8	0.090
29	P26040	Ezrin	-1.4	0.265	1.1	0.556	**2.1**	**0.034**
11	P19001	Keratin, type I cytoskeletal 19	-1.9	0.005	**-2.0**	**0.004**	-1.7	0.007
48	P19001	Keratin, type I cytoskeletal 19[b)]	1.1	0.814	1.5	0.104	**2.1**	**0.031**
6	P11679	Keratin, type II cytoskeletal 8	1.2	0.488	-1.5	0.244	**-2.0**	**0.048**
31	P11679	Keratin, type II cytoskeletal 8	**-3.4**	**0.008**	**-2.1**	**0.037**	-1.3	0.300
47	P11679	Keratin, type II cytoskeletal 8[b)]	-1.9	0.005	**-2.2**	**0.001**	-1.1	0.647
57	P37804	Transgelin	-1.3	0.203	**-2.0**	**0.003**	-1.7	0.037
19	Q99PT1	Rho GDP-dissociation inhibitor 1	-1.6	0.031	**-2.2**	**0.004**	-1.0	0.930
35	P60710	Actin, cytoplasmic 1	-1.2	0.438	-1.5	0.090	**-2.1**	**0.008**
26	P60710	Actin, cytoplasmic 1[a)]	1.7	0.066	**2.7**	**0.004**	**2.8**	**0.016**
10	P63268	Actin, gamma-enteric smooth muscle	1.3	0.311	1.1	0.821	-1.8	0.074
20	P63268	Actin, gamma-enteric smooth muscle[b)]	1.1	0.855	**2.1**	**0.038**	2.2	0.071
28	P63268	Actin, gamma-enteric smooth muscle[b)]	**2.4**	**0.000**	**3.0**	**0.001**	**2.1**	**0.011**
Ungrouped								
54	P16015	Carbonic anhydrase 3	2.2	0.053	**2.4**	**0.004**	**2.2**	**0.017**
9	Q9R111	Guanine deaminase	**-2.8**	**0.000**	**-2.4**	**0.000**	**-2.4**	**0.001**
60	P55050	Intestinal fatty acid-binding protein	-1.6	0.000	**-2.0**	**0.000**	-1.3	0.030
43	Q9R1P4	Proteasome subunit alpha type 1	**-2.3**	**0.005**	**-2.1**	**0.006**	-1.6	0.089
50	Q9QWG7	Sulfotransferase family cytosolic 1B	-1.6	0.005	**-2.0**	**0.001**	-1.3	0.281
55	O88569	Heterogeneous nuclear ribonucleoproteins	-1.1	0.376	**-2.3**	**0.000**	**-2.1**	**0.000**
21	Q9JM14	5'(3')-deoxyribo-nucleotidase, cyt.	-1.9	0.007	-1.5	0.024	1.1	0.683
53	Q8R0F8	Fumarylacetoacetate hydrolase domain containing protein 1	**-2.1**	**0.012**	**-3.5**	**0.003**	-1.9	0.043
Mixtures								
3	P56480	ATP synthase beta chain, mit.	**-2.6**	**0.016**	**-2.5**	**0.018**	-1.7	0.074
	P20029	78-kDa glucose-regulated protein[a)]	**-2.6**	**0.016**	**-2.5**	**0.018**	-1.7	0.074
14	P63268	Actin, gamma-enteric smooth muscle[b)]	1.5	0.118	1.2	0.548	-1.7	0.077
	P31001	Desmin[a)]	1.5	0.118	1.2	0.548	-1.7	0.077

Cyt., cytosolic; mit., mitochondrial

a) C-terminal fragment

b) N-terminal fragment

Biological pathways changed during starvation

Data of differentially expressed protein spots were analyzed using GenMAPP and MAPPFinder software to explore biological processes which are changed during starvation. Results obtained for the 'local MAPPS' in GenMAPP point to the glycolysis and gluconeogenesis pathway. In total, six proteins (represented by eight protein spots) of this pathway significantly changed their expression levels in response to starvation (Figure 3). Most of these protein spots showed decreased expression levels during starvation compared to the fed condition, and they were identified as fructose-bisphosphate aldolase B, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate mutase 1, lactate dehydrogenase A, and cytoplasmic malate dehydrogenase. Triosephosphate isomerase displayed an

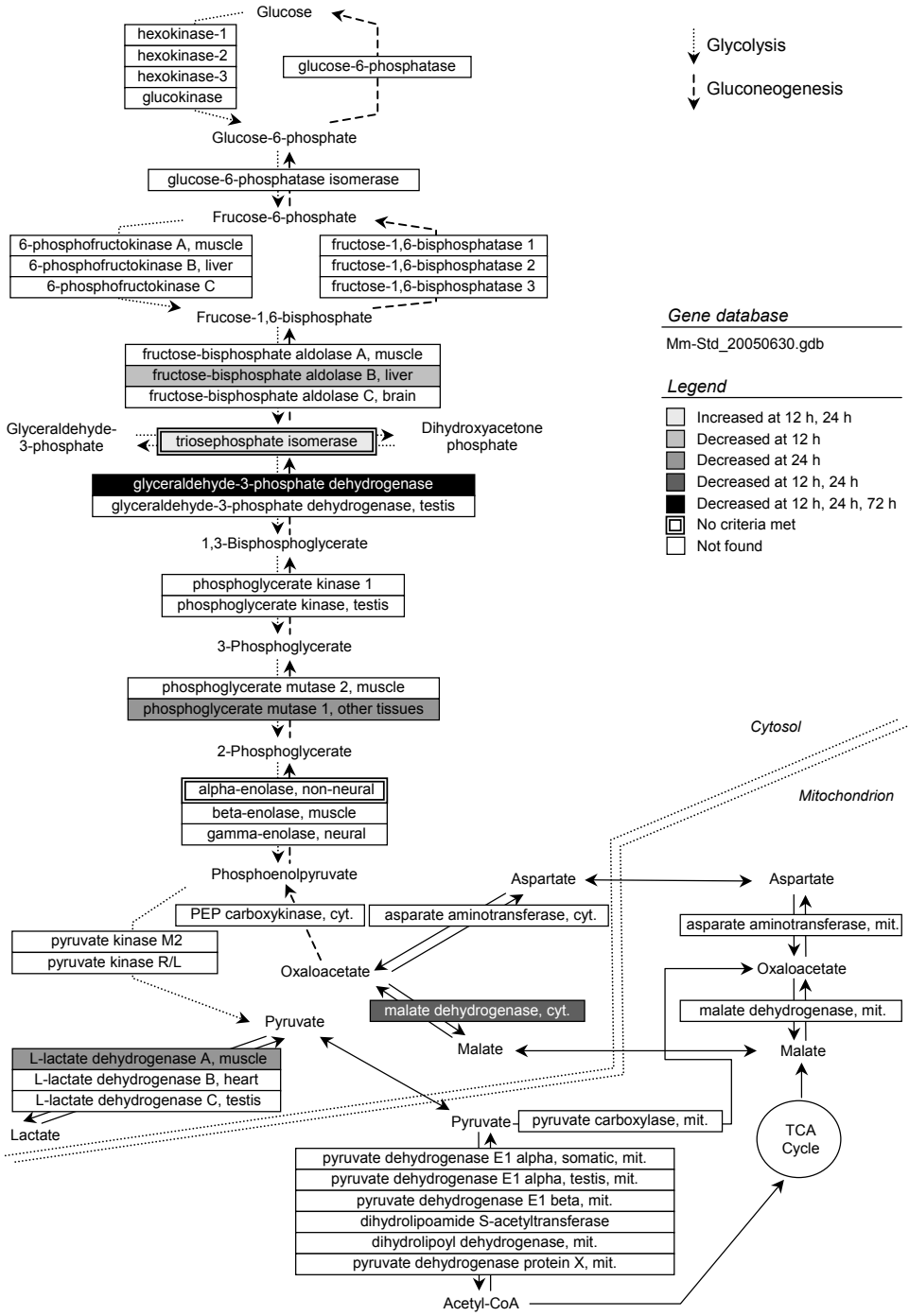

Figure 3. Glycolysis/gluconeogenesis pathway adapted from GenMAPP in which each box represents a protein. The MAPP is color-coded with expression data of intestinal proteins in response to starvation for 12, 24, and 72 h. Only significant changes (p<0.05) of at least 2-fold are indicated. Cyt., cytosolic; mit., mitochondrial; PEP, phosphoenolpyruvate.

increased expression level during starvation. Another protein spot identified as triosephosphate isomerase showed a decrease in expression levels after 12 and 24 h, but only for 1.6- and 1.9-fold ($p<0.05$), respectively (data not shown).

Functional characteristics of proteins with changed expression levels during different phases of starvation

Next to glycolysis proteins, we found other proteins that differed in expression level after a certain starvation period. These are grouped according to their functional characteristics (spot mixtures with two identified proteins are excluded). Data of mRNA levels (mentioned below) are available for several changed proteins and these data were obtained by microarray technology as described by Sokolović *et al.* (submitted).

Proteins involved in energy metabolism

Expression levels of ubiquinol-cytochrome-c reductase complex core protein I, ATP synthase beta chain, nucleoside diphosphate kinase B, and creatine kinase B were reduced in response to a 12-h fasting period. A C-terminal part of the ATP synthase beta chain was up-regulated in response to fasting at all time points.

Alcohol-metabolizing enzymes

Alcohol dehydrogenase [NADP+] and aldehyde dehydrogenase 1 family member B1 showed reduced expression levels after 12 h of fasting.

Protein folding and processing

After 12 and 24 h of starvation, peptidyl-prolyl cis–trans isomerase A (N-terminal part) and protein disulfide-isomerase (C-terminal part) were up-regulated. Full-length peptidyl-prolyl isomerase cis–trans isomerase A was decreased ($p<0.05$) after 24 and 72 h of starvation, but not 2-fold (data not shown). Calreticulin (spot 2, Figure 2, Table 1) was up-regulated upon starvation for 12 and 24 h. Another spot identified as the same protein (spot 1, Figure 2, Table 1) was down-regulated after 72 h. Microarray data showed comparable findings for calreticulin mRNA that was up-regulated after 12 h of starvation and down-regulated after 72 h of starvation. After 24 and 72 h of starvation, T-complex protein 1 subunit alpha B showed increased expression levels. Heat shock cognate 71-kDa protein (N-terminal part) was up-regulated after 72 h of starvation.

Proteins involved in protein synthesis

Elongation factor 1-beta and 2 (EF-1-beta and EF-2) showed decreased expression levels upon 24 h of starvation, EF-2 remained down-regulated after 72 h of starvation (see Figure 4). EF-2 mRNA levels were shown to be increased at all time points of starvation. The discrepancy between mRNA and protein levels of EF-2 was already demonstrated before in liver and muscle of 48-h-starved mice [14].

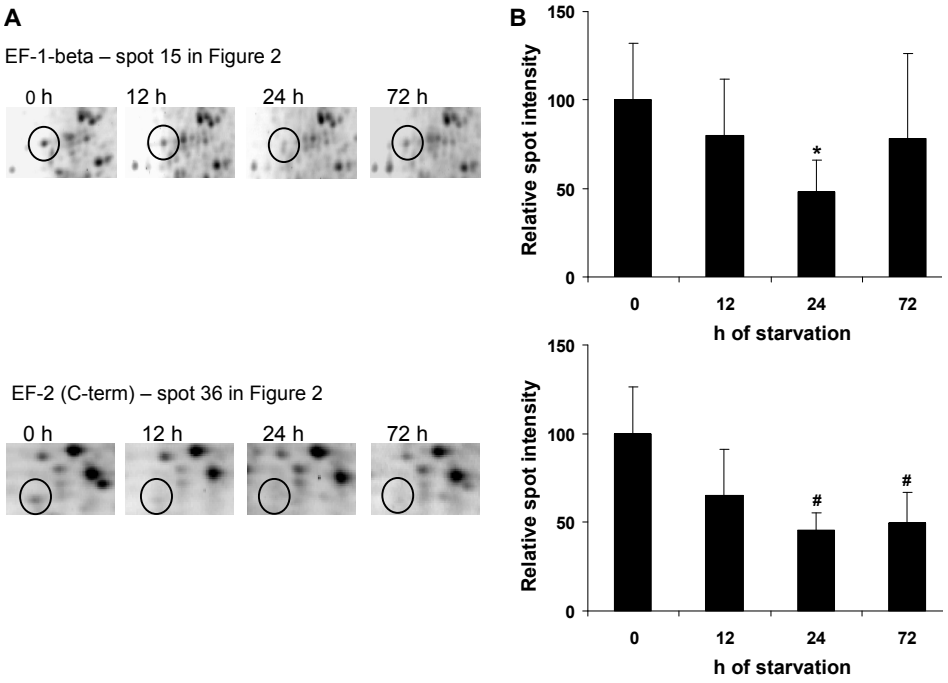

Figure 4. A. Examples of differentially expressed protein spots (spot 15 and 36 in Figure 2). Both spots, identified as EF-1-beta and EF-2, respectively, show a decrease in intensity after 24 h of starvation, which remains significant for EF-2 after 72 h of starvation. **B.** Graphical presentation of the expression patterns of spots depicted in A. The relative spot intensity of the control condition is set to 100. Values are means + SD of six biological replicates, * and # indicate significant differences compared to control group with $p<0.01$ and $p<0.005$, respectively (obtained from Student's t-test).

Proteins involved in amino acid metabolism

Expression levels of OAT and its C-terminal part were reduced after 24 and 72 h of starvation. This finding was confirmed with immunohistochemical staining of OAT protein in intestinal sections after 0 and 72 h of starvation. OAT levels are clearly reduced after starvation, especially at the tips of the villi (Figure 5). Microarray data showed also a down-regulation of OAT mRNA levels after 12, 24, and 72 h of starvation of 2.4-, 1.5- and 4.1-fold respectively. For arginase-2, a 2-

fold significant increase (p=0.020) was found comparing the 72-h-starved group with the 12-h-starved group.

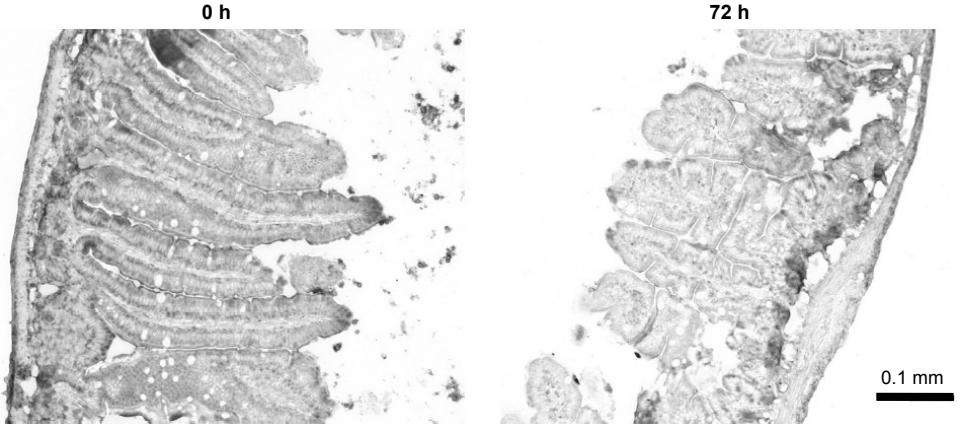

Figure 5. Immunohistochemical staining of OAT in section of the small intestine of fed (0 h) and 72-h-starved mouse (10x magnification).

Proteins with a role in the extracellular space

Glutathione peroxidase 3 (GPx-3) protein expression was gradually increased with prolonged fasting periods (significant from 24 h), whereas mRNA levels were increased after 12 and 24 h (2.3- and 1.7-fold, respectively). Lithostathine 1 and 2 were strongly up-regulated after 24 h of fasting. They remained up-regulated after 72 h of fasting (see Figure 6). Expression levels of pancreatic alpha-amylase (N-terminal part) were also increased compared to control levels (spot 51 after 24 h and spot 40 after 72 h, Figure 2, Table 1). Proteins with significantly decreased expression levels upon starvation are anterior gradient protein 2 homolog (after 12 and 24 h) and intelectin-1a (after 24 and 72 h). Fragments of serum albumin were up-regulated (spot 18, all time points; spot 13, 12 and 24 h).

Cytoskeleton-related proteins

Expression levels of desmin, transgelin, villin-1 (C-terminal part), and rho GDP-dissociation inhibitor 1 decreased after 24 h of starvation whereas ezrin was up-regulated after 72 h of starvation. Cytoplasmic actin was down-regulated after 72 h of starvation whereas its C-terminal part was up-regulated after 24 and 72 h of starvation. Gamma-enteric smooth muscle actin (N-terminal part) was up-regulated and full-length keratin type I cytoskeletal 19 and type II cytoskeletal 8 were down-regulated in response to starvation.

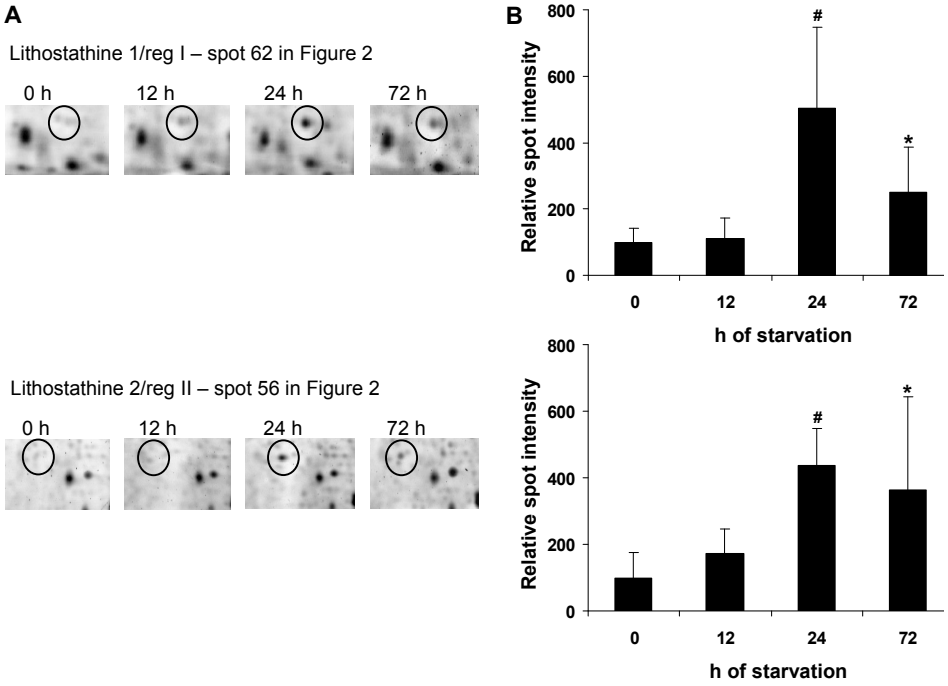

Figure 6. A. Examples of differentially expressed protein spots (spot 56 and 62 in Figure 2). Both spots, identified as lithostathine 2 (reg II) and 1 (reg I), respectively, show a strong increase in intensity after 24 h of starvation, which remains significant after 72 h of starvation. **B.** Graphical presentation of the expression patterns of spots depicted in A. The relative spot intensity of the control condition is set to 100. Values are means + SD of six biological replicates, * and # indicate significant differences compared to control group with $p<0.005$ and $p<0.05$, respectively (obtained from Student's t-test).

Ungrouped proteins

Proteins down-regulated upon 24 h of starvation are intestinal fatty acid-binding protein and sulfotransferase family cytosolic 1B member 1. Fumarylacetoacetate hydrolase domain-containing protein 1 was down-regulated after both 12 and 24 h of fasting. After 24 and 72 h of fasting, protein levels of proteasome subunit alpha type 1 and the N-terminal part of heterogeneous nuclear ribonucleoproteins A2/B1 were decreased and levels of carbonic anhydrase 3 was increased. Guanine deaminase showed reduced expression levels during the whole starvation period.

DISCUSSION

The gastrointestinal tract is a metabolically active organ consuming considerable amounts of energy [15]. When food supply, and thereby energy supply, is

restricted, structural and functional changes occur rapidly and energy-saving mechanisms are initiated resulting in a decline in metabolism [1]. It was shown, however, that the basic morphology of the mouse intestine remained unaffected, even after 72 h of starvation. The enterocytes and the smooth muscle cells represented approximately 71 and 24% of the intestinal tissue, respectively. Moreover, goblet cells were visualized and none of these cell types showed a significant change in its contribution to the entire small intestine during fasting (Sokolović *et al.*, submitted).

Using a proteomics approach, we showed here that early in the starvation period several proteins involved in glycolysis are down-regulated. This is in agreement with starvation-induced decreases in the activity of some glycolytic and citric acid-cycle enzymes in the intestine [16]. Furthermore, the down-regulation of glyceraldehyde-3-phosphate dehydrogenase and aldolase B can be explained by lysosomal proteolysis as both are substrates for chaperone-mediated autophagy, a process activated during nutrient limitation [17], and probably also during starvation. We speculate that the reduction in the level of glycolytic enzymes is a direct effect of the diminished glucose supply to the intestine.

Another mechanism involved in energy supply is ATP generation via oxidative phosphorylation. First, ubiquinol-cytochrome-c reductase complex core protein I, a constituent of the mitochondrial electron transport chain complex III, and ATP synthase beta chain, a subunit of the catalytic portion F_1 of the ATP synthase complex, are more than 2-fold reduced. The energy derived from the passage of electrons through complexes I, III, and IV of the respiratory chain is coupled to the synthesis of ATP. Mitochondrial ATP synthase catalyzes the ATP synthesis in the presence of a proton gradient across the inner mitochondrial membrane. Thus, the observed changes in protein expression in response to fasting indicate a decrease in ATP synthesis. A similar process has been described in the liver of 18-h-starved rats, where a 40% decrease in expression of the beta-F_1 subunit of ATP synthase was shown [18]. Finally, a reduced expression level of creatine kinase B-type was observed in the early phase of the starvation response. This enzyme catalyzes the reversible reaction of creatine and ATP forming phosphocreatine and ADP and plays a significant role in energy homeostasis of cells. As the ATP levels decrease, no excess ATP is available to be stored as phosphocreatine and consequently the enzyme levels decrease.

Another protein with reduced expression levels after 12 h of starvation is anterior gradient protein 2 homolog, also known as AG-2 or gob-4 protein, which is expressed in the goblet cells of the intestine. Goblet cells secrete mucus that covers the absorptive surface of the intestine, and gob-4 protein is suggested to have a role in the mucus-secreting function [19]. The mucus layer acts as a barrier between the luminal contents and the absorptive cells of the intestine and

comprises the first line defense against exogenous or endogenous luminal pathogens and irritants. Hence, decreases in proteins involved in this mucus secretion, like gob-4 expression during fasting, could have adverse effects on the intestinal mucus layer and its protective function.

Fragments of serum albumin are increased in response to starvation. Albumin is known to be synthesized by the liver; however, a recent study showed that nonhepatic tissues have also this capacity, and that albumin is expressed in the intestine of bovine [20]. Accordingly, intestinal albumin synthesis may be up-regulated when mice are subjected to starvation. Although, another explanation may be an increase in serum albumin catabolism, because only fragments of this protein were up-regulated [21].

In the subsequent phase of starvation (24 h), the change in protein expression particularly directs to protein and amino acid metabolism and cellular protection mechanisms. We observed a down-regulation of two key players involved in protein translation, EF-1-beta and EF-2, which suggests inhibition of protein synthesis during starvation [22]. Translation requires high amounts of metabolic energy and a known regulatory pathway inhibiting this process is via ATP depletion associated with fasting, resulting in an increased AMP/ATP ratio which consecutively results in AMPK activation. AMPK directly phosphorylates EF-2 kinase, which in turn inactivates EF-2 by phosphorylation [23]. In this manner, AMPK and EF-2 kinase may provide an important link between cellular energy status and the inhibition of protein synthesis, which seems a reasonable way to preserve energy during starvation.

In response to a 24-h starvation period, we also found a down-regulation of nucleoside diphosphate kinase B, which suggests a decrease of synthesis of nucleoside triphosphates other than ATP, such as GTP, that is involved in many metabolic and cellular processes such as protein synthesis and G-protein signaling [24].

With respect to amino acid metabolism, OAT was found to be down-regulated. This key enzyme is present predominantly in the small intestine and is involved in the conversion of glutamine to ornithine. Ornithine is a precursor for polyamines, known for their involvement in cell proliferation, cell differentiation, and repair for intestinal cells [25]. The decrease of this ornithine-synthesizing protein might be a glutamine-preserving mechanism, which adversely affects the intestinal integrity during starvation. Although arginase-2 is not differentially expressed in the 24-h-starvation response, the protein is discussed here because of its role in ornithine metabolism. The enzyme catalyzes the hydrolysis of arginine to ornithine and urea and is highly expressed in the small intestine of mice. It is co-localized with OAT and ornithine decarboxylase, suggesting a role in the synthesis of proline and polyamines [26]. Arginase-2 shows a biphasic response, and the levels gradually

increase in the period after 12 h of starvation. This might be a secondary response to the decreased OAT levels, which restores ornithine concentrations in the intestinal cells.

With respect to cellular protection, we observed a gradual increase of GPx-3 protein expression associated with increasing fasting times (24 and 72 h). This protein is responsible for the protection of cells against oxidative damage by catalyzing the reduction of hydrogen peroxide and lipid peroxides by glutathione and is an important reactive oxygen species scavenging enzyme. The mouse small intestinal epithelial cells synthesize GPx-3 and secrete it in the extracellular space [27]. Higher levels of oxidized glutathione were detected in the gut mucosa of 72-h-starved rats compared to controls, resulting from starvation-induced oxidative stress [2]. Another up-regulated protein with a role in oxidative stress handling is carbonic anhydrase 3 [28]. Up-regulating the expression of GPx-3 and carbonic anhydrase 3 in the small intestine coinciding prolonged fasting could play a role in the local antioxidant defense of intestinal cells.

Our data suggest that cellular protection coincides with preservation of intestinal integrity during later phases of starvation, as lithostathine 1 and 2 (also called reg I and reg II) are strongly up-regulated after 24 h starvation. They belong to the regenerating gene (reg) family within the superfamily of C-type lectin. The regeneration of pancreatic beta-cells seems to be mainly regulated by the expression of the reg I gene and led to the protein name 'reg protein' [29]. More research supported the hypothesis that reg protein has a trophic effect on islet cells [30]. In the rat stomach reg protein has been localized mainly in enterochromaffin-like cells. Levels are increased during regeneration of gastric mucosal cells, and the protein has a trophic effect on gastric epithelial cells [31,32]. The reg protein is expressed in human small intestinal cells localized in the crypts of Lieberkuhn and not in mature villous cells [33]. Therefore, reg protein may be associated with growth, and could have a role early in the differentiating process of intestinal epithelium [34]. The protein acts as an autocrine/paracrine growth factor for beta-cell regeneration via a cell surface reg receptor and this receptor is also found in the gastric fundic mucosa and many more tissues of rat, including the small intestine [35,36]. Several factors have been shown to enhance reg gene expression, for example gastrin in normal gastric mucosa and proinflammatory cytokines after gastric mucosal injury [32,37]. Reg II has only been described in mice [38]. We now identified starvation as a potent inducer of reg I and reg II protein expression, and postulate a protective effect of these proteins in the fasting gut.

Intelectin and some cytoskeleton-related proteins are down-regulated upon starvation and may be involved in the detrimental effects of fasting. Intelectin is a member of the lectin family and is expressed in the small intestine, colon, heart, and thymus. In the small intestine, the expression of intelectin is restricted to the

Paneth cells that are located in the lowest region of the crypts and may play a role in the defense against microorganisms [39]. Human intelectin has been proposed to play a role in the innate immune response to microbes containing the bacterium-specific carbohydrate galactofuranose [40]. A decreased intelectin expression, accompanied with starvation, may count partly for the increased susceptibility to infections during such stressful conditions [41].
Expression of ezrin, a component of the microvilli of intestinal epithelial cells, is induced by prolonged starvation (72 h). Ezrin-deficient mice revealed that ezrin functions to organize the apical terminal web and associated apical junctions that mediate cell-cell communication during villus morphogenesis. This leads to incomplete villus segregation and abnormal villus morphology [42]. The up-regulation of ezrin in the last starvation phase (phase III) might be an adaptive response to sustain villus function. In that phase of the starvation, an increase in mucosal mass and a decrease in apoptosis is observed in rats [5]. It is hypothesized to be a reaction to permit rapid food assimilation immediately after refeeding and thus, the restoration of the whole body condition [5]. This might be essential for survival since animals have reached a critical depletion level in their lipid reserves and body proteins.

Conclusion

Our results point to an adaptation of the intestine to the absence of nutrients supply during the early phase of starvation. In the first 12 h, mainly proteins involved in glycolysis and energy metabolism are decreased. This is followed by a down-regulation of proteins involved in protein synthesis and amino acid metabolism during 24 h. Simultaneously, the stressful conditions in the intestine seems counteracted by up-regulation of proteins with a protective role such as reg I and II, GPx-3, and carbonic anhydrase 3. The last starvation phase is characterized by increased ezrin expression, which may enhance villus morphogenesis critical for survival. In conclusion, our results contribute to a further understanding of the molecular events in the mouse intestine during starvation. This may benefit nutritional support during conditions characterized by malnutrition.

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SUPPORTING INFORMATION

Detailed protein identification characteristics. This material is available free of charge via the Internet at http://pubs.acs.org.

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CHAPTER 6

COMPARATIVE PROTEOMIC ANALYSIS OF CELL LINES AND SCRAPINGS OF THE HUMAN INTESTINAL EPITHELIUM

Kaatje Lenaerts, Freek Bouwman, Wouter Lamers, Johan Renes and Edwin Mariman

Submitted

ABSTRACT

In vitro models are indispensable study objects in the fields of cell and molecular biology, with advantages such as accessibility, homogeneity of the cell population, reproducibility, and growth rate. The Caco-2 cell line, originating from a colon carcinoma, is a widely used *in vitro* model for small intestinal epithelium. Cancer cells have an altered metabolism, making it difficult to infer its representativity for the tissue from which it is derived. This study was designed to compare the protein expression pattern of Caco-2 cells with the patterns of intestinal epithelial cells from human small and large intestine. HT-29 intestinal cells, Hep G2 liver cells and TE 671 muscle cells were included too, the latter two as negative controls.

Two-dimensional gel electrophoresis was performed on each tissue and cell line. Profiles of Caco-2 cells and small intestinal cells showed a considerable overlap in the type of proteins expressed. Principal component and cluster analysis, however, revealed that global expression of intestinal epithelial scrapings differed from that of intestinal epithelial cell lines. Since all cultured cell lines clustered together, this finding was ascribed to an adaptation of cells to culture conditions and their tumor origin, and responsible proteins were identified by mass spectrometry.

Caco-2 cells retained expression characteristic for intestinal epithelium, and the small intestine expressed biologically significant proteins which were also present in Caco-2 cells, indicating the usability of this model. Nevertheless, defined characteristics of the Caco-2 proteome indicate that care should be taken to prevent over- or misinterpretation of *in vitro* obtained findings when translating them to the *in vivo* situation.

INTRODUCTION

In vitro cell models play an important role in understanding cellular events related to (patho)physiological conditions in humans. Many advantages such as accessibility, homogeneity of the cell population, reproducibility, growth rate and hence the amount of material for analysis make them indispensable study objects in the search for molecular mechanisms. The differentiation process of intestinal cells, pathologies related with inflammatory conditions in the intestine, but also the adaptation of intestinal cells to certain nutritional conditions is often studied by an *in vitro* approach using intestinal epithelial cell models [1-5].

The human Caco-2 and HT-29 cell lines are commonly used models representing features of the human intestinal epithelium. Caco-2 cells were derived from a human colon adenocarcinoma, and they differentiate spontaneously *in vitro* under standard culture conditions thereby exhibiting enterocyte-like structural and functional characteristics [6]. In differentiated state, they mimic typical characteristics of the human small intestinal epithelium, like a well-developed brush border with associated enzymes, such as alkaline phosphatase and sucrase isomaltase [6]. Nevertheless, the Caco-2 cell model is different from the small intestine in several aspects, and their phenotype is dependent on the time in culture [7,8]. The HT-29 cell line is also of human colon adenocarcinoma origin, and cannot differentiate spontaneously *in vitro* under standard conditions, representing undifferentiated colonic epithelial cells [9].

Caco-2 cells, HT-29 cells, and many other widely used cell lines originate from tumour tissue, which alters the cellular metabolism drastically compared to physiological conditions. Therefore, it is difficult to interpret how representative a specific cell line is for the tissue from which it is derived. Furthermore, in comparison with the *in vivo* situation with influences from different neighboring cells, (neuro)endocrine regulators and blood flow, the *in vitro* model is relative simple. Although this is ideal for specific research questions, it complicates the translatability of *in vitro* results to *in vivo* situations.

Above all, it is not clear if cells in culture retain the gene expression profiles of their *in vivo* counterparts. A study regarding the gene expression patterns in 60 human cancer cell lines, including colon cancer cell lines, revealed that the tissue from which the cells are derived is the primary factor accounting for the variation in gene expression [10]. Gene expression was also investigated at a large scale in Caco-2 and HT-29 cells using microarray technology [11]. The current study aims to investigate similarities and differences in protein expression between cultured intestinal epithelial cells and epithelial scrapings obtained from human intestine. Protein isolates of cultured Caco-2 cells and HT-29 were subjected to two-dimensional gel electrophoresis (2-DE) and compared with the 2-DE patterns generated from normal epithelium from the small and large intestine. As a negative

control, protein profiles of the Hep G2 hepatoma cell line (liver) and the TE 671 rhabdomyosarcoma cell line (muscle) were used.

MATERIALS AND METHODS

Materials

The Caco-2 and Hep G2 cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA). The HT-29 and TE 671 cell lines were kindly provided by Dr. J. Keijer (RIKILT, Wageningen, The Netherlands) and Dr. M. De Baets (Dept. Neurology, Academical Hospital Maastricht, Maastricht, The Netherlands), respectively. Dulbecco's modified Eagle's medium (DMEM) and cell culture supplements were purchased from Invitrogen (Carlsbad, CA, USA) except fetal calf serum (FCS) which was from Bodinco (Alkmaar, The Netherlands). Dithiothreitol (DTT), 3-[(3-cholamidopropyl)dimethylamonio]-1-propanesulfonate (CHAPS), iodoacetamide, α-cyano-4-hydroxycinnamic acid, alcohol dehydrogenase and adrenocorticotropic hormone fragment 18-39 were from Sigma (St. Louis, MO, USA). Urea, SYPRO Ruby Protein Stain and all reagents for SDS-PAGE were from Bio-Rad Laboratories (Hercules, CA, USA). Immobiline Dry Strips (pH 3-11, nonlinear) and immobilized pH gradient (IPG) buffer (pH 3-10, nonlinear) were from Amersham Biosciences (Little Chalfont, England). Sequencing grade modified trypsin was from Promega (Madison, WI, USA).

Cell culture

All cells were cultured in DMEM supplemented with 1% (v/v) nonessential amino acid solution, 100 units/mL penicillin and 100 μg/mL streptomycin. For the Caco-2 cell line, 20% (v/v) FCS was added to this medium, whereas 10% (v/v) FCS was added for the other cell lines. Caco-2 cells were grown until confluence for 6 days onto 24 mm Transwell bicameral systems (Corning, Aston, MA, USA) with collagen-coated membranes (0.4 μm pore size, 4.7 cm2 surface area). After this period, cells were maintained in culture to differentiate for 5 and 15 days, representing a partially and a fully differentiated enterocytic phenotype, respectively. Hep G2 and HT-29 cells were cultured until confluence. The human muscle cell line TE 671 underwent myogenic differentiation for 5 days with 10^{-7} M 12-O-tetradecanoylphorbol-13-acetate (TPA) [12,13]. We also tested TE 671 cells not differentiated with TPA, but 2-D patterns were similar and for that reason left out of all analyses.

Sample preparation

Cell lines: the cells were washed three times with phosphate-buffered saline (PBS). Proteins were isolated from the cell lines by scraping them in ice-cold PBS, and centrifuging the obtained cell suspensions at 350 x *g* for 5 min at 4°C. Cell pellets were dissolved in a cell lysis buffer containing 8 M urea, 2% (w/v) CHAPS, 65 mM DTT, 0.5% (v/v) IPG buffer supplemented with Complete protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). This mixture was subjected to three cycles of freeze thawing, vortexed thoroughly and centrifuged at 20000 g for 30 min at 10ºC. Supernatant was collected and stored at –80 ºC until further analysis.

Epithelial scrapings: small intestine and colon (sigmoid colon) epithelial scrapings were obtained from patients who underwent Whipple pancreatico-duodenectomy and colectomy as a treatment for pancreas and colon cancer, respectively. An informed consent was obtained from the patients. The epithelial scrapings were derived from healthy tissue at least 10 cm removed from the tumor. The samples were snap frozen in liquid nitrogen and stored at –80 ºC until proteins were isolated. For protein isolation, samples were grinded in liquid nitrogen, after which cell lysis buffer (see above) was added. The rest was performed as described for the cell line material.

The protein concentrations of all protein samples were determined by a Bradford based protein assay [14].

2-DE

The 2-DE procedure was performed as described with minor modifications [15]. Briefly, 200 μg of total protein was separated by isoelectric focusing using Immobiline Dry Strips (24 cm, pH 3-11, nonlinear) according to the following protocol: 12 h at 30 V, 1 h at 500 V, 1 h at 1000 V, 2 h gradient from 1000 to 8000 V, 35 kVh at 8000 V. Strips were equilibrated and placed onto 12.5% SDS-polyacrylamide gels for protein separation in the second dimension. Electrophoresis was conducted at 200 V constantly for 6 h in a 24 cm Protein Dodeca Cell (Bio-Rad Laboratories). The gels were stained with SYPRO Ruby Protein Stain according to the manufacturer's protocol and the proteins were visualized by scanning gels with the Molecular Imager FX (Bio-Rad Laboratories).

Gel image and data analysis

Spot detection, matching and the examination of differentially expressed proteins was performed using PDQuest v7.3 (Bio-Rad Laboratories). Three technical

replicates were made per condition and formed one replicate group with average normalized spot intensities. A spot was regarded as significantly differentially expressed between groups if the average spot intensity differed 1.4-fold or more and if $p<0.05$ (Student's t-test) for at least one comparison. PCA and hierarchical clustering (GeneMaths XT software, Sint-Martens-Latem, Belgium) were applied as exploratory data analysis tools to compare protein spot patterns derived from the used tissues and cell lines.

In-gel digestion

For identification, protein spots were excised from the gel using an automated spot cutter (Bio-Rad Laboratories) and processed on a MassPREP digestion robot (Waters, Manchester, UK). A solution of 50 mM ammonium bicarbonate in 50% (v/v) acetonitrile was used for SYPRO Ruby destaining. Cysteines were reduced with 10 mM DTT in 100 mM ammonium bicarbonate for 30 minutes followed by alkylation with 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 20 minutes. Spots were washed with 100 mM ammonium bicarbonate to remove excess reagents and were subsequently dehydrated with 100% acetonitrile. Trypsin (6 ng/μl) in 50 mM ammonium bicarbonate was added to the gel plug and incubation was preformed at 37 °C for 5 h. The peptides were extracted with 1% (v/v) formic acid/2% (v/v) acetonitrile.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

For MALDI-TOF MS 1.5 μl of each peptide mixture and 0.5 μl matrix solution (2.5 mg/ml α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA) was spotted automatically onto a 96 well-format target plate. The spots were allowed to air dry for homogeneous crystallization. Spectra were obtained using an M@LDI-LR mass spectrometer (Waters). The instrument was operated in positive reflector mode. Acquisition mass range was 800-4000 Da. The instrument was calibrated on 10-12 reference masses from a tryptic digest of alcohol dehydrogenase. In addition, a near point lockmass correction for each sample spot was performed using adrenocorticotropic hormone fragment 18-39 (MH+ 2465.199) to achieve maximum mass accuracy. Typically 120 shots were combined and background subtracted. A peptide mass list was generated for the subsequent database search [16,17].

Database search

The peptide mass list generated with MassLynx v4.0.5 was searched with ProteinLynx Global Server v2.0.5 (Waters) or Mascot search engine (http://www.matrixscience.com) against the Swiss-Prot database (http://expasy.ch/sprot) for protein identification. Taxonomy was set to *Homo sapiens* and Mascot probability scores were calculated using 30 mass peaks or less with the highest signal intensity, trypsin and keratin peaks were excluded. One miss-cleavage was tolerated; carbamidomethylation was set as a fixed modification and oxidation of methionine as an optional modification. The peptide mass tolerance was set to 100 ppm. No restrictions were made on the protein molecular weight and the isoelectric point. A protein was regarded identified when it had a significant Mascot probability score (scores greater than 60 correspond to $p<0.05$), and at least four matched peptides, from which different forms of the same peptide were excluded.

RESULTS

Protein expression profiles of the intestinal scrapings and cell lines

A dendogram (Figure 1) generated by unsupervised hierarchical clustering of the averaged expression data of 2-D patterns revealed that the profiles of the epithelial scrapings (small intestine and large intestine) were separated from those of the cell lines. In the cell line cluster, profiles of cells of epithelial origin (Caco-2, HT-29 and Hep G2) were separated from the profile of cells of mesenchymal origin (TE 671).

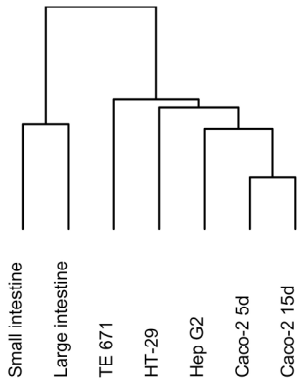


Figure 1. Unsupervised hierarchical clustering of protein profiles. A dendogram, generated by unsupervised hierarchical clustering of averaged protein expression data of 2-D profiles of protein isolates from small and large intestinal scrapings, partially and fully differentiated Caco-2 cells, HT-29 cells, Hep G2 cells and TE 671 cells.

To get further insight in how 2-D patterns of intestinal Caco-2 cells (partially and fully differentiated) and HT-29 cells are related to profiles of small and large intestinal epithelial scrapings, a principal component analysis (PCA) was performed. PCA allows for grouping of cells with overall similar protein expression characteristics and for identifying proteins which are responsible for the differences between the groups. The plot shown in Figure 2A is a simple scatter plot of the first two principal components (PC). PC1 and PC2 explain 48.9% and 13.5% of the total variation, respectively. Triplicate gels cluster together which indicates that the biological variation is responsible for the clear separation of the different samples. Along PC1, three distinct groups are visible, of which one corresponds to all cultured cell lines, independent of tissue origin. In the cluster of the cell lines, a sub-cluster exists along PC2 of profiles from cell lines of epithelial origin (Caco-2, HT-29 and Hep G2). When blood-derived proteins were removed from the data set, the PCA plot is very similar but PC1 explains only 30.3% of the total variation, largely because profiles of large intestinal cells move closer towards small intestinal cells along PC1, whereas PC2 explains 18.3% (data not shown).
Protein data of all *in vitro* cell cultures were used to generate a set of protein spots (within a circle of 25% from the plot center), which have the same relative abundance in all types of cell lines studied. These proteins are present in all *in vitro* cultured cells included in this analysis, and seem not much contributory to the specific phenotype of a certain cell line. Therefore, another PCA was performed, in which expression data of the 25% proteins common for *in vitro* cell cultures were subtracted from the data set (without blood proteins), so proteins that make a cell line unique contribute more to the overall pattern. The outcome of the analysis, displayed as a scatter plot of PC1 and PC2, revealed that protein expression patterns of scrapings and cell lines are still separated (Figure 2B). Along PC1 (explaining 27.9% of the variation), the 2-D profiles of the intestinal cell lines, Caco-2 cells and HT-29 cells, are visible at a similar range, but most distant from the profiles of the epithelial scrapings. Along PC2 (explaining 15.5% of the variation) the profiles of Caco-2 cells appear at a similar position as those of the small intestinal cells, whereas HT-29 appear at a similar position as those of the large intestinal cells.

Protein expression in the intestinal scrapings and cell lines

In PDQuest, 2-D patterns of all protein isolates under study were compared and resulted in 133 differentially expressed proteins. In addition, 6 protein spots were selected that showed a similar expression pattern in all isolates. Out of 139 protein spots, 94 spots (68%) were identified by MALDI-TOF MS.

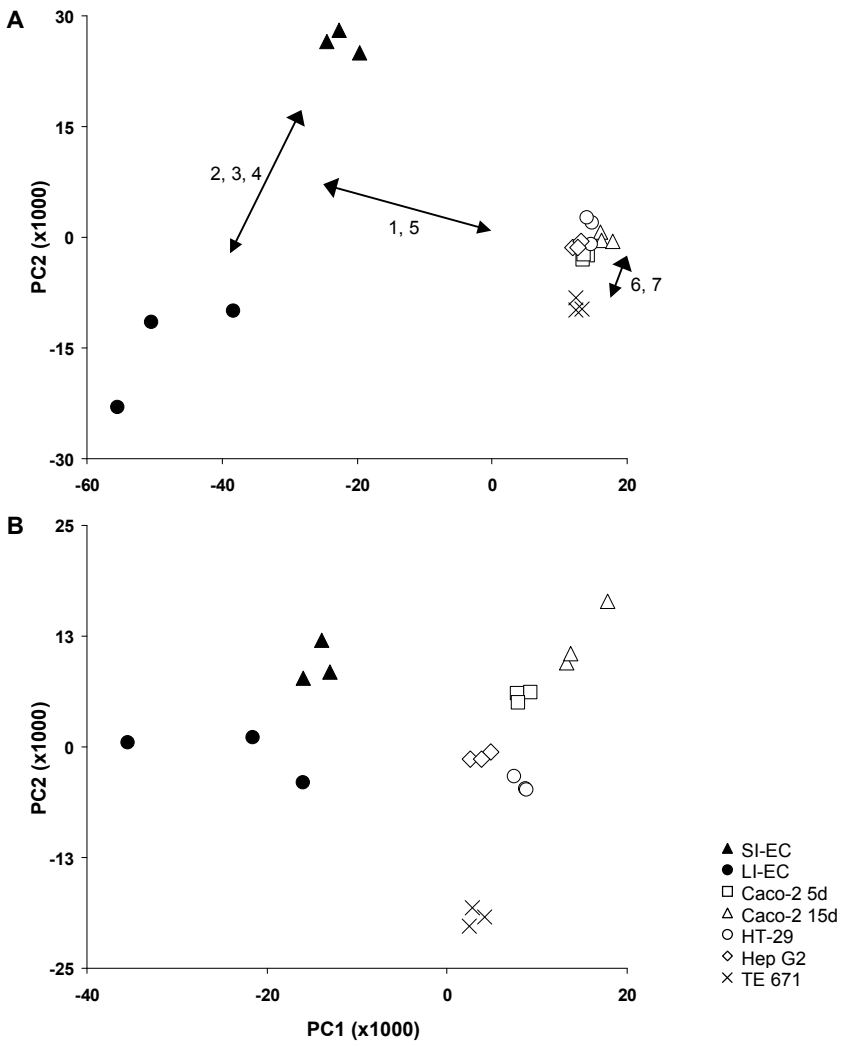
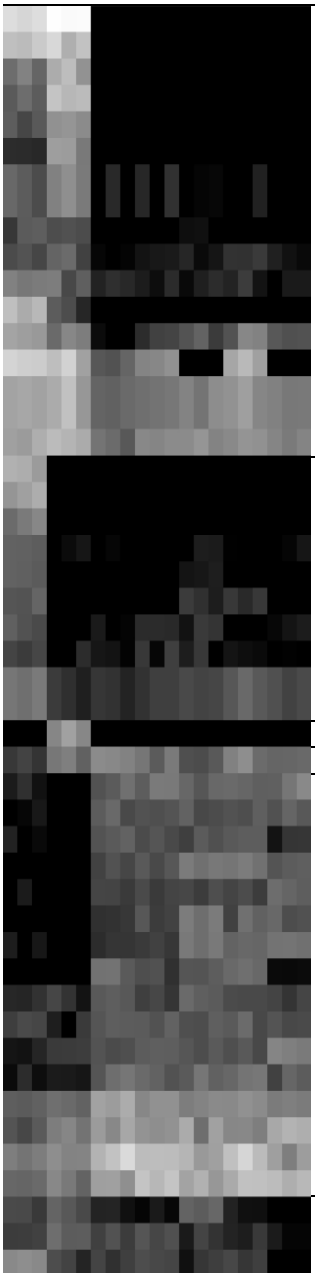
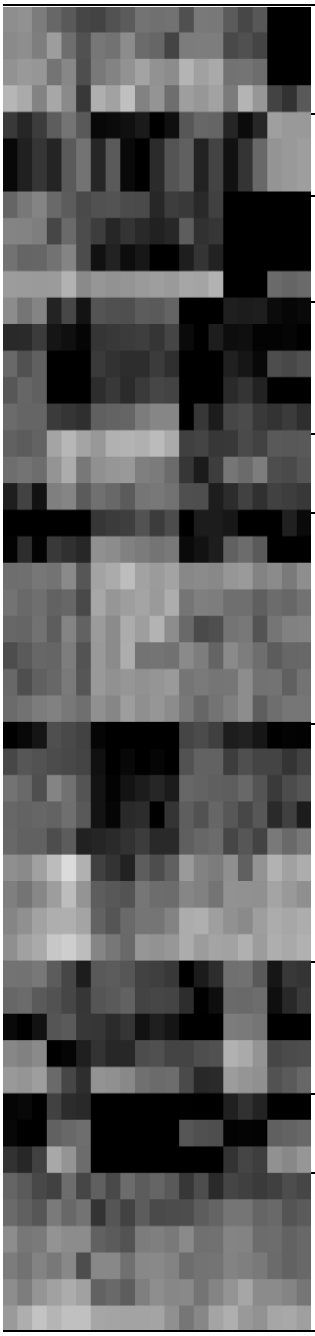


Figure 2. A. PCA projection of protein profiles obtained by 2-DE in a scatter plot using the entire protein data set. PC1 and PC2 explain 48.9% and 13.5% of the total variation, respectively. The cluster numbers containing proteins which are mainly responsible for separation between groups are depicted next to the arrows. **B.** PCA projection of protein profiles obtained by 2-DE in a scatter plot using the data set after subtracting data points corresponding to proteins common for in vitro cell cultures. PC1 and PC2 explain 27.9% and 15.5% of the total variation, respectively. SI, small intestine; LI, large intestine; EC, epithelial cells.

Identified proteins were grouped in 15 clusters according to their expression pattern in the analyzed samples. These clusters of protein spots are shown in Table 1, in which triplicate log-transformed expression values in different samples are shown by a grayscale. The spot numbers correspond to those indicated in Figure 3.

Table 1. Clustering of identified protein spots according the expression levels in analyzed samples. Identified protein spots grouped in different clusters according to the expression level in different samples. Triplicate spot intensities are indicated by a grayscale in which white, gray, and black represent high, average, and low levels of protein expression.

Expression pattern[a)] 1 2 3 4 5 6 7	Spot	Swiss- Prot accession number	Protein name	Cl. number
	92	P68871	Hemoglobin subunit beta	1
	93	P69905	Hemoglobin subunit alpha	1
	21	P02768	Serum albumin	1
	56	P02768	Serum albumin	1
	45	P00915	Carbonic anhydrase 1	1
	85	P02766	Transthyretin	1
	18-1	P30101	Protein disulfide-isomerase A3	1
	18-2	P12955	Xaa-Pro dipeptidase	1
	43	P28062	Proteasome subunit beta type 8	1
	33	P14550	Alcohol dehydrogenase [NADP+]	1
	61	P35232	Prohibitin	1
	86	P12104	FABP, intestinal	1
	44	P30086	Phosphatidylethanolamine-binding protein	1
	87	P07148	FABP, liver	1
	65-1	P30085	UMP-CMP kinase	1
	65-2	P32119	Peroxiredoxin 2	1
	90	P23528	Cofilin-1	1
	83	P50120	RBP II, cellular	2
	36	P05062	Fructose-bisphosphate aldolase B	2
	35	P05062	Fructose-bisphosphate aldolase B	2
	52	P50053	Ketohexokinase	2
	75	P28065	Proteasome subunit beta type 9	2
	81	P00167	Cytochrome b5	2
	51	P78417	Glutathione transferase omega 1	2
	11	Q9Y2T3	Guanine deaminase	2
	49-1	P30039	MAWD binding protein	2
	49-2	P25786	Proteasome subunit alpha type 1	2
	48	P02768	Serum albumin	3
	57	Q13162	Peroxiredoxin 4	4
	3	P11021	78-kDa glucose-regulated protein	5
	27	P14866	HnRNP L	5
	82	Q9Y2B0	MIR-interacting saposin-like protein	5
	4	P07437	Tubulin beta-2 chain	5
	29	P14866	HnRNP L	5
	2	P07237	Protein disulfide-isomerase	5
	74	P13693	Translationally-controlled tumor protein	5
	80	P60660	Myosin light polypeptide 6	5
	19	P61979	HnRNP K	5
	25	P49411	Elongation factor Tu, mit.	5
	38	P22626	HnRNPs A2/B1	5
	7	Q15084	Protein disulfide-isomerase A6	5
	62	P35232	Prohibitin	5
	37	P22626	HnRNPs A2/B1	5
	9	P10809	60-kDa HSP, mit.	5
	94	P61604	10-kDa HSP, mit.	5
	72	P31947	Epithelial cell marker protein 1	6
	34	Q16762	Thiosulfate sulfurtransferase	6
	60	P50224	Monoamine-sulfating phenol sulfotransferase	6

Expression pattern[a)] 1 2 3 4 5 6 7	Spot	Swiss- Prot accession number	Protein name	Cl. number
	59	P09525	Annexin A4	6
	79	P60660	Myosin light polypeptide 6	6
	17	P05787	Keratin, type II cytoskeletal 8	6
	78	P62158	Calmodulin	6
	54	P04792	Heat-shock protein beta-1	7
	8-1	P08670	Vimentin	7
	8-2	P05787	Keratin, type II cytoskeletal 8	7
	32	P54868	Hydroxymethylglutaryl-CoA synthase, mit.	8
	16	P35900	Keratin, type I cytoskeletal 20	8
	10	P35900	Keratin, type I cytoskeletal 20	8
	64	P09211	Glutathione S-transferase P	8
	66	Q9NRV9	Heme-binding protein 1	9
	23	P31040	Succinate dehydrogenase, mit.	9
	50	P50225	Sulfotransferase 1A1	9
	28	Q16822	Phosphoenolpyruvate carboxykinase, mit.	9
	26	P04181	Ornithine aminotransferase, mit.	9
	40	Q13268	Dehydrogenase/reductase SDR 2	10
	41	P17931	Galectin-3	10
	14	P12277	Creatine kinase B-type	10
	22	P13674	Prolyl 4-hydroxylase alpha-1	11
	70	P07858	Cathepsin B precursor	11
	1	P27797	Calreticulin	11
	53	P30084	Enoyl-CoA hydratase, mit.	11
	39	P21796	Voltage-dependent anion-selective channel 1	11
	20	P38646	Stress-70 protein, mit.	11
	55	P30048	Thioredoxin-dependent peroxide reductase	11
	5	P06576	ATP synthase beta chain, mit.	11
	73	P15374	Ubiquitin carboxyl-terminal hydrolase L3	12
	71	P28066	Proteasome subunit alpha type 5	12
	69	P52565	Rho GDP-dissociation inhibitor 1	12
	24	P00352	Retinal dehydrogenase 1	12
	63	P28070	Proteasome subunit beta type 4	12
	91	P07737	Profilin-1	12
	42	Q06830	Peroxiredoxin 1	12
	88	P62937	Peptidyl-prolyl cis-trans isomerase A	12
	89	P62937	Peptidyl-prolyl cis-trans isomerase A	12
	77	P07237	Protein disulfide-isomerase	13
	13	P07237	Protein disulfide-isomerase	13
	67	P02753	Plasma RBP	13
	58	P07339	Cathepsin D precursor	13
	76	P07237	Protein disulfide-isomerase	13
	30	O60701	UDP-glucose 6-dehydrogenase	14
	6	Q15293	Reticulocalbin-1 precursor	14
	84	P09382	Galectin-1	14
	15	P31930	Ubiquinol-cytochrome-c reductase I, mit.	15
	46	P18669	Phosphoglycerate mutase	15
	47	P60174	Triosephosphate isomerase	15
	68	O75947	ATP synthase D chain, mit.	15
	31	P06733	Alpha-enolase	15
	12	P60709	Beta-actin	15

a) 1, Small intestine; 2, Large intestine; 3, Caco-2 cells (5-day-differentiated); 4, Caco-2 cells (15-day-differentiated); 5, HT-29 cells; 6, Hep G2 cells; 7, TE 671 cells.

Data are log-transformed to obtain a better visualization of the color range indicating protein expression.

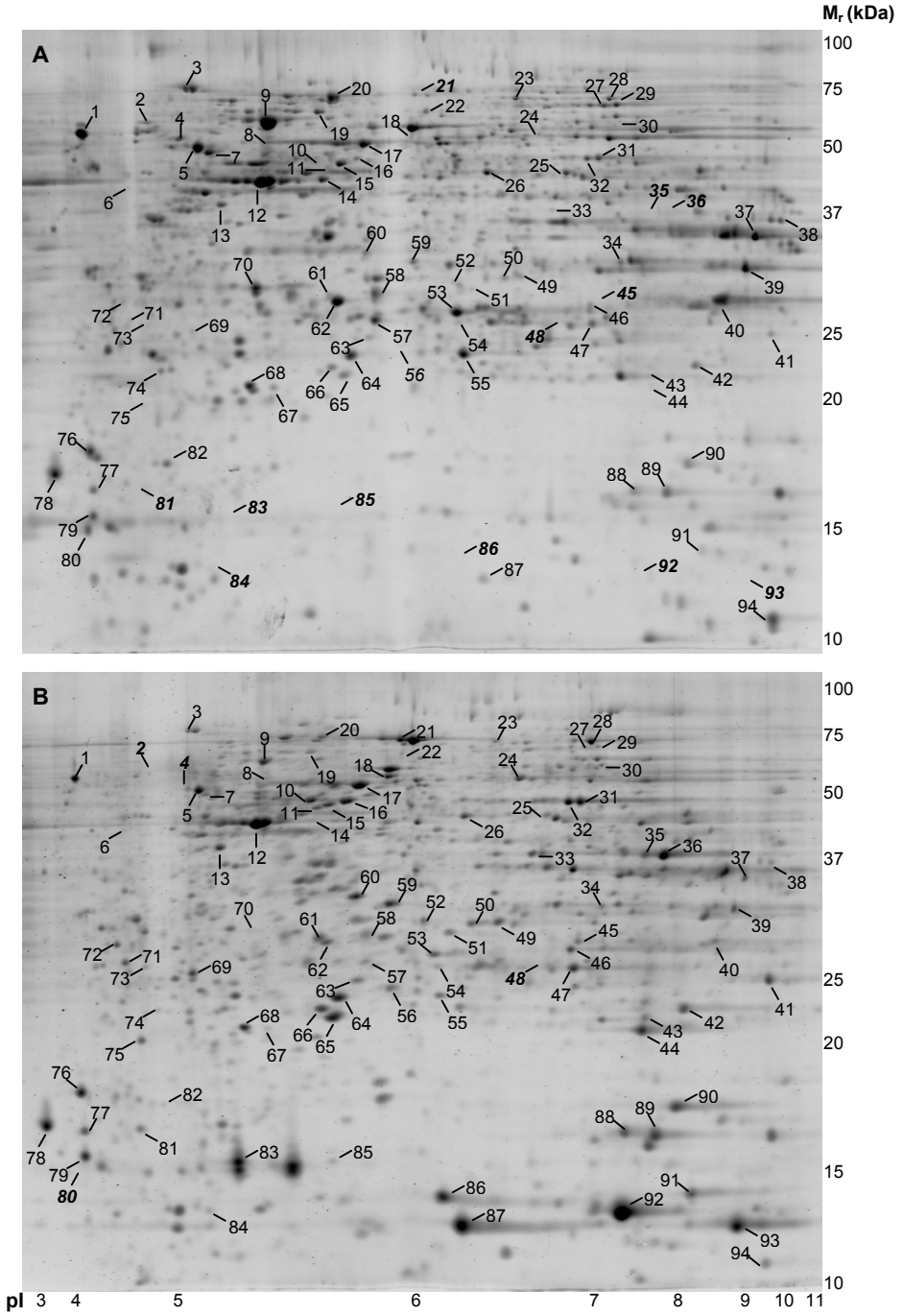


Figure 3. 2-D protein profiles of 5-day-differentiated Caco-2 cells (**A**) and human small intestinal epithelial cells (**B**). Identified protein spots are indicated with spot numbers corresponding to those in Table 1. When a spot number is indicated in bold/italic, this means that the spot could not be observed in the particular pattern.

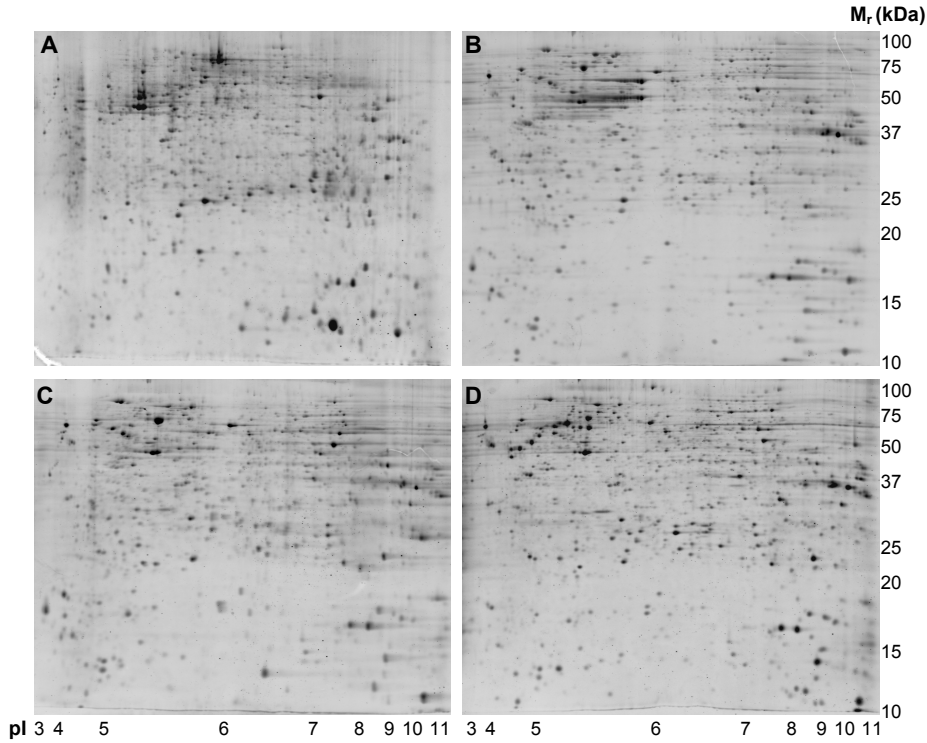


Figure 4. 2-D protein profiles of human large intestinal epithelial scrapings (**A**), HT-29 cells (**B**), Hep G2 cells (**C**) and TE 671 cells (**D**).

Figure 3 shows a 2-D pattern of 5-day-differentiated Caco-2 cells (Figure 3A), and a 2-D pattern of a small intestinal epithelial scraping (Figure 3B) to give an indication of the overlap of the profiles. The spot numbers of spots which were not present in one of these patterns are indicated in bold, italic. Figure 4 shows 2-D patterns of large intestinal epithelium, HT-29, Hep G2 and TE 671 cells to obtain a general view on the differences and similarities of the different patterns.
Cluster 1 contains proteins which show a high expression in both intestinal epithelial scrapings relative to the *in vitro* cell cultures. Cluster 2 and 3 consist of proteins with a high expression level in small and large intestinal scrapings, respectively, whereas one protein with a low expression level in the small intestine is displayed in cluster 4. Proteins with low expression levels in the intestinal scrapings are grouped in cluster 5. Cluster 6 contains proteins with a high expression in all cells of epithelial origin, and cluster 7 contains proteins with a low expression in those cells. Cluster 8 shows proteins with a high expression in all protein isolates of intestinal origin, namely small and large intestinal epithelium, partially and fully differentiated Caco-2 cells and HT-29 cells. Protein spots with a

high expression in small intestinal epithelium and Caco-2 cells are shown in cluster 9, and proteins with a high expression in large intestinal epithelial cells and Caco-2 cells are shown in cluster 10. Cluster 11 and 12 represent proteins with a high and low expression in Caco-2 cells, respectively. Cluster 13 contains proteins mainly expressed in Hep G2 but with co-expression in some other cell types, the same applies for cluster 14, but then in large intestinal cells. The last cluster, cluster 15, shows proteins with a relatively similar expression pattern in all protein isolates.

DISCUSSION

The current proteomics approach aims to obtain a better insight in the expression patterns of human intestinal epithelial cells and the commonly used *in vitro* models for these cells, Caco-2 cells and HT-29 cells, both derived of human intestinal adenocarcinoma. This information is highly relevant to researchers using such models, and aiming for translating data to the *in vivo* situation.

When comparing the 2-D pattern of Caco-2 cells and small intestinal cells, it becomes apparent that many protein spots are present in both profiles, indicating a great deal of overlap in the type of proteins expressed, supporting the usability of this *in vitro* model. However, several high abundant proteins in human epithelial cells of the small intestine are not expressed or expressed at low levels in Caco-2 cells, and *vice versa*. This might be caused by the transformed status of Caco-2 cells and an adaptation of their metabolism to growth conditions outside the body. This is supported by the clustering data, which revealed a separation of the epithelial scrapings and all *in vitro* cell lines, irrespective of their tissue origin, suggesting that the protein expression profiles were quite similar among each of the cell lines, but different from the protein profiles of epithelium *in vivo*.

When exploring the clusters explaining the main variation in expression, noticeably, typical proteins with a higher expression in the scrapings are hemoglobin alpha and beta, which seem to originate from blood cells. More possible contaminants are serum albumin and transthyretin, both uniquely expressed in the scrapings, although with the highest expression in cells from the large intestine, indicating that this sample is probably more contaminated with blood. This finding demonstrates the difficulty of obtaining pure cell isolates, and points to the advantage of using *in vitro* cell lines, which consist of a homogenous cell population.

Other proteins which are responsible for the distinction of the *in vitro* and *in vivo* proteomes are involved in lipid and carbohydrate metabolism, with, in general, a more pronounced expression in the small intestinal epithelial cells. On the other hand, heat shock proteins (HSPs) and heterogeneous nuclear ribonucleoproteins (hnRNPs) are higher expressed in the *in vitro* protein profiles.

Intestinal fatty-acid binding protein (FABP) expression is only observed in the protein profiles of intestinal scrapings, and the expression is markedly higher in small intestinal epithelium compared to large intestinal epithelium. This was observed in another study as well, in which levels were tested at different segments of the human intestinal tract [18]. Liver FABP is highly expressed in intestinal scrapings, but shows expression in liver Hep G2 cells too. In Caco-2 cells, its expression is less, and increases with differentiation, which is in agreement with a previous study [19]. Another member of the FABP family is cellular retinol-binding protein (RBP) II, and this protein is solely expressed in the small intestinal epithelial scrapings. This vitamin A-binding protein is abundant in the human small intestinal mucosa and is localized to the villus-associated enterocytes by immunohistochemistry [20]. In adult rats, expression of cellular RBP II is restricted to villus-associated enterocytes in the proximal small intestine [21]. This protein may be important for intestinal absorption and/or metabolism of retinol. It is also reported in literature that Caco-2 cells express the protein, and that the expression increases with differentiation [22]. However, we failed to identify the protein in the profiles of these cells in the current study, probably because its abundance is beyond the detection limit of our method.

Two proteins involved in fructose metabolism, ketohexokinase and fructose-bisphosphate aldolase B, are also higher expressed in the small intestine, of which the latter one is even exclusively expressed in the small intestinal scrapings. This enzyme is known to be primarily expressed in liver, small intestine and kidney [23]. In Hep G2 cells, aldolase B mRNA was shown to be weakly expressed [24]. In comparison, differentiated Caco-2 cells express fructose-bisphosphate aldolase A and C mRNA [25]. Recently, we identified the fructose-bisphosphate aldolase A protein in Caco-2 cells [26].

To recapitulate, several proteins with a role in the metabolism of lipids and carbohydrates are higher expressed, or exclusively expressed in the *in vivo* intestinal material. This may be a reflection of the intestine's capacity in absorbing and metabolizing complex mixtures of nutrients. *In vitro* cells, on the contrary, are probably adapted to the culture medium used, which has a rather simple composition.

Prohibitin is present as two spots in the gels, from which one spot (no. 61, cluster 1) shows a strong expression whereas the other spot (no. 62, cluster 5) shows a low expression in the epithelial scrapings, compared to the cultured cells. Similarly, two spots were identified as myosin light polypeptide 6 (no. 80, cluster 5 and no. 79, cluster 6) from which one spot is absent in the epithelium *in vivo*, while the other spot is as abundant as in most cell lines. These data indicate that gene products might be processed via alternative pathways in *in vitro* cell cultures.

Next to specific forms of prohibitin and myosin light polypeptide 6, we identified more proteins with a typically higher expression in all cultured cells tested here. Compared to normal tissue, the higher expression levels of proteins discussed below might be explained by the tumoral origin of the *in vitro* cell lines tested. One should be aware of this phenomenon when making use of such cell lines. HnRNPs K, L and A2/B1 belong to a family of proteins with central roles in DNA repair, telomere elongation, cell signaling and in regulating gene expression at transcriptional and translational level. Through these key cellular functions, individual hnRNPs have a variety of potential roles in tumor development [27]. Several hnRNPs, under which hnRNP K and A2/B1 are up-regulated in various cancers [27]. Translationally-controlled tumor protein is also found to be higher expressed in *in vitro* cultured cells. It is not a tumor-specific protein, although its expression level tends to be higher in tumors, compared to the corresponding normal tissue [28]. The protein is highly conserved and widely expressed in all eukaryotic organisms, and is thought to be important for cell growth and division [29]. Several HSPs, like 78-kDa glucose-regulated protein, 10-kDa HSP and 60-kDa HSP belong to this group. It was demonstrated before that HSPs, under which 78-kDa glucose-regulated protein, are expressed in increased amounts in many tumours [30]. Recently, 10-kDa HSP was shown to be increased in colorectal cancer tissue compared to normal tissue [31]. Elevated HSP expression has a cytoprotective role. For example, individual HSPs are able to block the pathways of apoptosis, and hence appear to play a role in tumor pathogenesis.

A set of proteins was higher expressed in all cells of epithelial origin included in this analysis. Two enzymes with a role in detoxification, monoamine-sulphating phenol sulfotransferase and thiosulfate sulfurtransferase, show such an expression pattern. Expression of monoamine-sulphating phenol sulfotransferase was already demonstrated in the human jejunal mucosa and the Caco-2 cell line [32, 33]. Thiosulfate sulfurtransferase activity was present in the small intestine, colon and rectum of human [34], but high activity of this enzyme was also shown in liver and kidney of rat [35]. Annexin A4 shows a comparable expression pattern. The protein belongs to a ubiquitous family of Ca2+-dependent membrane-binding proteins thought to be involved in membrane trafficking and membrane organization within cells and is found at high levels in many epithelial cells, where it is closely associated with the apical region [36]. Epithelial cell marker protein 1, also called stratifin or 14-3-3 sigma, was also highly expressed in the epithelial cells but was not observed in the mesenchymal TE 671 cells. In contrast, HSP beta-1 and a protein spot containing both vimentin and keratin type II cytoskeletal 8 were highest expressed in TE 671 cells. These data indicate that the epithelial character is retained in Caco-2 cells.

Proteins with a higher expression in the intestinal cell lines Caco-2 and HT-29 and in both the small intestine and colon epithelial scrapings compared to negative controls Hep G2 and TE 671 are hydroxymethylglutaryl-CoA synthase, keratin type 1 cytoskeletal 20, and glutathione S-transferase P. These proteins could have an important role in gut epithelial cells, as all profiles of intestinal origin, being from *in vitro* cultures or scrapings, express them. Keratin type 1 cytoskeletal 20 is such a protein identified as a major cytoskeletal polypeptide of the human intestinal epithelium [37], and is thought to have a role in the intermediate filament organization in intestinal epithelium [38]. Hydroxymethylglutaryl-CoA synthase is a control enzyme in the synthesis of ketone bodies, and is highly expressed in liver and colon, and low in testis, heart, skeletal muscle, and kidney [39]. Expression in the human small intestine is reported here for the first time.

Next, Caco-2 cells share common characteristics with both small and large intestinal epithelial scrapings. Proteins with a higher expression in Caco-2 cells and small intestinal cells were identified as heme-binding protein 1, succinate dehydrogenase, and ornithine aminotransferase, and hence they can be used as markers for the small intestinal epithelial phenotype. Succinate dehydrogenase and mitochondrial phosphoenolpyruvate carboxykinase are also shown in this cluster, but they show also expression in TE 671 and Hep G2 cells, respectively. Ornithine aminotransferase appears to be an important protein in the intestine, and is down-regulated by glutamine depletion in Caco-2 cells and starvation in mouse small intestine [5, 40]. This key enzyme is present predominantly in the small intestine and is involved in the conversion of glutamine to ornithine. Ornithine is a precursor for polyamines, known for their involvement in cell proliferation, cell differentiation and repair for intestinal cells [41]. Mitochondrial phosphoenolpyruvate carboxykinase has an important role in gluconeogenesis, where it catalyzes the conversion of oxaloacetate to phosphoenolpyruvate. The gene is expressed in various human tissues, but mainly in liver, kidney, pancreas, intestine, and fibroblasts [42], which fits with our observation. In contrast with the cytosolic counterpart, this protein is constitutively expressed and not inducible by glucagon [43]. We found that glutamine depletion in Caco-2 cells resulted in a decreased expression of this protein [15]. As mentioned before, Caco-2 cells share also protein expression characteristics with colonic scraping, namely a higher expression of dehydrogenase/reductase SDR 2, galectin-3, and creatine kinase B. We can deduce from this that the Caco-2 cells express a mixture of the colonic and enterocytic phenotype [7], and protein markers for these phenotypes are provided.

Proteins with a higher or lower expression in Caco-2 cells compared to all other protein isolates were also observed, and this information needs to be considered when studying processes in which one of these proteins play an important role. Mostly, protein expression of partially differentiated Caco-2 cells equals that of

fully differentiated Caco-2 cells. However, some proteins like profilin-1, peroxiredoxin 1 and peptidyl-prolyl cis-trans isomerase A (cluster 12) show higher expression in fully differentiated Caco-2 cells, and hence resemble expression levels in the small intestine more closely. This counts also for mitochondrial stress-70 protein (cluster 11) which shows higher expression in partially differentiated Caco-2 cells, compared to the rest of the cells. Conversely, the protein level of ornithine aminotransferase in the small intestine equals that of partially differentiated Caco-2 cells. However, generally considered, data suggest that protein expression in 15-day-differentiated Caco-2 cells correlates more with expression in the small intestinal epithelial scraping, but differences are only minor compared to 5-day-differentiated cells.

Hep G2 cells have the highest plasma RBP levels, which is in line with the liver being the major site for plasma RBP synthesis, where it is secreted in the blood for retinol transport to the peripheral tissues. However, other sites of plasma RBP secretion are reported [44]. Epithelial scrapings of the small and the large intestine contain this protein as well; however, we cannot exclude the possibility that it is derived from blood. The finding that plasma RBP is expressed at comparable levels in Caco-2 cells supports the idea that intestinal cells can synthesize the protein too. Recently, we have shown that plasma RBP was down-regulated considerably by glutamine deficiency in Caco-2 cells [5], and this may have relevance for the intestinal condition *in vivo*.

Proteins with a role in cytoskeletal function and proteasome activity are present throughout different clusters, which makes it difficult to interpret data concerning these central cellular processes. Some proteins involved in carbohydrate metabolism, namely triosephosphate isomerase, phosphoglycerate mutase and alpha enolase are similarly expressed in all samples (cell lines and scrapings) tested.

CONCLUSIONS

This study was designed to compare expression profiles of widely used cell intestinal cell models with their *in vivo* counterparts. PCA revealed that global protein expression of intestinal epithelial scrapings differed considerably with that of intestinal epithelial cell lines. The co-analysis of protein expression in liver and muscle cell lines was required to prevent misclassification of intestine-specific proteins, since all 2-D patterns showed a considerable number of overlapping proteins. Data showed that Caco-2 cells retained expression characteristics for intestinal epithelium, and the small intestine expressed biologically significant proteins which were also present in Caco-2 cells, indicating the usability of this *in vitro* model. Nevertheless, care should be taken to prevent over- or

misinterpretation of *in vitro* obtained findings when translating them to the *in vivo* situation.

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CHAPTER 7

GENERAL DISCUSSION

INTRODUCTION

Nutritional deficiencies often occur in states of critical illness, thereby altering the intestinal condition. The maintenance of intestinal epithelial cell integrity and function is essential in the defense against pathogens, and prevents the development of a more severe catabolic state like multiple organ failure that may be caused by gut-derived sepsis.

Glutamine and arginine are two conditionally essential amino acids during catabolic disease states [1]. Since the physiological importance of these amino acids for promoting and maintaining intestinal cell function is now generally accepted, strong interest has focused on the amino acids as nutraceuticals to enhance bowel function and integrity in a clinical setting. The molecular mechanisms and pathways underlying the health-promoting potential of glutamine and arginine in the intestine remain unclear, which prompted us to the study of protein expression at a large scale in human intestinal Caco-2 cells either exposed to or deprived from glutamine or arginine.

Critically ill patients often exhibit symptoms of malnutrition and require nutritional support. Studies in humans and animals showed that parenteral nutrition is associated with gut atrophy, loss of gut barrier, increased bacterial adherence, and increased apoptosis, and hence seems detrimental for intestinal function [2]. To understand the effects of malnutrition on the small intestine on a molecular basis, and to pinpoint the progression of events, a study was performed in which mice were subjected to fasting for several periods. Protein expression of the small intestine was analyzed to increase the knowledge about molecular effects, which might ultimately lead to a nutritional intervention which better assists intestinal function.

All studies described in this thesis focused on the protein entities in the cells, as they primarily carry out the biological functions in a cell. In addition, changes in mRNA levels are not always correlated with changes in protein levels [3]. The relative change in abundance of individual proteins was analyzed under several applied conditions. The up- and down-regulation of certain proteins could be linked to cellular processes with potential importance for gut health.

PROTEOMICS TOOLS FOR IDENTIFICATION OF NUTRIENT-GENE REGULATIONS

The techniques for large-scale transcriptome analysis are already widely used by the nutrition research community, in contrast to the techniques for proteome analysis [4]. Nevertheless, the value of proteomics for nutritional sciences has been recognized for several years [5]. Therefore, the studies described in this thesis contribute to a further insight in nutrient-gene regulations at the protein level.

Gel-based proteomics was used in all studies described in this thesis to investigate the effects of dietary compounds on the protein profiles of intestinal cells, and compare protein abundances at a large scale under different applied conditions. Two-dimensional gel electrophoresis (2-DE) combined with matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) for identification of differentially expressed proteins was used to gain information regarding biological processes which are altered under a certain condition. For all purposes, large gels were used to obtain a high resolution. Essential in the multi-step process of gel-based proteomics is to perform each step in a standardized way in order to maximize reproducibility between the experiments.
Array-based proteomics, in particular the use of the antibody array technology, was also tested in chapter 4 and provided a high-throughput technique for proteome analysis. The arrays were composed of 500 monoclonal antibodies and allowed the analysis of many cytosolic, as well as membrane-bound proteins in one experiment. The technique is however restricted by the quality and quantity of the antibodies available.

AMINO ACID DEPRIVATION

Cellular glutamine uptake and incorporation of glutamine into intestinal proteins

In chapter 2 it was shown that fully differentiated Caco-2 cells have a diminished glutamine uptake, compared to partially differentiated cells. Glutamine uptake across the basolateral membrane consistently exceeded uptake across the apical side of the cells, which corresponded to the higher incorporation of labeled glutamine into proteins when the amino acid was delivered via the basolateral side. When a protein showed significant incorporation of labeled amino acid, it was newly synthesized during the time period that the label was present in the culture medium of the cells. The longer the time period, the higher the amount of 'labeled' protein relative to the total pool of that particular protein. Despite the difference in incorporation rate of labeled glutamine in the cellular proteins when delivered from apical or basolateral side, it was shown that the same proteins were labeled irrespective of the side of glutamine administration. Therefore, it was concluded that glutamine, taken up from either the apical or the basolateral side of the cells, enters a common pool. Differences in the effects of 'luminal' versus 'serosal' glutamine observed in several studies, could hence not be explained by a different inducing effect on the synthesis of particular proteins. Actively synthesized

proteins in Caco-2 cells were involved in cell-cell and cell-extracellular matrix interactions, redox status, and glutamine metabolism.

Glutamine-mediated regulation of intestinal proteins

Intracellular glutamine concentrations failed to decline after withdrawal of glutamine from the medium, and cells kept their proliferative activity and normal protein synthesis rates [6]. Compared to normal human intestinal cells, Caco-2 cells have a high glutamine-synthesizing capacity [6, 7], which explains the limited influence of exogenous glutamine on the Caco-2 proteome as described in chapter 2. This prompted us to inhibit endogenous glutamine synthesis in Caco-2 cells by adding L-methionine sulfoximine (MSO) to the medium. In this manner, the glutamine synthetase activity was inhibited selectively and irreversibly, and the cells mimic a more human physiologic situation. With this procedure we created a glutamine-deficient state and observed a number of glutamine-dependent proteins in Caco-2 cells.

The identified proteins were, however, not those with a high labeling rate with glutamine that were identified in chapter 2. The fact that several proteins with a high synthesis rate and glutamine content did not show decreased expression under glutamine-deprived conditions suggests a more specific regulation for the observed changes in protein expression under this condition. This issue is further addressed in 'Mechanisms of amino acid-dependent regulation of protein expression'.

The results described in chapter 3 revealed that proteins which had a potential health-promoting effect in the intestinal cells were decreased under glutamine-limiting culture conditions. One of these proteins is ornithine aminotransferase (OAT) which is down-regulated in glutamine-deprived Caco-2 cells. It catalyzes the reversible conversion of ornithine and alpha-ketoglutarate to glutamic-gamma-semialdehyde and glutamate. As the intestine is a glutamine-metabolizing tissue under physiological conditions [8], it is suggested that the balance directs towards the synthesis of alpha-ketoglutarate and ornithine.

An interesting aspect of glutamine-mediated regulation of OAT expression is its involvement in polyamine synthesis. Ornithine is namely used as a precursor of polyamines via ornithine decarboxylase (ODC). Polyamines (putrescine, spermidine, and spermine) are important molecules for gastrointestinal mucosal growth and the maintenance of the integrity of the intestinal epithelial lining by their effect on intestinal cell proliferation, differentiation and migration [9]. This was also evidenced in Caco-2 cells by the finding that blocking of polyamine synthesis in Caco-2 cells by alpha-difluoromethylornithine (DMFO), inhibited cell growth. Adding spermidine together with DMFO was able to rescue the growth

inhibition in the cells [10]. In addition, polyamines are needed to maintain epithelial integrity by their effect on expression of several tight junction proteins [11]. That glutamine is involved in the regulation of polyamine synthesis is shown in a porcine jejunal enterocyte cell line, in which glutamine could stimulate ODC activity in a time- and dose-dependent manner [12]. ODC is the first and key regulatory enzyme in the synthesis of polyamines, and it is known that putrescine content in Caco-2 cells varied in accordance with the changes in ODC activity [13]. Down-regulation of OAT during glutamine-deficient states in Caco-2 cells is thought to be a direct effect of the diminished substrate supply. By the postulated consequences described above, a glutamine deficiency can be detrimental for the integrity and function of the intestine. It must be mentioned, however, that not only OAT enzyme activity results in ornithine production, but also by the action of arginase, ornithine is released.
Ornithine is also used as a precursor for arginine via ornithine carbamoyltransferase (OCT), argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL). The activities of the latter two enzymes are shown to be repressed in several cell lines when arginine is present at mM concentrations in the medium [14]. However, there is clear evidence that glutamine can increase ASS mRNA and activity in Caco-2 cells, suggesting that the arginine-synthesizing capacity of Caco-2 cells is more effective when glutamine is present in the cells [15].

Arginine-mediated regulation of intestinal proteins

Initially, preconfluent Caco-2 cells that are actively proliferating were used as a model to evaluate the effects of arginine deprivation on intestinal epithelial cells. This was done because arginine is shown to be effective in stimulating intestinal repair mechanisms, which involves cell proliferation. When arginine was deprived from the culture medium, Caco-2 cell growth was completely inhibited. Cells effectively restored their proliferative activity when arginine was added again.
The study described in chapter 4 demonstrated that under conditions where the supply of arginine to the intestinal epithelial cells was prohibited, proteins involved in processes like cell proliferation, heat shock response and apoptosis were changed. These processes are critical in intestinal repair mechanisms, but also in physiological situations, since the maintenance of gut mucosal homeostasis and integrity depends on a balance between cell proliferation and cell death. When proliferation is impaired and the susceptibility to apoptosis is increased, the balance is disturbed which might lead to a loss of the integrity. Heat shock proteins have a cytoprotective effect on the intestinal epithelium [16], and thus a decreased expression of them is likely to be harmful for the intestinal cells. Therefore, an

arginine-deficient state, observed during periods of rapid growth and critical illness in case that nutrition does not provide sufficient arginine, negatively influences the intestinal epithelial function.

Both arginine and citrulline supplementation are able to normalize the changes induced by arginine deficiency, suggesting that citrulline can be used as a substitute for arginine. Recently, the clinical potential of citrulline to correct arginine deficiency has been studied [17, 18]. As arginase activity is very high in the liver, this organ would consume most of the portal supply of dietary arginine, and convert it into urea. The gut reduces this possibility by converting dietary arginine to citrulline, which effectively bypasses the liver and is resynthesized to arginine in the kidney [19]. When the intestine is not properly functioning, or after massive small bowel resection, the main site of citrulline production is significantly reduced, leading to reduced plasma levels of citrulline, and consequently of arginine. In this situation, arginine supplementation would seem logical, however, this might cause excessive ureagenesis. Therefore, citrulline supplementation would have a potential advantage over arginine supplementation since it is not taken up by the liver and is the major precursor of arginine [17].

Our study suggests that this would not be harmful for the intestinal cells, since they can use citrulline efficiently via the conversion to arginine (by ASS and ASL) during periods of arginine deprivation. In this manner, cell proliferative capacity and normal protein levels are maintained. Possibly, citrulline can act directly on protein synthesis in a similar way as arginine, but this is unknown and a subject for further research.

Chapter 4 is mainly focused on the effects of arginine on the protein profile of preconfluent Caco-2 cells, as arginine is known to have a role in intestinal repair mechanisms. However, effects of arginine deprivation on protein expression that might influence intestinal function were also shown in postconfluent, differentiated Caco-2 cells. Furthermore, the study suggested that arginine regulated the protein expression differently in preconfluent and postconfluent cells.

This might be explained by the proliferative state of the preconfluent cells, in which cells have a higher arginine requirement [20]. Several proteins which were differentially expressed in the preconfluent cells were not expressed, or only very low expressed in the postconfluent cells. An example of such a protein is nuclear deoxyuridine 5'-triphosphate nucleotidohydrolase (dUTPase). This protein has a role in the formation of dUMP from dUTP and is thought to function primarily to limit the pool of dUTP levels in the cells, thereby preventing the incorporation of uracil into DNA during the replication process [21]. When Caco-2 cells are confluent and contact inhibition slows proliferation, expression of this protein is probably not longer required. In contrast, a protein with changed expression levels

in postconfluent cells, which is not expressed in preconfluent cells, is protein NDRG1 (also called differentiation-related gene 1 protein). The NDGR1 mRNA was shown to be induced approximately 20-fold during *in vitro* differentiation of the colon carcinoma cell lines HT29-D4 and Caco-2 [22]. Since the preconfluent cells did not yet enter the stage of differentiation, this protein seems not yet relevant which is reflected in the expression level.
However, most proteins were expressed in both pre- and postconfluent cells, and a clear explanation cannot be provided based on the available data. In addition, it must be noted that the conditions were not exactly comparable, as the preconfluent cells were deprived of arginine for 48 h, and the postconfluent cells for 24 h. The latter condition was chosen with respect to the comparison between glutamine- and arginine-deprived differentiated Caco-2 cells in an identical setting, to obtain indications about the specificity of the amino acid deprivation response (see next paragraph).

Common and opposite effects of glutamine and arginine deprivation on intestinal protein expression

The response of arginine deficiency and glutamine deficiency in differentiated Caco-2 cells was evaluated in an identical setting, which made it possible to compare the protein changes. In chapter 3 and 4 it was shown that the proliferative capacity was completely inhibited when Caco-2 cells were deprived of arginine (exogenously) and glutamine (endogenously plus exogenously). These conditions were then applied to postconfluent Caco-2 cells. The resultant was a change in expression levels of several proteins, most of them being specific for the amino acid which was deficient. However, two proteins showed a similar response to single amino acid deprivation, independent of the type of amino acid being either glutamine or arginine.
The first one was identified as apolipoprotein A-I (apoA-I) which was down-regulated in both cases. Hence, both amino acids seem to be necessary for optimal apoA-I synthesis in intestinal cells. Recently, two other amino acids aspartate and glutamate were shown to exert antiatherogenic properties in an *in vivo* rabbit model fed a cholesterol-rich diet. The diet with the amino acids was able to maintain apoA-I levels in blood, whereas the control group showed a significant decrease in apoA-I levels [23]. The potential of certain amino acids in sustaining intestinal apoA-I secretion, and the mechanisms by which these amino acids exert this beneficial effect need to be explored further.
The other protein with a similar response is the 44-kDa form of reticulocalbin-1. This is a Ca^{2+}-binding protein localized in the lumen of the ER and expressed in various cell types which suggests a role in protein synthesis, modification, and

intracellular transport, but at present its function remains unknown [24]. It remains to be elucidated how amino acid deficiency can up-regulate the protein expression of reticulocalbin-1, and what the consequence is for intestinal cell function. For both proteins, the fold change in protein expression between the deprived and the supplemented condition was greater for cells submitted to glutamine deprivation, and this is not related with the amino acid content (glutamine or arginine) of the protein sequence.

Another protein, identified as chromobox protein homolog 3, was down-regulated by glutamine deprivation and arginine deprivation. Noticeably, glutamine deprivation for 24 h induced this response in differentiated cells, whereas arginine deprivation for 48 h regulated this protein in preconfluent cells. Mitochondrial phosphoenolpyruvate carboxykinase (PEPCK), a protein involved in gluconeogenesis, was regulated by deprivation of both amino acids as well, but in the opposite direction. Glutamine deprivation in postconfluent Caco-2 cells induced a down-regulation of this protein, whereas arginine deprivation up-regulated the protein in preconfluent Caco-2 cells. It is known that arginine can modify the metabolism of energy substrates via its metabolite NO. When arginine is absent, nitric oxide (NO) production is prohibited in intestinal cells since arginine is the only precursor. Physiological levels of NO stimulate glucose oxidation in insulin-sensitive organs and inhibit gluconeogenesis [25]. As the intestine is also an insulin-sensitive organ, these processes may also be influenced by arginine in this tissue [26]. Glutamine is the main gluconeogenic substrate in the small intestine [27], probably leading to a substrate-dependent adaptive down-regulation of mitochondrial PEPCK expression during glutamine-limiting conditions. In a similar way, mRNA expression of glutaminase, the enzyme that catalyzes the initial step in glutamine degradation, in the intestine was increased in parenterally fed rats supplemented with glutamine [28].

Mechanisms of amino acid-dependent regulation of protein expression

In mammals, the plasma concentration of amino acids is affected by nutritional or pathological conditions. Besides their role as a substrate for protein synthesis, amino acids have multiple important functions. They can act as glucogenic substrates, nitrogen carriers, neurotransmitters, and regulators of enzyme activity, and they are precursors of signal transducers, nucleotides, and many more molecules. Consequently, the supply of free amino acids to the tissues plays an important role in the maintenance of protein levels at organ and whole body level. It is well established that amino acids are involved in the control of gene expression. Next to the substrate-dependent regulation of proteins that are involved in the metabolism of the particular amino acid under study, other

proteins were shown to be differentially expressed by the amino acid-limiting conditions described in this thesis. The molecular mechanisms involved in the amino acid-dependent regulation of mammalian gene and protein expression have not been completely elucidated to date.

The expression level of a certain protein is determined by an interplay between synthesis and degradation of that protein, and these events are highly regulated in a cell. The process of protein degradation in the liver can be influenced by amino acid availability, and different effects were observed for glutamine and leucine [29]. Furthermore, the process of protein synthesis is well-known to be regulated by the availability of amino acids. Mammalian cells have two recognized pathways for monitoring and responding to amino acid availability, by which they change the rate of protein synthesis in opposite ways. First, the mammalian target of rapamycin (mTOR) pathway functions to confirm a sufficient level of amino acids to support protein synthesis and cell growth [30]. Leucine and arginine have the ability to activate the mTOR signaling pathway in rat intestinal epithelial cells [31]. The second known amino acid response pathway is designed to detect amino acid deficiency. Limiting the availability for any single amino acid initiates this signaling cascade, also called the 'general amino acid control' response. When the extracellular supply of an essential amino acid is limited, or the synthesis of an otherwise nonessential one is blocked, this will result in an accumulation of uncharged tRNAs, which leads to the activation of the kinase general control nonderepressible protein 2 (GCN2) [32]. Several stress-responsive kinases, under which GCN2, can phosphorylate eukaryotic translation initiation factor 2 subunit alpha (eIF2alpha), which leads to a reduction of protein synthesis [33, 34]. Expression of activating transcription factor 4 (ATF4) is selectively up-regulated under this condition, and is known to activate other genes through amino acid response elements (AAREs). Such consensus sequences have been identified in the promotor regions of C/EBP homologous protein (CHOP) and asparagine synthetase [35, 36]. Recently, it has been shown that an AARE is involved in the expression of two amino acid transporters [37, 38].

In MSO-treated Caco-2 cells, intracellular glutamine concentrations were reduced and the overall protein synthesis rate was down-regulated [6]. The mechanism of regulation was not elucidated in that particular study, but based on above findings the 'general amino acid control' pathway might have been involved in here. Such a response is not yet revealed for glutamine limitation, but in Caco-2 cells, starvation of several other individual amino acids, under which leucine and lysine, could activate the CHOP promotor [35]. Phosphorylation of eIF2alpha was not measured in that study, but the effect on the CHOP promotor suggests the involvement of the 'general amino acid control'. It must be mentioned that there

are graded differences observed in response to depletion of a particular amino acid within a given cell line [39], and amino acid specificity appears to play a role [35].
The study described in chapter 3 aimed to determine if glutamine deprivation exerted only a general effect on protein synthesis or a more gene-specific effect in MSO-treated Caco-2 cells. Therefore, the synthesis rate of individual proteins (both differentially and not-differentially expressed spots) was measured semiquantitatively by mass spectrometry. That study showed that there was no correlation between the protein synthesis rate during glutamine-sufficient periods and the change in expression level during glutamine-deficient periods. The abundance of several proteins with a high protein synthesis rate and average to high glutamine content was not decreased under glutamine-limiting conditions. In addition, some proteins showed an increase in expression level under this experimental condition.
In case of 48-h arginine deprivation of preconfluent Caco-2 cells, the unphosphorylated form of eIF2alpha tends to decrease (p=0.055). Subsequent arginine exposure increased the eIF2alpha expression significantly, indicating that in this phase translation initiation is promoted, and could provide a direct link between cellular arginine levels and protein synthesis. Despite this relation, nine protein spots were down-regulated by both arginine and citrulline supplementation under this condition, as is shown in chapter 4.
Based on the above findings, and on the fact that most proteins are not regulated in a similar manner under glutamine- and arginine-limiting conditions in Caco-2 cells, we propose that these amino acids regulate the expression of certain proteins via a more specific mechanism. This points to the possibility that these amino acids can act as signaling molecules, and that the processes they regulate are most likely to be influenced by the nutritional status.

FOOD DEPRIVATION

Effect of food deprivation on intestinal proteins

In chapter 5, the mouse was used as a model organism to study the effect of total food deprivation on the protein expression of the small intestine. For this purpose, the use of the whole small intestine was preferred above the use of epithelial scrapings. Several reasons justified this choice. First, over 70% of the mouse small intestine consists of epithelial cells. Second, sequential isolation of enterocytes is time-consuming and, hence, entails a risk of protein degradation. And last, the scraping procedure harvests villi more efficiently than crypts [40], whereas both cell populations are of interest for this study. It was also shown that no significant

changes occurred with respect to the proportion of cellular components (epithelium, muscle cells and goblet cells) during starvation.

The study revealed that during starvation several proteins were up-regulated in order to protect intestinal cells, whereas other protein changes negatively influence gut health. The first group includes proteins such as reg I and II (two members of the regenerating gene multifamily), glutathione peroxidase 3, and carbonic anhydrase 3. In wild-type mice, reg I mRNA is expressed in the gastrointestinal tract at highest level in the small intestine [41], suggesting a critical role in this tissue. Only recently, a study with reg I-knockout mice was performed to clarify the role of reg I in the normal mouse mucosa. A growth-promoting activity of reg I was demonstrated in the small intestine, next to a promoting effect on cell migration towards the villus tip [42]. In chapter 5, up-regulation of reg I and reg II expression was postulated to exert a protective effect on the fasting intestine. After 72 h of starvation, increased ezrin expression was observed which might enhance villus morphogenesis. In that phase of starvation, an increase in mucosal mass and a decrease in apoptosis were observed in rats [43]. It was hypothesized to be a reaction for the preservation of enterocytes during this critical fasting period in order to optimize nutrient absorption as soon as food is available, and thus, to rapidly restore body mass [43]. Increased ezrin expression might serve the same goal.

Changed proteins that likely influence the intestinal health in a negative way were also observed during starvation. For example, gob-4 protein expression is down-regulated during starvation and is thought to have a role in mucus secretion [44]. The mucus layer acts as a barrier between the luminal contents and the absorptive cells of the intestine and is a component of the first line defense. Another down-regulated protein with a role in the immune system is intelectin-1a. Intelectin has been proposed to play a role in the innate immune response to microbes containing a bacterium-specific carbohydrate galactofuranose in humans [45]. In addition, proteins responsible for ornithine production, namely arginase-2 and OAT, were down-regulated at 12 h of starvation. Arginase-2, OAT and ornithine decarboxylase show co-localization in the small intestine of mice, suggesting a role in the synthesis of proline and polyamines [46]. The OAT levels remained decreased during prolonged fasting, while arginase-2 levels increased again. The reason for the maintenance of decreased OAT expression and not of arginase-2 expression after 72 h of starvation might be that glutamine is used for gluconeogenesis during prolonged starvation [27]. Expression levels of glutaminase were decreased in the fasting intestine, whereas glutamine synthetase levels were increased, suggesting that intestinal cells try to maintain cytosolic glutamine levels (Sokolović *et al.*, submitted). Decreased OAT levels correspond with this finding,

but it might as well be an adaptation to a relative glutamine-deficient state coinciding starvation.
OAT appears to be very sensitive to the nutritional status both *in vitro* (see chapter 3) and *in vivo* in the intestine, and thus a clear example of a nutrient-gene regulation is provided for this enzyme. Another example of an intestinal protein that was regulated by nutritional factors both *in vivo* and *in vitro* is fructose-bisphosphate aldolase (aldolase A *in vivo*, and aldolase B *in vitro*).

Mechanisms for protein regulation during food deprivation

Gastrointestinal tissues account for a large fraction of whole-body protein synthesis and turnover. Rates of protein synthesis in the intestinal mucosa are among the highest in the body and can be more than an order of magnitude greater than those observed in peripheral tissues such as the skeletal muscle. The high rate of protein synthesis in the mucosa reflects likely the turnover of whole cells, in addition to the intracellular turnover of proteins [47].
The rate of protein synthesis in the jejunal mucosa was affected by starvation for 2 days in rats, and was decreased for approximately 30% [48]. Data shown in chapter 5 reveal that two elongation factors (EFs), EF-1-beta and EF-2, were down-regulated in mice submitted to starvation. These proteins are two key players involved in protein translation [49], and a diminished expression is expected to result in an impairment of translation in the intestinal cells. Probably, this is an adaptation to preserve energy during nutrient restriction, since translation consumes a high amount of energy. AMP-activated protein kinase (AMPK) can phosphorylate EF-2 kinase, thereby inactivating EF-2 by phosphorylation [50]. EF-2 protein levels were also found reduced in muscle and liver of 48-h starved mice [51].
Another mechanism by which starvation can mediate protein expression is via the activation of a selective pathway of lysosomal proteolysis. This was demonstrated in liver lysosomes derived from rats that had been fasted. The activity of this pathway was shown to increase progressively by starvation for up to 88 h [52]. Substrate proteins for this pathway include glyceraldehyde-3-phosphate dehydrogenase, a protein that we identified as significantly decreased in the intestine during the whole time period of starvation. More substrates of this pathway are known, and their expression can be influenced by starvation in this manner.

EVALUATION OF THE CACO-2 CELL MODEL

The Caco-2 cell line is a widely used *in vitro* model for the intestinal epithelium and originates from tumor tissue which, compared to physiological conditions, alters the cellular metabolism. To obtain information about the representativity of this cell line for the tissue from which it is derived, the expression pattern of Caco-2 cells and human epithelial cells were compared in chapter 6. Principal component analysis of the protein data revealed that global protein expression of intestinal epithelial scrapings differed considerably from that of intestinal epithelial cell lines, and from *in vitro* cell lines in general. Compared to the *in vivo* situation, several groups of proteins with a higher as well as lower expression in cell lines were observed. These differences reflect probably the tumoral origin and the adaptation of these cells to *in vitro* culture conditions.

Despite above findings, it appeared from the study that Caco-2 cells express proteins which are characteristic for the intestinal epithelial phenotype. Furthermore, the small intestine showed expression of proteins that were found regulated in response to glutamine deprivation in Caco-2 cells. For example, glutamine deficiency influences the expression of OAT in Caco-2 cells (chapter 3). In the study described in chapter 6, OAT was identified as a marker for the small intestinal phenotype of the Caco-2 cells, since these cells showed similar OAT protein expression levels compared to small intestinal tissue. Epithelial cells of the large intestine showed a lower expression, and this corresponds with the finding that OAT activity is much higher in the small intestine compared to the large intestine in pigs [53]. Some proteins were, however, similarly expressed in Caco-2 and large epithelial cells, and this finding supports the idea stated before that the Caco-2 cells represent features characteristic to the colonic and enterocytic phenotype [54].

Another protein affected by glutamine deprivation in Caco-2 cells is plasma RBP. Matching the proteomes of Caco-2 cells and human intestinal epithelial scrapings showed that the latter cells also express the plasma retinol-binding protein (RBP), although at lower levels, which indicates the possible relevance for the *in vivo* situation. A link between plasma glutamine levels and plasma retinol-binding protein levels was demonstrated before in patients receiving parenteral nutrition [55]. However, the intestine's contribution to the level of plasma RBP in blood remains to be determined.

The study described in chapter 2 provided also relevant biological information about characteristics of Caco-2 cells. For example, it was shown that they synthesize LI-cadherin. CDX2 and LI-cadherin expression is tightly coupled in tissue of the gastrointestinal tract [56]. The CDX2 protein is known to play a role in a broad range of functions from differentiation to maintenance of the intestinal epithelial lining in both the small and large intestine. *In vitro,* CDX2 negatively

regulates proliferation and promotes the acquisition of a mature enterocyte phenotype [57]. CDX2 protein expression is not detectable in subconfluent Caco-2 cells, and shows a very high expression at 6 days postconfluence [58]. This is in line with our findings regarding active LI-cadherin synthesis in 5-day differentiated Caco-2 cells.

As described in the chapter 1, Caco-2 cells exhibit morphologic characteristics which resemble those of small intestinal cells, such as a brush border, consisting of tightly packed, uniform, apical microvilli with associated hydrolases. Typical small intestinal brush-border proteins were not observed in the 2-D patterns of Caco-2 cells, but also not in the patterns of human intestinal epithelial cells. Hence, the method applied in this study did not allow the comparison of such characteristic features of the small intestine. Possible explanations are the high molecular weight of these proteins, and their close association with the membrane, both leading to an underrepresentation in 2-D gels.

CONCLUSIONS

This thesis shows the successful application of proteomics technologies in nutrition research, since novel proteins and molecular pathways were found to be regulated by the availability of glutamine and arginine in intestinal Caco-2 cells, and by (prolonged) fasting in the mouse small intestine, which gave explanations for the detrimental effects of nutritional deficiencies. It was shown that both glutamine and arginine are required to maintain expression of proteins with health-promoting capacities in intestinal cells. This can provide an explanation for the observed positive effects of the supplementation of these amino acids when conditionally essential. Hence, state-of-the-art proteomics techniques can provide novel biological insights that would remain undiscovered using a conventional one-protein-at-a-time hypothesis driven technique, such as Western blotting. This indicates the importance of proteomics as a discovery science.

REMARKS FOR FUTURE RESEARCH

It is well established that amino acids can regulate the expression of mammalian genes, and this is also supported by our research. However, it remains to be elucidated which molecular mechanisms are responsible for the gene-nutrient regulations described in this thesis. In this respect, the possible involvement of the amino acid response element (AARE) or other nutrient-sensing elements in promotor regions of regulated genes is a subject for further research.

In the analysis of protein expression changes, the fold-change criteria are often set arbitrarily. However, when the expression level of a protein changes 2-fold, this may have little physiological consequence, whereas a 20% change may be critical, depending on the role of the protein in a particular pathway. Furthermore, the activity of the particular protein cannot always be derived from the level at which the protein is expressed, and some differentially expressed proteins catalyze reversible reactions. Therefore, it would be worthwhile to determine protein activity and the related metabolites. In addition, the analysis of protein modification, localization and interaction in complexes is of significance.

Another remark is that the number of proteins visible on a 2-D gel is limited in comparison to the expected number of proteins present in a cell. New emerging techniques are being developed with a greater sensitivity, for example quantitative liquid chromatography tandem mass spectrometry (LC-MS/MS), which will allow further in-depth proteome research.

The exact function of several proteins described in this thesis is still unknown, and several speculations are made with respect to their significance in intestinal biology. A possible way to obtain information about the function of a certain protein in cell culture systems is via RNA interference (RNAi), a biological mechanism by which double-stranded RNA (dsRNA) induces gene silencing by targeting complementary mRNA for degradation. For *in vivo* studies, the development of a knockout mouse model for the protein of interest is a possible approach.

In conclusion, the approaches used in this thesis allowed the exploration of food-gut interactions under strictly controlled conditions. However, extrapolating the knowledge of *in vitro* cell studies or *in vivo* animal studies to the situation in humans has yet to be performed, and this remains one of the major challenges in future research.

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SUMMARY

SUMMARY

In healthy individuals, the processes of digestion and absorption of food in the gastrointestinal tract provide sufficient nutrients for the body's metabolism. Consequently, a good functioning of the small intestinal absorptive epithelial cells is required to maintain an adequate nutritional status. Since the mucosa of the intestine is directly exposed to the external environment with potentially harmful agents, it should also prevent the entry of such agents in the human body. Therefore, the intestinal epithelial layer has two major functions, namely digesting and absorbing nutrients, and acting as a barrier which is critical to the host defense. The gut becomes injured following critical illness, which is manifested by alterations of intestinal function, such as increased permeability of the epithelial lining and increased villous injury or atrophy. Hence, there is interest in nutritional compounds that can sustain gut integrity and function during such pathological states.

The amino acids glutamine and arginine are considered conditionally essential in patients with catabolic diseases, and ongoing studies are defining potential therapeutic roles for these amino acids in gut mucosal turnover, repair and barrier function. However, more studies are needed to define the targets through which both nutrients may exert beneficial effects on the intestinal tissue *per se*. Accordingly, one of the objectives of the studies described in this thesis was to identify molecular mechanisms and pathways that are responsible for the health-promoting potential of these amino acids in the gut. For this purpose, the Caco-2 cell line was used as an *in vitro* model for the intestine. This cell line is derived from a human colorectal carcinoma, and in differentiated state, Caco-2 cells share many characteristics with epithelial cells of the small intestinal villi. Protein expression of these intestinal cells was analyzed under several conditions, mostly by means of gel-based proteomics technology.

In chapter 2, the effect of varying glutamine concentrations delivered from the apical (≈luminal) or basolateral (≈serosal) side of the Caco-2 monolayer was assessed by two-dimensional gel electrophoresis (2-DE). From that study, it appeared that a change in the exogenous glutamine supply did not alter the proteome of Caco-2 cells visibly. Despite this lack of significant differences, it was observed that exogenous glutamine is taken up by the cells, and that they actively incorporate this glutamine into their proteome when delivered from either side of the monolayer. Basolateral delivery of labeled glutamine resulted in a higher uptake and incorporation rate, but the same proteins were labeled, indicating that glutamine enters a common pool irrespective of the side of delivery. Reported differences in the effects of 'luminal' versus 'serosal' glutamine could hence not be explained by a different inducing effect on the synthesis of particular proteins.

The intestinal glutamine synthetase activity is shown to be very low in humans, indicating that these cells are dependent on exogenous glutamine for their supply. On the contrary, human Caco-2 cells have a high glutamine synthesis capacity. By blocking the endogenous glutamine production of Caco-2 cells, they may resemble the *in vivo* human intestinal cells more closely. This approach has led to the discovery of glutamine-dependent proteins in intestinal cells as described in chapter 3. The differential change in the expression levels of those proteins was not correlated with their rate of synthesis, excluding the possibility that glutamine deprivation acts only on general protein synthesis. Some proteins have potential health-promoting effect on the human intestine, and hence, their decreased expression could be responsible for the adverse effects on the intestine when glutamine is conditionally essential. An example of such a protein is ornithine aminotransferase (OAT), an enzyme that is involved in the synthesis of ornithine from glutamine. Ornithine is a precursor of polyamines, *i.e.* molecules that are important for gastrointestinal mucosal growth and the maintenance of the integrity of the epithelial lining by their effect on intestinal cell proliferation, differentiation and migration. Arginine synthesis from glutamine, glutamate and proline occurs also via ornithine. Several other proteins of which the expression was changed by varying glutamine levels were detected, of which plasma retinol-binding protein, apolipoprotein A-I (apoA-I), mitochondrial 3-hydroxy-3-methylglutaryl-CoA and acyl-CoA synthase 5 may act beneficially on the intestinal cells.

Chapter 4 focuses mainly on the effects of arginine deficiency on the proteome of proliferating intestinal Caco-2 cells, as arginine is known to favorably affect intestinal repair, a process in which cell proliferation is involved. The study revealed that the expression of proteins involved in cell proliferation, heat shock response and apoptosis was regulated by the availability of arginine to the intestinal cells. Exposure of the cells to arginine and citrulline after a period of arginine deprivation could normalize the expression changes of those proteins. The fact that citrulline exerts such a response suggests that this amino acid can be used, at least partly, by intestinal cells as a substitute for arginine. Citrulline is thought to be a better agent to correct arginine deficiency since it effectively bypasses the liver and is converted to arginine in the kidneys. Our data imply that citrulline can be used in such cases without detrimental effects on the intestinal cells, as cell proliferative capacity and normal protein expression levels can be maintained. Arginine-dependent proteins were also explored in differentiated, postconfluent Caco-2 cells. Only a minor overlap was observed in the changes induced by arginine-deficiency in preconfluent and postconfluent cells. This can be explained by the difference in active processes that depend on the stage in which the cells are at that moment.

The effects of glutamine and arginine on postconfluent monolayers of Caco-2 cells were studied in a similar setting as shown in chapter 3 and 4. Single amino acid deprivation of glutamine or arginine for 24 h resulted in a decreased apoA-I expression and an increased reticulocalbin-1 expression. This finding indicates that sufficient glutamine and arginine levels appear to be necessary for optimal apoA-I synthesis in intestinal cells. ApoA-I is the major protein component of the high-density lipoprotein (HDL) particles, also called the 'good cholesterol'. The impact of the increased expression of reticulocalbin-1 under the influence of glutamine or arginine deprivation is not clear. Reticulocalbin is localized in the lumen of the endoplasmatic reticulum, and is suggested to have a role in the synthesis and processing of proteins.

Besides interest in nutritional compounds that support optimal gut function, this thesis deals also with the early and late progressive changes of the intestine under malnourished condition. Understanding the intestinal adaptation to nutrient limitation at the molecular level might indeed benefit the development of improved nutritional support during clinical conditions characterized by malnutrition. To address this research question, mice were starved for 12, 24 and 72 h, and the proteome of the small intestine was studied. The results, which are described in chapter 5, show that the expression profile of the intestinal tissue is altered quite significantly due to starvation. Based on the protein changes, some general processes, such as glycolysis and energy metabolism, were found to be down-regulated after 12 h of starvation. These changes reflect probably a direct response to the diminished nutrient supply to the intestine. At 24 h, levels of two elongation factors that play an essential role in protein synthesis were down-regulated. Despite this inhibitory effect on general protein synthesis, expression levels of reg I, reg II, glutathione peroxide 3 and carbonic anhydrase 3 were up-regulated, and were postulated to have a protective effect on the intestinal cells. Starvation for 24 h resulted in decreased levels of OAT, a protein that was also influenceable by glutamine deprivation for 24 h in Caco-2 cells. Up-regulated ezrin expression, detected at 72 h of starvation, might be an adaptive response to sustain the villus function of the intestine at such critical depletion levels.

Two widely used *in vitro* cell models for the intestinal epithelial lining are Caco-2 cells and HT-29 cells. These and many other cell lines originated from tumor tissue, which suggests that the metabolism of these cells is altered considerably compared to the *in vivo* situation. As a cell's metabolism depends on the expression and activity of several gene products, an initial effort was undertaken to evaluate these widely used models in terms of protein expression as described in chapter 6. Therefore, protein profiles of the *in vitro* models were compared to the profiles of their *in vivo* counterparts. Two negative controls were included in the study, namely the Hep G2 liver cell line and the TE 671 muscle cell line, which are also derived

from human tumor tissue. Principal component analysis and hierarchical clustering of the protein data revealed that the protein expression of intestinal epithelial scrapings differed considerably from that of intestinal epithelial cell lines, and from *in vitro* cell lines in general. Typical proteins with a higher expression in epithelial scrapings were hemoglobin alpha and beta, serum albumin and transthyretin. These proteins probably originate from contamination with blood, pointing to the difficulty of obtaining pure cell isolated, and the advantage of *in vitro* cell lines, which consist of a homogenous cell population. Other proteins with a clear expression difference in *in vitro* cultures as compared to scrapings were observed, and especially proteins reflecting the tumoral origin of the cell lines and their adaptation to culture conditions belong to this group. Despite above findings, 2-D patterns showed a considerable number of overlapping proteins between Caco-2 cells and small intestinal epithelial cells. In addition, the study allowed the identification of several protein markers, such as OAT, in Caco-2 cells that seem characteristic for the small intestinal epithelial phenotype. Overall, the data point to the usability of Caco-2 cells for increasing the understanding of the epithelial lining of the intestine, but of course, extrapolating the knowledge of *in vitro* cell studies to the human situation remains one of the major challenges in future research.

To conclude, this thesis demonstrates the applicability of proteome technology in nutrition research, as novel proteins and molecular pathways were found to be regulated by the availability of selected nutrients to the intestinal cells. The identified nutrient-gene regulations may contribute to a further understanding of the nutritional needs for maintaining and improving gut health in human. However, more functional research is needed to establish the importance of the regulated proteins in the gut.

SAMENVATTING

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De voedingsstoffen die gebruikt worden voor de stofwisseling in het menselijk lichaam worden gehaald uit de verterings- en resorptieprocessen van voedsel in het maagdarmstelsel. Een goede conditie van de darmcellen die hiervoor verantwoordelijk zijn, ook wel resorberende epitheelcellen genoemd, is noodzakelijk voor het behoud van een optimale voedingsstatus. Daarbij komt dat de mucosa van de dunne darm rechtstreeks wordt blootgesteld aan de uitwendige omgeving, waarin mogelijk gevaarlijke stoffen aanwezig zijn. De darm heeft dus een dubbele functie, namelijk het verteren en opnemen van voldoende nutriënten en het vormen van een barrière die essentieel is voor het immuunsysteem van de gastheer. Kritisch zieke patiënten vertonen veranderingen in hun darmfunctie, zoals een verhoging van de permeabiliteit van het darmepitheel en een toename van schade of atrofie van de darmvlokken. Daarom bestaat er interesse in voedingsfactoren die de darmintegriteit en -functie kunnen ondersteunen in zulke omstandigheden.
De aminozuren glutamine en arginine worden conditioneel essentieel geacht bij patiënten die lijden aan katabole ziekten. Verschillende studies hebben aangetoond dat deze aminozuren mogelijk een gunstig effect hebben op het herstel van beschadigd darmepitheel en de integriteit van de darm in het algemeen. Er zijn echter meer studies nodig om de onderliggende moleculaire mechanismen en 'pathways' van het effect van deze aminozuren op de darmcellen te kunnen begrijpen. Om dit te onderzoeken werd er gebruik gemaakt van een *in vitro* model voor de darm, namelijk de humane coloncarcinoom cellijn Caco-2. In gedifferentieerde toestand vertonen de Caco-2 cellen zowel morfologische als functionele eigenschappen van de epitheelcellen van de darmvlokken. Ze vormen een aaneensluitende cellaag en vertonen een borstelzoom (microvilli) op hun apicale (≈luminale) oppervlak waarmee verschillende verteringsenzymen geassocieerd zijn. Nadat deze cellen werden blootgesteld aan verschillende condities, werd hun proteoom, *i.e.* het geheel aan eiwitten dat op een bepaald moment in de cel aanwezig is, geanalyseerd. Dit werd meestal gedaan met behulp van tweedimensionale gelelektroforese (2-DE), dit is een techniek waarmee verschillen in eiwitexpressie tussen twee condities gedetecteerd kunnen worden. Dergelijke eiwitten worden vervolgens geïdentificeerd door middel van massaspectrometrie.
In hoofdstuk 2 werd het effect van glutamine op het proteoom van Caco-2 cellen onderzocht. Hierbij werd glutamine in een verschillende hoeveelheid toegevoegd aan het apicale dan wel basolaterale (≈serosale) medium waaraan de cellen werden blootgesteld. Deze studie toonde aan dat een veranderde glutamineconcentratie niet in staat is het proteoom van Caco-2 cellen zichtbaar te veranderen. Er werd wel aangetoond dat glutamine opgenomen wordt door de Caco-2 cellen, en dat ze dit glutamine ook inbouwen in hun eiwitten tijdens de synthese, ongeacht via

welke zijde van de cellaag dit aminozuur wordt aangeboden. Echter, wanneer glutamine via de basolaterale zijde wordt aangeboden is de opname en inbouw in eiwitten groter dan wanneer glutamine via de apicale zijde wordt aangeboden. Dezelfde eiwitten worden gelabeld onafhankelijk van de zijde van blootstelling van het aminozuur, wat erop wijst dat het glutamine in dezelfde pool wordt opgenomen. Gerapporteerde verschillen tussen het effect van 'luminaal' en 'serosaal' glutamine kunnen dus niet verklaard worden door een verschillend stimulerend effect op de synthese van bepaalde eiwitten.

De glutamine synthetase activiteit is zeer laag in de epitheelcellen van de dunne darm *in vivo*, wat aangeeft dat de darmcellen afhankelijk zijn van exogeen glutamine voor hun voorziening. In tegenstelling tot normale darmcellen hebben Caco-2 cellen een grote capaciteit om glutamine aan te maken. Door het blokkeren van deze glutamineproductie lijken de Caco-2 cellen mogelijk meer op de normale darmcellen. Deze onderzoeksbenadering heeft geleid tot de ontdekking van eiwitten, waarvan het expressieniveau afhankelijk is van de hoeveelheid glutamine die beschikbaar is voor de cellen, zoals beschreven in hoofdstuk 3. Hierbij valt op te merken dat er geen verband bestaat tussen de verandering in eiwitexpressie en de mate waarin het eiwit in fysiologische omstandigheden nieuw wordt gesynthetiseerd. Vermits glutamine een aminozuur is dat vereist is voor de aanmaak van eiwitten, wijst dit feit erop dat deprivatie van glutamine niet uitsluitend een invloed heeft op de algemene eiwitsynthese, maar dat er een meer specifiek regelmechanisme speelt. Dit mechanisme is tot op heden niet bekend. Sommige gereguleerde eiwitten hebben mogelijk een gezondheidsbevorderend effect op de dunne darm. Een verlaagde expressie van deze eiwitten kan dus mede verantwoordelijk zijn voor de negatieve effecten die de darm ondervindt bij een tekort aan glutamine. Een voorbeeld van een dergelijk eiwit is ornithine aminotransferase (OAT), een enzym dat betrokken is bij de omzetting van glutamine naar ornithine. Ornithine is een precursor voor polyamines, welke moleculen zijn die een trofisch effect hebben op de darmmucosa en die belangrijk zijn voor het behoud van de integriteit van de epitheellaag van de darm. Dit resultaat wordt bewerkstelligd door een effect op celproliferatie, celdifferentiatie en migratie. Meerdere eiwitten die veranderd tot expressie komen onder invloed van de beschikbaarheid van glutamine, werden gedetecteerd, waarvan plasma retinol-bindend eiwit, apolipoproteïne A-I (apoA-I), mitochondrieel 3-hydroxy-3-methylglutaryl-CoA en acyl-CoA-synthase 5 evenees een gunstig effect kunnen hebben op de darm.

Hoofdstuk 4 behandelt voornamelijk het effect van een tekort aan het aminozuur arginine op het proteoom van prolifererende Caco-2 cellen. In deze studie is in eerste instantie gekozen voor cellen die zich nog delen, aangezien arginine het proces van darmherstel gunstig beïnvloedt en proliferatie hierbij een belangrijke rol

speelt. De resultaten van de studie kwamen overeen met een dergelijke bevinding, aangezien vooral eiwitten met een rol in proliferatie, apoptose en de 'heat shock' respons veranderd tot expressie kwamen bij een argininetekort in de darmcellen. Deze veranderingen in eiwitexpressie werden tenietgedaan nadat de cellen opnieuw werden blootgesteld aan arginine of citrulline. De bevinding dat citrulline een dergelijke respons vertoont, suggereert dat dit aminozuur arginine (gedeeltelijk) kan vervangen. Daar gedacht wordt dat supplementatie met citrulline een betere therapie is dan supplementatie met arginine bij een tekort aan dat laatste, kan voorgaande bevinding klinische implicaties hebben. In tegenstelling tot arginine kan citrulline namelijk de lever passeren zonder afgebroken te worden en wordt het vervolgens omgezet tot arginine in de nieren. De studie in hoofdstuk 4 laat zien dat dit geen negatieve effecten voor de darmcellen teweegbrengt op het vlak van eiwitexpressie en celproliferatie. Ook gedifferentieerde Caco-2 cellen werden blootgesteld aan medium zonder arginine en vervolgens werd het proteoom vergeleken met dat van cellen die wel arginine via het medium kregen. Slechts weinig eiwitten vertoonden een gelijkaardige respons ten gevolge van een argininetekort in prolifererende en gedifferentieerde Caco-2 cellen. Dit kan te wijten zijn aan het feit dat in deze cellen andere processen actief zijn die karakteristiek zijn voor het stadium waarin ze zich bevinden.

Het effect van glutamine en arginine op Caco-2 cellen werd bestudeerd in een vergelijkbare setting, zoals beschreven in hoofdstuk 3 en 4. Het afzonderlijk weghalen van glutamine en arginine uit het medium van deze cellen gedurende een periode van 24 uur leidt tot een stijging van de expressie van reticulocalbine-1 en een daling van de apoA-I-expressie. Dat laatste suggereert dat er voldoende glutamine en arginine voorhanden moet zijn in darmcellen, willen ze een normale apoA-I-productie bewerkstelligen. ApoA-I is het belangrijkste structurele eiwit van de anti-atherogene HDL-lipoproteïnen, ook wel de goede cholesterol genoemd. De impact van de toegenomen reticulocalbine-1-expressie ten gevolge van een tekort aan glutamine of arginine is nog onduidelijk. Reticulocalbine is gelokaliseerd in het lumen van het endoplasmatisch reticulum, en zou mogelijk een rol hebben bij de synthese en processing van eiwitten.

Naast de interesse in voedingsfactoren die een gunstig effect hebben op de darm, richt dit proefschrift zich op de vroege en late veranderingen in de darm ten gevolge van vasten. Het beter begrijpen van de moleculaire adaptatie van de darmen, die veroorzaakt wordt door een gebrek aan voeding, kan uiteindelijk leiden tot een verbetering van de voedingsondersteuning bij patiënten die lijden aan pathologieën welke geassocieerd zijn met ondervoeding. Om deze onderzoeksvraag te beantwoorden werden muizen gehongerd voor 12, 24 en 72 uur waarna hun dunne darmen werden onderzocht op eiwitniveau. De resultaten, beschreven in hoofdstuk 5, tonen aan dat het expressiepatroon van het

darmweefsel significant verandert ten gevolge van hongeren. Op basis van de verandering in eiwitexpressie werden processen als glucose- en energiemetabolisme geclassificeerd als verlaagd na 12 uur vasten. Dit is waarschijnlijk een directe reactie op de verminderde aanvoer van voedsel. Na 24 uur vasten kwamen twee elongatiefactoren, die een belangrijke rol spelen bij het proces van eiwitsynthese, verminderd tot expressie. Ondanks dit remmende effect op de algemene eiwitsynthese werden de expressieniveaus van reg I, reg II, glutathionperoxidase 3 en koolzuuranhydrase 3 opgereguleerd. Verondersteld wordt dat deze eiwitten een beschermende rol hebben in de darmcellen. Vasten gedurende 24 uur resulteerde in een verlaging van de expressie van OAT, vergelijkbaar als bij deprivatie van glutamine voor 24 uur in Caco-2 cellen. Verhoogde expressie van ezrine werd gedetecteerd na 72 uur vasten en dit duidt op een poging de villusfunctie te handhaven bij een dergelijke depletie van de energiereserves.

Twee veelgebruikte celmodellen voor het epitheelweefsel van de humane dunne en dikke darm zijn Caco-2 en HT-29 cellen, zo ook in dit proefschrift. Deze en vele andere cellijnen zijn van tumorale oorsprong, wat impliceert dat het metabolisme van de cellen veranderd is in vergelijking met de fysiologische *in vivo* situatie. Het metabolisme van een cel wordt bepaald door de expressie en activiteit van meerdere genproducten. Daarom werden deze celmodellen geëvalueerd op het niveau van eiwitexpressie in hoofdstuk 6. Om dit te realiseren werden de eiwitprofielen van deze humane *in vitro* modellen vergeleken met de eiwitprofielen van epitheelcellen afkomstig van schraapsels van de dunne en de dikke darm. Twee negatieve controles werden toegevoegd aan de studie, namelijk de levercellijn Hep G2 en de spiercellijn TE 671, beiden afkomstig van humaan tumorweefsel. Statistische analyses zoals 'principal component analysis' en 'hierarchical clustering' werden toegepast op de eiwitdata en onthulden dat de eiwitexpressie van de schraapsels van het darmepitheel behoorlijk verschilde van die van de darmcellijnen en van cellijnen in het algemeen. Typische eiwitten die hoger tot expressie kwamen in de schraapsels zijn hemoglobine alfa en bèta, serumalbumine en transthyretine. Deze eiwitten zijn hoogstwaarschijnlijk afkomstig van bloed, wat erop wijst dat het, ondanks het zorgvuldig schrapen, vrij moeilijk is om zuivere isolaten van epitheelcellen te verkrijgen. Meteen is ook het voordeel van cellijnen kracht bijgezet, daar zulke celculturen uit een homogene celpopulatie bestaan. Meer eiwitten die verschillen in expressie tussen *in vitro* celculturen en de epitheelschraapsels, werden gedetecteerd. Vooral eiwitten, die de tumorale oorsprong van de cellijnen en hun aanpassing aan kweekcondities reflecteren, behoren tot deze groep. Ondanks bovenstaande bevindingen vertonen de 2-D-patronen van Caco-2 cellen en epitheelcellen van de dunne darm veel overlap. Daarenboven heeft de studie geleid tot de identificatie van enkele eiwitmerkers, zoals OAT, in Caco-2 cellen die karakteristiek lijken voor het fenotype van het

epitheel van de dunne darm. Algemeen beschouwd tonen de data, beschreven in hoofdstuk 6, aan dat de Caco-2 cellen bruikbaar zijn voor het vergroten van de kennis over het darmepitheel. Wel moet rekening gehouden worden met het feit dat het extrapoleren van zulke kennis, verkregen door middel van het gebruik van een celmodel, naar de humane *in vivo* situatie gecompliceerd is en stof voor verder onderzoek.

Concluderend demonstreert dit proefschrift de toepasbaarheid van proteomics-technologie in het voedingsonderzoek, daar nieuwe eiwitten en moleculaire 'pathways' werden gevonden die gereguleerd worden door de beschikbaarheid van bepaalde nutriënten. De geïdentificeerde nutriënt-genregulaties kunnen een verdere bijdrage leveren aan het begrijpen en onderbouwen van de behoefte van bepaalde nutriënten voor het handhaven en verbeteren van de darmgezondheid in de mens. Niettegenstaande is meer functioneel onderzoek vereist om het belang van de gereguleerde eiwitten in de darm kracht bij te zetten.

ABBREVIATIONS

Abbreviations

1-DE	one-dimensional gel electrophoresis
2-DE	two-dimensional gel electrophoresis
AARE	amino acid response element
Ab	antibody
ApoA-I	apolipoprotein AI
ASL	argininosuccinate lyase
ASS	argininosuccinate synthase
CAS	cellular apoptosis susceptibility protein
CHAPS	3-[(3-cholamidopropyl)dimethyl-amonio]-1-propanesulfonate
CBB	Coomassie Brilliant Blue
DMEM	Dulbecco's modified Eagle's medium
DTT	dithiothreitol
dUTPase	deoxyuridine 5'-triphosphate nucleotidohydrolase
EF	elongation factor
eIF2alpha	eukaryotic translation initiation factor 2 subunit alpha
eNOS	endothelial NOS
ER	endoplasmatic reticulum
FABP	fatty-acid binding protein
FCS	fetal calf serum
GCN2	general control nonderepressible protein 2
GI	gastrointestinal
GPx-3	glutathione peroxidase 3
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
hnRNP	heterogeneous nuclear ribonucleoprotein
HSF	heat shock transcription factor
HSP	heat shock protein
IEF	isoelectric focusing
iNOS	inducible NOS
IPG	immobilized pH gradient
LI-cadherin	liver-intestine cadherin
LPS	lipopolysaccharide
m/z	mass-to-charge ratio
MALDI-TOF	matrix-assisted laser desorption/ionization-time of flight
MS	mass spectrometry
MSO	L-methionine sulfoximine
NASP	nuclear autoantigenic sperm protein
nNOS	neuronal NOS
NO	nitric oxide
NOS	NO synthase

OAT	ornithine aminotransferase
OCT	ornithine carbamoyltransferase
P5C	L-Δ^1-pyrroline-5-carboxylate
PBS	phosphate-buffered saline
PC	principal component
PCA	principal component analysis
PEPCK	phosphoenolpyruvate carboxykinase
PMF	peptide mass fingerprint
RBP	retinol-binding protein
SF2/ASF	splicing factor arginine/serine-rich 1
TCA	tricarboxylic acid
TPA	12-O-tetradecanoylphorbol-13-acetate

DANKWOORD

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Dit proefschrift is het eindresultaat van meer dan vier jaar onderzoek. Het ging niet altijd van een leien dakje (zeker de eerste twee jaar), maar toch heb ik het als een heel aangename tijd ervaren waar ik met veel plezier aan terugdenk. Ik heb er veel van opgestoken, maar ook op niet-wetenschappelijk vlak was het een fijne periode. Ik wil iedereen bedanken die direct of indirect aan dit alles heeft bijgedragen.

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PUBLICATIONS

PUBLICATIONS

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CURRICULUM VITAE

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Kaatje Lenaerts was born on June 20, 1978 in Bilzen (Belgium). In 1996, she received her diploma Modern Languages-Sciences at the Provincial Secondary School of Voeren. The same year, she started her studies in Biomedical Sciences at the University of Antwerp, which resulted in a licentiate degree in Biochemistry, with specialization Medical Biochemistry at the University of Antwerp in June 2000. The title of her thesis was 'Implementation of the comparative genomic *in situ* hybridization technique: a molecular-cytogenetic approach'. One year later, she completed her Academic Teacher Training at the Catholic University of Leuven. In April 2002, she started her PhD study which was part of the project 'An integrated genomics approach towards gut health' at the department of Human Biology at the Maastricht University under the supervision of Prof. dr. E. Mariman and Dr. J. Renes. In December 2006, she was granted a 'Talent for the Future' scholarship from the Faculty of Health Sciences of Maastricht University to continue her work in the field of nutrigenomics with emphasis on proteomics technology.