Starvation Induces Phase-Specific Changes in the Proteome of Mouse Small Intestine

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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.

Keywords: short-term food deprivation • long-term food deprivation • protein profiling • intestinal adaptation • lithostathine 1 and 2 (reg I and II) • ezrin expression

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.

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Materials and Methods

Materials. Zirkonia-Silica beads (2.5 mm) were from BioSpec Products (Bartlesville, OK, USA). CHAPS, DTT, goat anti-rabbit Ig-alkaline phosphatase, and levamisole (Tetramisolehydrochloride) were obtained from Sigma (St. Louis, MO). NBT/BCIP solution was from Roche Applied Science (Penzberg, Germany). Urea and SYPRO Ruby Protein Stain were from Bio-Rad Laboratories (Hercules, CA). 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, 50−60% 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 20 000 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). 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) 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 anti-rabbit 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) ($\geq$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.

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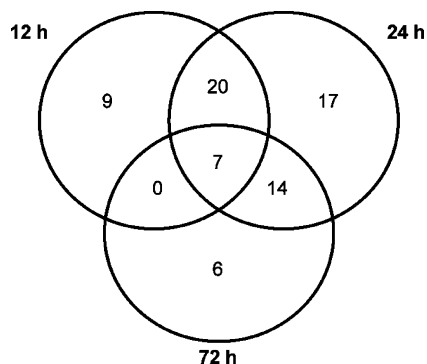
Figure 1. Venn diagram of 73 differentially expressed protein spots (12, 24, or 72 h fasting compared to control group). The intersections indicate the spots that are changed at more than one time point.

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 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.
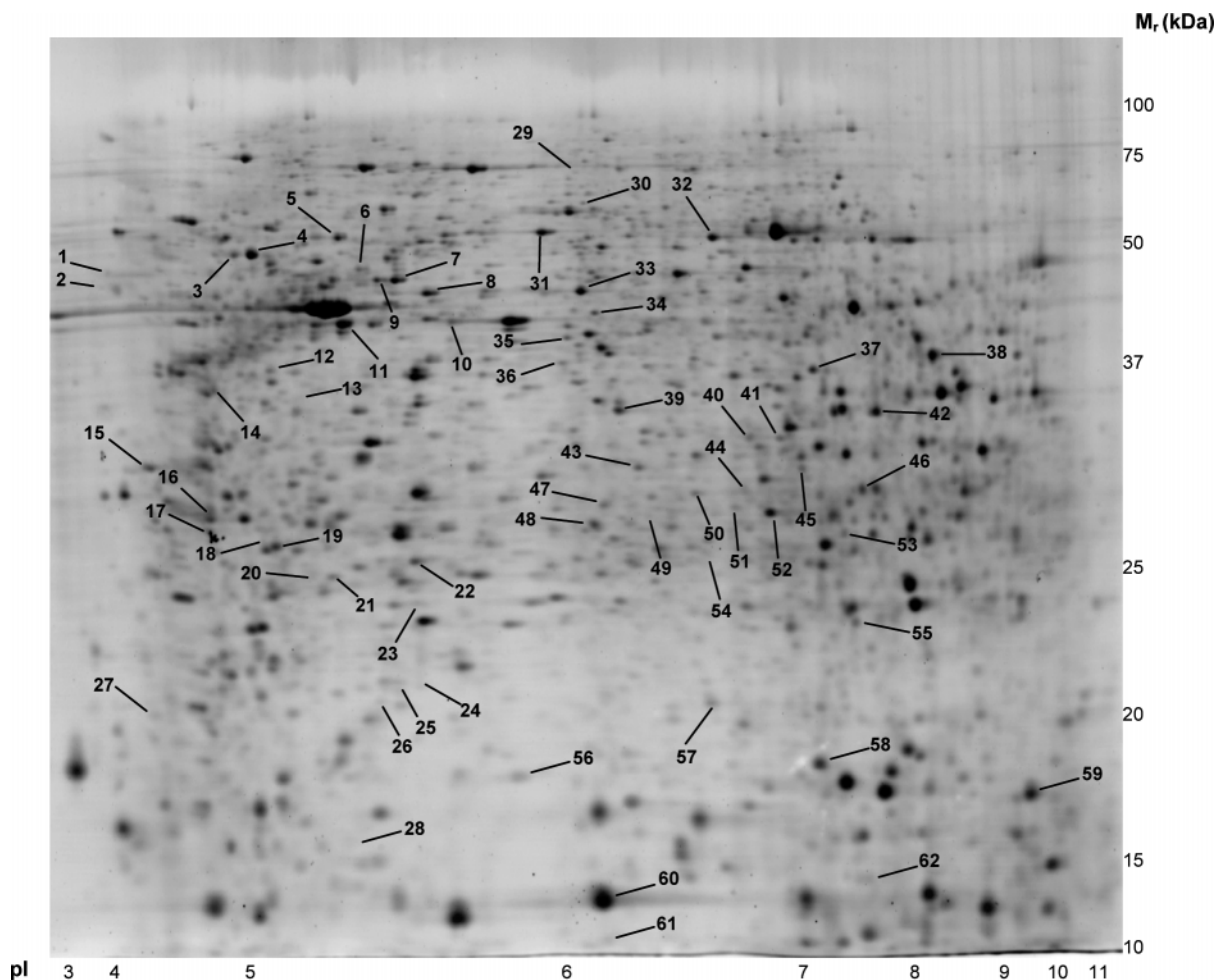

Figure 2. Example of a 2-D pattern of proteins extracted from a mouse small intestine (at 12 h of fasting). The gel was stained with SYPRO Ruby Protein Stain. Expression levels of numbered protein spots were changed between the different conditions (0, 12, 24, and 72 h of starvation), and their identity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

Table 1. Spot Numbers, Corresponding to Figure 2, and Identity of Changed Protein Spots During Starvation[a]

spot no.	Swiss-Prot accession no.	protein ID	0 h vs 12 h		0 h vs 24 h		0 h vs 72 h	
			FC	p-value	FC	p-value	FC	p-value
		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[b]	**−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 complex core protein I[c]	**−2.2**	**0.022**	−1.3	0.398	−1.3	0.379
4	P56480	ATP synthase beta chain[c]	**−2.7**	**0.030**	−2.1	0.064	−1.0	0.961
17	P56480	ATP synthase beta chain[c,d]	**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, member B1	**−2.9**	**0.009**	−1.9	0.056	−1.4	0.285
		Protein folding and processing						
27	P09103	protein disulfide-isomerase[d]	**2.2**	**0.023**	**2.8**	**0.001**	2.1	0.089
61	P17742	peptidyl-prolyl cis−trans isomerase A[e]	**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[e]	1.6	0.139	1.9	0.004	**2.3**	**0.002**
		Protein synthesis						
15	O70251	EF-1-beta	−1.3	0.298	**−2.1**	**0.006**	−1.3	0.377
36	P58252	EF-2[d]	−1.5	0.044	**−2.2**	**0.001**	**−2.0**	**0.003**
		Amino acid metabolism						
33	P29758	OAT[c]	−1.9	0.008	**−2.0**	**0.002**	**−2.0**	**0.003**
16	P29758	OAT[c,d]	−1.2	0.406	**−2.3**	**0.002**	**−3.3**	**0.000**
34	O08691	arginase-2[c]	−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[e]	1.9	0.052	**2.6**	**0.003**	2.7	0.108
40	P00688	pancreatic alpha-amylase[e]	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[d]	**4.4**	**0.005**	**6.5**	**0.004**	**5.9**	**0.000**
13	P07724	serum albumin[d]	**3.2**	**0.001**	**3.4**	**0.009**	2.5	0.141
22	P07724	serum albumin[e]	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
12	Q62468	villin-1[d]	−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[e]	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[e]	−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[d]	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[e]	1.1	0.855	**2.1**	**0.038**	2.2	0.071
28	P63268	actin, gamma-enteric smooth muscle[e]	**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 member 1	−1.6	0.005	**−2.0**	**0.001**	−1.3	0.281
55	O88569	heterogeneous nuclear ribonucleoproteins A2/B1[e]	−1.1	0.376	**−2.3**	**0.000**	**−2.1**	**0.000**
21	Q9JM14	5′(3′)-deoxyribo-nucleotidase[b]	−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

Table 1 (Continued)

spot no.	Swiss-Prot accession no.	protein ID	0 h vs 12 h		0 h vs 24 h		0 h vs 72 h	
			FC	*p*-value	FC	*p*-value	FC	*p*-value
		Mixtures						
3	P56480	ATP synthase beta chain[c]	**−2.6**	**0.016**	**−2.5**	**0.018**	−1.7	0.074
	P20029	78 kDa glucose-regulated protein[d]	**−2.6**	**0.016**	**−2.5**	**0.018**	−1.7	0.074
14	P63268	actin, gamma-enteric smooth muscle[e]	1.5	0.118	1.2	0.548	−1.7	0.077
	P31001	desmin[d]	1.5	0.118	1.2	0.548	−1.7	0.077

[a] 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 or more and $p < 0.05$. [b] Cytosolic. [c] Mitochondrial. [d] C-terminal fragment. [e] N-terminal fragment.

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]

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.

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.

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. Goblet cells were also visualized, and none of the mentioned 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
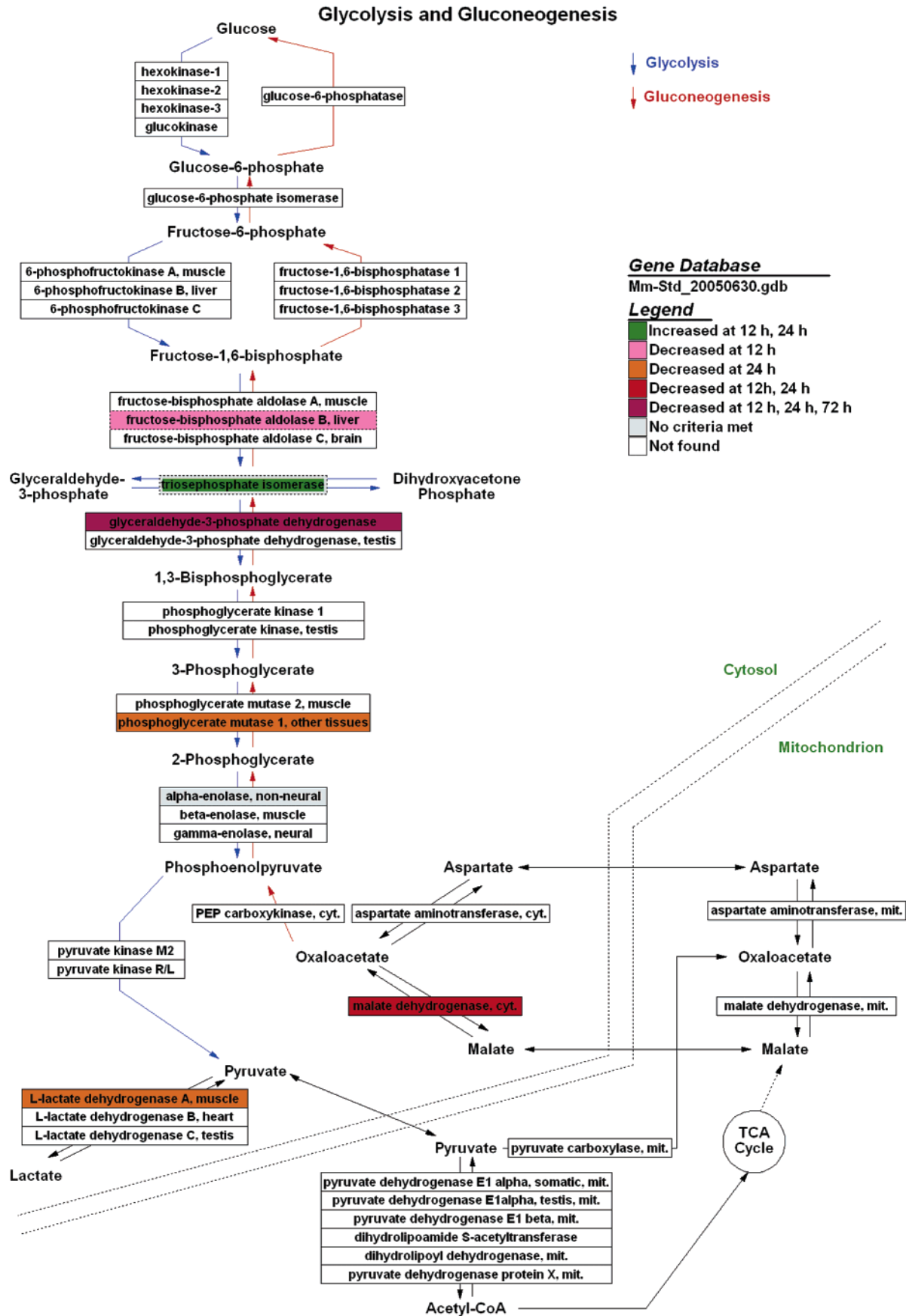
Glycolysis and Gluconeogenesis

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.
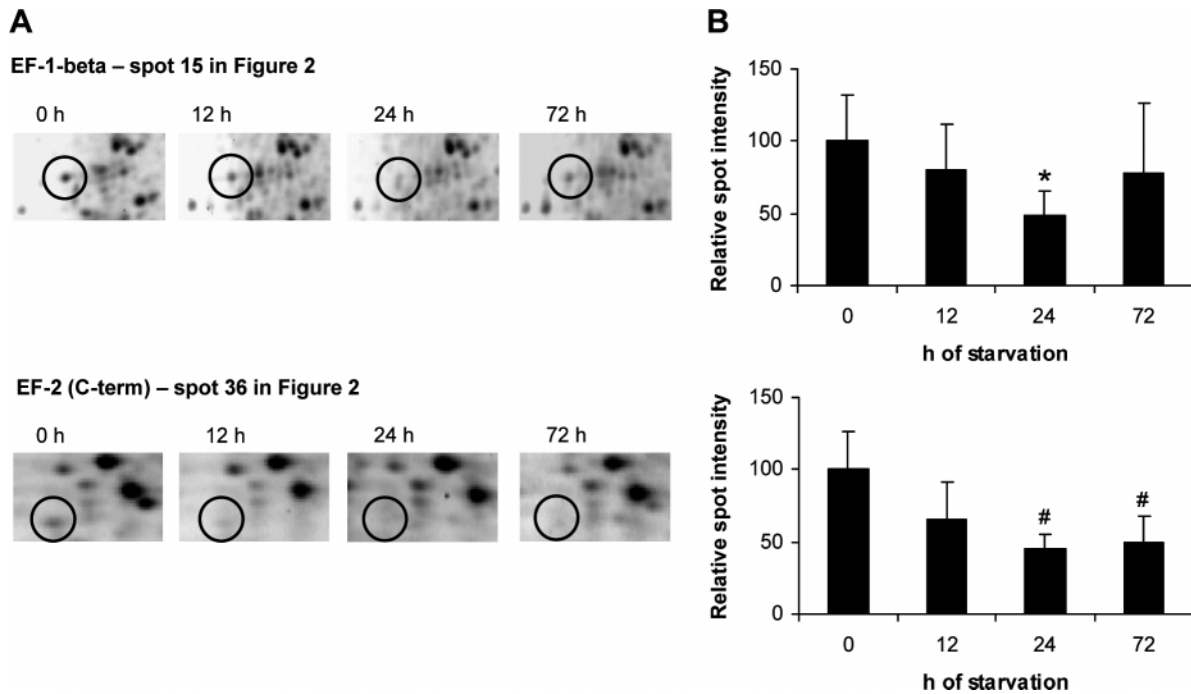
Figure 4. (A) Examples of differentially expressed protein spots (spot 15 and 36 in Figure 2). Both spots, identified as EF-1-beta and EF-2, respectively, show a decrease in intensity after 24 h of starvation, which remains significant for EF-2 after 72 h of starvation. (B) Graphical presentation of 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).
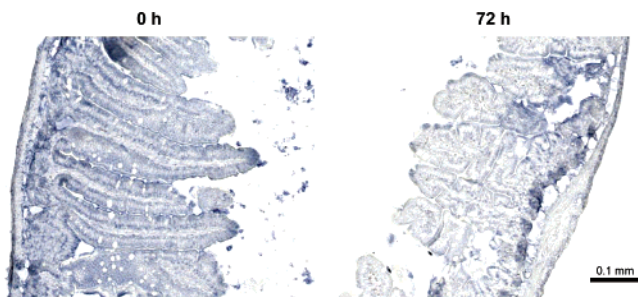
Figure 5. Immunohistochemical staining of OAT in section of the small intestine of fed (0 h) and 72-h-starved mouse (10× magnification).

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.

A

Lithostathine 1/Reg I – spot 62 in Figure 2

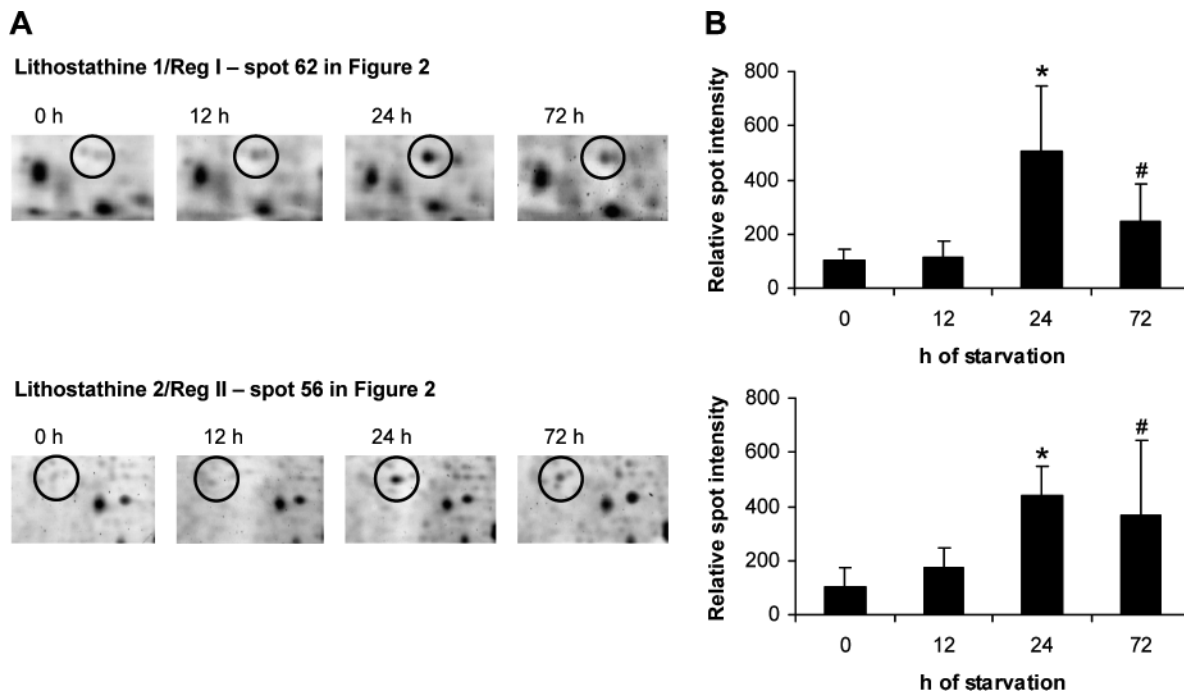0 h   12 h   24 h   72 h

B

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).

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 Lieberkühn 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.

Abbreviations: IPG, immobilized pH gradient; PBS, phosphate-buffered saline; 2-DE, two-dimensional gel electrophoresis; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; OAT, ornithine aminotransferase; EF, elongation factor; GPx-3, glutathione peroxidase 3.

Note Added after ASAP Publication: This paper was originally published on the Web (08/08/2006) with the authors in an incorrect order. The correct version was posted to the Web 08/10/2006.

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

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