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Fasting does not increase mRNA levels of proteolytic systems in small intestinal mucosa of the rat

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Fasting results in rapid and profound wasting of the small intestine. mRNA levels of genes encoding critical components of proteolytic systems were measured in small intestinal mucosa to indirectly assess the possible role that proteolysis plays in mediating this wasting. Male Sprague-Dawley rats (120 g; n = *6 per group) were either fed or fasted for 1 or 2 days. Small intestinal mucosal mass decreased by 19% and 31% after 1 and 2 days of fasting, respectively (*P < *0.05). Fasting did not significantly change mRNA levels for lysosomal (cathepsin B) or ubiquitin-proteasome-dependent (ubiquitin, 14-kDa ubiquitin-conjugating-enzyme E2, and the C8 and C9 proteasome subunits) systems. Northern hybridizations were also performed using membranes made with poly* A^+ *mRNA instead of total RNA. mRNA levels for these proteolytic systems and m-calpain did not significantly change with fasting. These data clearly demonstrated that fasting does not increase expression of genes encoding critical components of proteolytic systems in the small intestinal mucosa, suggesting that increased proteolysis cannot explain wasting of the small intestinal mucosa during brief fasting in young rats.* (J. Nutr. Biochem. 11: 496–499, 2000)

Keywords: starvation; proteolysis; protein degradation; wasting; intestine

Introduction

Rapid and profound wasting of the small intestine occurs after fasting in young and adult animals.[1–3] Twenty-five and 50% of small intestinal protein mass is lost in adult rats after 1 and 5 days of fasting, respectively.[3] Decreased food intake is also a prominent feature of cancer cachexia, infection, and trauma. Small intestinal protein losses due to decreased food intake may compromise digestive and absorptive functions, barrier integrity, and immune competence.[4] A clear understanding of the mechanisms causing wasting in the small intestine is needed to facilitate the development of therapies to inhibit or limit such losses.

Research from several groups has shown that decreased protein synthesis cannot explain the bulk of this wasting.[1–3,5,6] For example, we previously showed that in whole small intestine, although protein synthesis was only 10% lower ($P > 0.05$) in fasted rats, protein mass was 25% lower.[3] To explain wasting of small intestine in the face of relatively unchanged protein synthesis, we decided to investigate the role that proteolysis might play in mediating wasting.[3] The measurement of intestinal protein degradation is problematic, as there are no published methods for its measurement either in vivo or in vitro. To indirectly assess proteolysis, we measured the mRNA levels for the three known major proteolytic systems (lysosomal, calcium activated, and ubiquitin-proteasome-dependent proteolytic pathways) in whole small intestine from rats. We found that starvation seemed to co-ordinately increase mRNA levels for components of these three systems, suggesting that increased proteolysis contributed to wasting of small intestine.

Our past studies[3] were completed in whole small intestine, a morphologically and functionally heterogeneous tissue. Rates of protein synthesis are much higher in the mucosa than in the muscularis-serosa.[1] Also, wasting of the mucosa is more likely to result in detrimental effects to the host, such as bacterial translocation and digestive disturbances. Thus, it is important to determine if the increased mRNA levels we observed specifically stemmed from the mucosal layer. Our objective in the present study, therefore,

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was to measure mRNA levels for components of the major proteolytic systems in the small intestinal mucosa from fasted rats.

Materials and methods

Animals, housing, and diet

All animal studies were completed in accordance with the guidelines of the Canadian Council of Animal Care. Male Sprague-Dawley rats (100 g, 4–5 weeks old), obtained from the University of British Columbia (UBC) Animal Care Centre (Vancouver, B.C. Canada), were housed in individual cages and maintained at 22–23°C on a 12-hr light/dark cycle starting at 8:00 AM. Rats were given 3 days to adjust to their new environment before treatments were imposed. Rats were given free access to water and food (standard rodent chow, UBC Animal Care Centre, Vancouver, B.C. Canada) with the exception of fasted rats that were only given free access to water.

Experimental design

Rats were randomly designated to one of three treatment groups: (1) fed, (2) 1-day fasted, and (3) 2-day fasted. Five to 6 rats were used per group. On Day 0 and Day 1, food was removed from the 2-day and 1-day fasted rats, respectively, at 10:00 AM. On Day 2, animals were killed at 10:00 AM using an overdose of halothane (MTC Pharmaceuticals, Cambridge, ON, Canada). The visceral cavity was immediately opened and plunged in ice-cold water. The small intestine was removed, flushed with ice-cold saline, and blotted. Starting 10 cm distal to the pyloric sphincter, a 1- to 2-cm sample was taken. The sample was cut transversely, placed on a chilled glass plate, and the mucosal layer scraped using a chilled microscope slide. The mucosa was immediately frozen in liquid nitrogen and stored at −70°C. These were used for RNA isolation. The mucosal layer from the rest of the small intestine was scraped and weighed.

Northern hybridization

Total RNA was isolated from small intestinal samples by phenol-chloroform extraction.[7] Poly A^+ mRNA was also isolated from total RNA using an Oligotex® mRNA kit according to the manufacturer's instructions (Qiagen Inc., Mississauga, ON, Canada). Northern hybridizations were performed as previously described.[8] Fifteen micrograms of total RNA was electrophoresed on a 1% agarose gel containing formaldehyde, and was electrophoretically transferred to a nylon membrane (GeneScreen, NEN Research Products, Boston, MA USA). For poly A^+ mRNA, 200 pg was electrophoresed. RNA was covalently bound to the membrane using a UV crosslinker (Pharmacia, Baie d'Urfé, P.Q. Canada).

The RNA on the membrane was then hybridized overnight at 65°C with [^{32}P]cDNA probes labeled by random priming.[8] The probes used encoded rat cathepsin B,[9] human m-calpain[10] (poly A^+ mRNA membranes only), chicken polyubiquitin,[11] rat 14 kDa ubiquitin-conjugating enzyme E2,[12] and C8[13] and C9[14] rat 20S proteasome subunits. These probes were selected because they encode proteases and/or cofactors of the major proteolytic systems (lysosomal, calcium-dependent, and ubiquitin-proteasome-dependent). Probed membranes were washed four times in 0.1% sodiumdocecylsulfate (SDS) at 65°C for 15 min and the stringency of washes varied between 2× SSC to 0.2× sodium chloride-sodium citrate (SSC), depending on the probe. Washed membranes were placed in a phosphorimaging cassette and analyzed using a phosphorimager (Molecular Dynamics, Sunnyvale, CA USA). After stripping the probes, membranes were reprobed with human β-actin to correct for loading/transfer differences. There were no differences in β-actin mRNA levels among treatments ($P > 0.05$). Unless stated otherwise, all chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Table 1 Body and small intestinal mucosal mass after brief fasting in rats

	Fed	1-day fasted	2-day fasted
Body weight (g)	139.2 ± 2.4[a]	114.5 ± 1.9[b]	96.0 ± 1.7[c]
Mucosal mass (g)	3.32 ± 0.15[a]	2.70 ± 0.16[b]	2.35 ± 0.16[b,c]

Values represent means ± SEM. Values within a line with different superscipts are significantly different ($P < 0.05$).

Statistics

The effect of treatment was tested by analysis of variance.[15] Differences among means were assessed using the Student's *t*-test. Variability was expressed as SEM. Differences were considered significant at $P < 0.05$.

Results

Body weight and tissue mass

Body weight was 18% and 31% lower ($P < 0.05$) in 1- and 2-day fasted rats, respectively, compared to fed rats. Small intestinal mucosal mass was 19% and 32% lower ($P < 0.05$) in 1- and 2-day fasted rats, respectively, compared to fed rats (*Table 1*).

Northern hybridizations using total RNA

Northern hybridizations of cathepsin B, ubiquitin, 14-kDa E2 ubiquitin-conjugating enzyme, and the C8 and C9 proteasome subunits are shown in *Figure 1*. Levels of mRNA encoding 14-kDa E2 ubiquitin-conjugating enzyme, ubiquitin, the C8 and C9 proteasome subunits, and cathepsin B from the mucosa of the small intestine in 1- and 2-day fasted rats were not significantly different from fed control rats, although cathepsin B mRNA levels tended to be lower (−29%; $P < 0.1$) in 2-day fasted rats.

Northern hybridizations using poly A^+ mRNA

The above results were unexpected, because we had previously shown increased expression of several genes encoding components of proteolytic systems in whole small intestine from adult fasted rats.[3] Total RNA content and capacity for protein synthesis (μg RNA/mg protein) decrease with fasting,[3] so we thought that fasting might cause a specific reduction in mRNAs relative to total RNA in the mucosa. To test this possibility, we performed northern hybridizations on membranes prepared using 200 pg poly A^+ mRNA. Fasting did not decrease mRNAs encoding cathepsin B, 14-kDa E2 ubiquitin-conjugating enzyme, ubiquitin, and m-calpain from the mucosa of the small intestine compared to fed control rats (data not shown). Although mRNA levels encoding the C8 and C9 proteasome subunits from fasted rats were not significantly different from fed control rats, they tended to be lower (−23% to −33%) in the 1-day fasted rats. β-actin expression was not different among treatments.

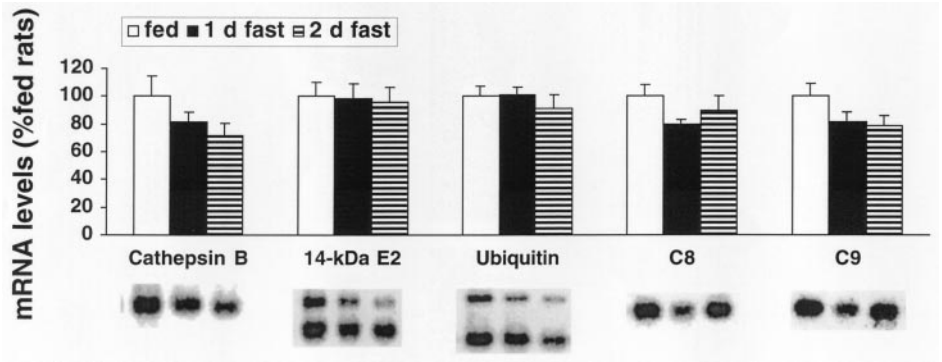

Figure 1 mRNA levels encoding various proteolytic systems in the mucosa from the small intestine of fasted rats using membranes made with 15 μg of total RNA per lane. Values are means ± SEM of phosphorimager signals corrected for β-actin mRNA abundance and expressed as a percentage of fed controls with $n = 5$ to 6 rats per group. Rats were fasted for either 1 or 2 days. No variation was significant ($P > 0.05$). Representative Northern blots are shown below the corresponding bars. 14-kDa E2, 14-kDa ubiquitin-conjugating enzyme E2; C8 and C9, C8 and C9 20S proteasome subunit.

Discussion

Intestinal wasting due to decreased food intake or due to conditions such as cancer, infection, and trauma, in which anorexia is a common feature, has a negative impact on the host. Thus, it was of interest to investigate the mechanisms that might lead to intestinal protein wasting. In this study, fasting resulted in rapid and profound body weight loss and depletion of the small intestinal mucosa in accordance with several studies.[1,2,6] However, these and other studies have shown that protein synthesis in mucosa or whole small intestine either does not change or modestly decreases.[1–3,5,6] Thus, we became interested in knowing if increased proteolysis could explain intestinal wasting. Unfortunately, the estimation of protein degradation in small intestine is elusive. There are no in vivo or in vitro techniques available for use in small intestine. An indirect approach that has been extensively used in skeletal muscle[8,16–23] is to measure the mRNA expression for various components of proteolytic systems. In rodent muscle, these mRNA levels correlate with rates of protein degradation measured in vitro[8,16,18–22] and with the activities of either m-calpain and cathepsins B and L[20] or 20S proteasome.[16] Furthermore, such increased mRNA levels are also observed in the muscles from cachectic patients and correlate with negative nitrogen balance[17,23] and with both enhanced rate of whole-body protein breakdown and increased urinary 3-methylhistidine excretion, which is an index of enhanced myofibrillar proteolysis.[17] Because the rate of protein degradation in the small intestine remains unknown, it is impossible to know the true value of relative changes in mRNA-encoding components of the major proteolytic systems. However, treatment differences between relative levels of mRNA may suggest directional changes in protein degradation as well as indicate transcriptional regulation or modulation of mRNA catabolism.

Overall, our study shows that fasting does not change gene expression for components of three major proteolytic systems (lysosomal, Ca^{2+}-dependent, and ubiquitin-proteasome-dependent) in the mucosa of the small intestine. In a related study, there was also unchanged or decreased proteolytic gene expression in small intestinal biopsy samples from fasted human volunteers (Corinne Bouteloup-Demange, Bernard Beaufrère, and Didier Attaix, unpublished observation). These suggest that increased proteolysis, assessed by mRNA expression, is unlikely to explain wasting in small intestinal mucosa in fasting, in accordance with the present findings.

In a prior study, we reported that starvation co-ordinately increased mRNA levels for components of these proteolytic systems in the small intestine from adult rats fasted for 1 and 5 days.[3] This suggested that proteolysis may play a role in mediating wasting of small intestinal proteins. This is in apparent contrast to our present findings. However, our previous studies[3] were completed in whole small intestine, a morphologically and functionally heterogeneous tissue. The muscularis-serosal layer of the small intestine contains myofibrillar proteins, which have been shown to be depleted during fasting.[1] Emery et al.[1] showed that protein degradation increased in intestinal smooth muscle from rats fasted for 1 to 3 days. During fasting, the expression of components of the ubiquitin-proteasome-dependent system only increases in striated muscle, but not in various other tissues, suggesting this adaptive response is skeletal muscle-specific.[18] However, muscular-serosa layer is composed of smooth muscle, which contains myofibrillar proteins. These suggest that the increase in mRNA for ubiquitin and the 14-kDa ubiquitin-conjugating enzyme E2 that we previously observed in whole intestine from fasted rats may have stemmed from muscularis-serosal layer of the gut, suggesting that the ubiquitin-proteasome proteolytic system was activated in that part of the gut. In our prior study, rats were adult; differences between the two studies may reflect age-dependent differences in proteolysis.

It is also possible that gene expression for proteolytic systems poorly correlates with protein breakdown rates in the small intestinal mucosa. Absorptive cells of the small intestine contain large numbers of proteasomes.[24] Proteasomes also play a key role in the regulation of cell growth,[25] and the high rate of cell proliferation is impaired in the intestinal mucosa during fasting.[26] Thus, the somewhat lower mucosal C8 and C9 proteasome subunit mRNA expression is not unexpected. Even in our previous studies on whole small intestine, the fasting-induced increases in 20S proteasome subunit expression failed to reach statistical significance, in contrast with the increased expression of ubiquitin, 14-kDa ubiquitin-conjugating-enzyme E2, m-calpain, and cathepsin B.[3]

The fact that neither decreased protein synthesis nor increased proteolysis can explain intestinal mucosal wasting during fasting in young rats poses the question, What causes the wasting? Protein mass in the small intestinal mucosa is dependent upon the rates of protein synthesis and degradation, but also upon the rate of cell loss into the lumen of the gut and secretion of digestive enzymes, glycoproteins,

immune proteins, and apolipoproteins.[4,27] Thus, cell turnover and protein secretion also contribute to the loss of intestinal protein mass; however, these processes have been reported or are believed to decrease during fasting.[26,28] Most export proteins are presumably degraded extracellularly, as are also large brush border enzymes, which are broken down by pancreatic proteases.[28] Recent evidence suggests that apoptosis may be involved in mucosal wasting during brief fasting in rats.[29] This would result in increased cell loss and could contribute to wasting. It is also possible that intracellular proteolysis may not play a key role in determining intestinal protein mass. However, there is recent evidence, although indirect, that supports a role for proteolysis in regulating intestinal protein mass.[30] Until quantitative methods for assessing intestinal proteolysis are developed, it is likely that the mechanism of wasting of the small intestine as a result of fasting will remain an enigma.

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