The Small Intestine Proteome Is Changed in Preterm Pigs Developing Necrotizing Enterocolitis in Response to Formula Feeding

Pingping Jiang, Jayda Lee Ann Siggers, Heidi Hoi-Yee Ngai, Wai-Hung Sit, Per T. Sangild, and Jennifer Man-Fan Wan

Abstract

Necrotizing enterocolitis (NEC) is the most common gastrointestinal emergency in newborn premature infants. Clinical studies show increased incidence of NEC in premature infants with enteral formula feeding; however, pathogenesis remains unclear. To identify the NEC-related proteins for molecular mechanisms, we applied proteomics analysis to characterize changes in the protein expression profile of newborn premature piglet intestines with NEC developed after enteral formula feeding for 24 h. Changes in protein expression were identified using 2-dimensional gel electrophoresis and peptide mass fingerprinting with MS as well as western blotting analysis. Nineteen differentially expressed proteins were identified and these have roles in oxidative stress, chaperone, signal transduction, protein folding and degradation, oxygen transport, signal transduction, and energy metabolism. Proteins with increased levels include manganese-containing superoxide dismutase and hemoglobin subunit and proteins with decreased expression include sorbitol dehydrogenase, mitochondrial aldehyde dehydrogenase 2, glucose-regulated protein 75, CRY protein, snail homolog 3, thyroid hormone-binding protein precursor, and DJ1 (Parkinson’s disease 7) etc. The data provided novel mechanistic insights into the pathogenesis of NEC and the insults of a formulated diet to the premature gut. J. Nutr. 138: 1895–1901, 2008.

Introduction

Necrotizing enterocolitis (NEC) is characterized by severe inflammatory-response gastrointestinal bleeding and hemodynamic instability in the intestine of preterm infants (1). It has an overall mortality of 25–30% with 25–50% of survivors requiring surgical intervention to remove affected intestinal sections (2). The proposed mechanisms involve the breakdown of intestinal mucosal barrier, translocation of bacteria and endotoxins, excessive production of proinflammatory mediators, nitrosative stress, and increased apoptosis that together lead to necrosis of enterocytes (3,4). The etiology of NEC is multifactorial and pathogenesis is currently unclear. Both premature and enteral nutrition are probably the major factors predisposing to the progression of NEC in preterm neonates (4).

The premature neonates require early nutritional support to provide sufficient nutrients so that their bodies grow at a rate similar to that in utero and to avoid adverse developmental and neurological outcomes. However, the introduction of an enteral diet into an immature lumen with underdeveloped digestive and absorptive capacity may induce mucosal dysfunction and predispose the neonates to malabsorption and nutrient fermentation and encourage microbial colonization (4). The intraluminal toxins generated can cause direct mucosal injury or initiate destructive bowel wall inflammation in the premature gut, leading to the development of NEC. Therefore, nutritional therapy of premature newborn infants remains a challenge to pediatricians. Research in this field is currently limited by the lack of human autopsied intestinal samples from the compromised population and relevant protein markers for the mechanistic study.

By using an enteral feeding-induced piglet model (4), with characteristics of human NEC, the aim of this study was to elucidate the mechanisms of NEC with a comparative proteomic approach. The application of high-throughput proteomic techniques can systemically identify and characterize the differentially expressed proteins in a time-efficient manner (5). The proteins identified can help generate novel mechanistic insights into the disease pathogenesis.

1 Supported by The University of Hong Kong and the Danish Research Council.
3 Supplemental Tables 1 and 2 are available with the online posting of this paper at jn.nutrition.org.
4 These authors contributed equally to the article.
5 Abbreviations used: Ctrl, group received no formula feeding; 2-DE, 2-dimensional gel electrophoresis; DTT, dithiothreitol; FF, formula feeding group; GRP75, glucose-regulated protein 75; IPG, Immobilized pH gradient; LAP, leucine aminopeptidase; MALDI-TOF MS, matrix-assisted laser desorption ionization-time of flight MS; NEC, necrotizing enterocolitis; OAT, o xo-acid aminotransferase; ROS, reactive oxygen species; SOD-2, superoxide dismutase 2; TCA, trichloroacetic acid; TPN, total parenteral nutrition.
6 To whom correspondence should be addressed. E-mail: jmfwan@hkusua.hku.hk.
In this study, we used 2-dimensional gel electrophoresis (2-DE) coupled with MS and database-analyzing techniques to investigate the global changes in protein expression of healthy and NEC-developed guts of premature piglets. Specific changes in protein expression were identified in inflamed compared with normal intestinal tissue. The differentially expressed proteins appear to be involved in antioxidation, metabolism, energy generation, chaperone, signal transduction, protein folding, and cellular proliferation. The results presented here help generate novel mechanistic insights into disease pathogenesis and the relationship to enteral formula feeding. It may also help to identify new therapeutic targets for treatment of NEC in preterm neonates.

Materials and Methods

Enteral formula feeding and piglet NEC model. The development of the enteral feeding induced-NEC premature piglet model has been previously described by us (4). Preterm piglets were delivered by caesarean section at 107–108 d of gestation from 2 different sows. They received total parenteral nutrition (TPN) for 24 h via the intravenous catheter as described before (4). This feeding protocol is based on a clinical setting, because TPN treatment often predisposes the premature infants to develop NEC in response to formula feeding (4). At the end of the TPN treatment, 4 piglets from the 108-d-gestation sow were killed for tissue collection and served as control (Ctrl). The 3 piglets from the other sow (107 d gestation) were immediately switched to an oral formula diet (15 mL/kg body weight) every 3 h for a period of 24 h and served as the formula feeding (FF) group. The formulation of the diet was designed to match the composition of sow milk during lactation as previously described (4,6). The National Committee on Animal Experiments in Denmark approved all procedures.

Sample preparation for proteomic analysis. The intestine tissue samples were embedded in paraffin and sectioned at 3 μm and stained with hematoxylin and eosin. The intestinal damage was examined by a light microscope (Orthoplan, Leitz) and saved as image files by NIH Image software (version 1.22c). The NEC scoring system was as follows: 0 = no damage; 1 = occasional areas of violaceous mucosa (0–25% affected); 2 = multiple areas of violaceous mucosa (25–50% affected); 3 = severe hemorrhagic mucosa (50–75% affected); and 4 = extensive hemorrhage mucosa (>75% affected) was used to characterize the extent of hemorrhage and/or necrosis as previously described (4). A score of ≥1 in any region of the gastrointestinal tract was deemed NEC.

Histology and NEC assessment. The paraffin-embedded-fixed samples were stained with hematoxylin and eosin. The intestinal damage was examined by a light microscope and was scored as previously described (4). Intestinal damage was evaluated by the extent of hemorrhage and necrosis.

Sample preparation for proteomic analysis. The intestine tissue samples were disrupted with a tissue teaser (Biospec Products) in a cocktail buffer [1% Triton X-100, 25 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L EDTA disodium salt, 1 mmol/L dithiothreitol (DTT)] with added Protease Inhibitor Cocktail Set III (AEBSF, aprotinin, bestatin, E-64, leupeptin hemisulfate, pepstatin A). The supernatant was stored at −80°C until 2-DE analysis. The protein solution was stored at −80°C until 2-DE analysis. Protein concentration was determined by Bradford assay (Bio-Rad).

2-DE and MS analysis. The 2-DE procedures used here have been previously described by us with modifications (5). All tissue samples were performed in duplicate and a total of 14 gels (8 for Ctrl and 6 for FF) were conducted. For the first dimensional electrophoresis of proteins, a fixed amount of 100-μg protein samples were mixed with 350 μL rehydration buffer (9.5 mol/L urea, 2% [3-(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 0.28% DTT, 0.5% Immobilized pH gradient (IPG) buffer, pH 3–10, 0.002% bromophenol blue) before being applied onto the Ettan IEGophor isoelectric focusing electrophoresis system. The samples were rehydrated for 7 h before isoelectric focusing via the following programs: 1) linear increase up to 500 V in 1 h; 2) held at 500 V for 3 h; 3) linear increase up to 10,000 V in 3 h; 4) linear increase up to 10,000 V in 3 h; and 5) finally held at 10,000 V to reach a total of 90,000 volt × h. Focused IPG gel strips were equilibrated for 15 min in a solution (50 mmol/L Tris-HCl, pH 8.8, 6 mol/L urea, 30% glycerol, 2% SDS, containing 20 mmol/L DTT) followed by incubation with the same buffer containing 20 mmol/L iodoacetamide for another 15 min. The 2nd dimensional separation was performed on 1.0-mm-thick 12.5% polyacrylamide gels and SDS-PAGE at a constant current of 30 mA for 30 min followed by a 60-mA current for the rest of the analysis. After electrophoresis, gels were fixed in solution (10% ethanol, 7% acetic acid in water) for 2 h before staining with Deep Blue Total Protein stain (GE Healthcare).

Image acquisition and analysis. The Typhoon 9410 Variable Mode Imager (GE Healthcare) was used to scan the 2-DE gels. Image Master 2D Platinum V6.0 (GE Healthcare) software, with function on background subtraction, spots detection, and volume normalization, was used for matching and analyzing protein spots on the 2-DE gels. Detected spots were normalized and assigned with a number referenced by a “virtual gel” or a reference gel (the best gel of the 14 gels) automatically. Any under-detected spots were manually assigned with the number according to the reference gel by the researcher. The expression level, expressed as percentage volume (% vol), was exported out for statistical analysis. Protein spots with differential expression between Ctrl and FF (P < 0.05) were selected for protein identification.

Protein identification. Spots with differential expression (P < 0.05) between Ctrl and FF were sent to the Genome Research Centre (The University of Hong Kong, Hong Kong) for protein identification. The proteins were digested with trypsin and applied to matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS on the Voyager-DE STR BioSpectrometry Workstation or MALDI-TOF/TOF MS analysis on 4800 MALDI TOF/TOF Analyzer (Applied Biosystems) for analysis. The match between the experimental data and mass values calculated from a candidate protein was carried out by Mascot peptide mass fingerprinting, a powerful search engine that uses MS data to identify proteins from (7) against the NCBI database with taxonomy limited to mammalia (mammals). Mascot reported the molecular weight search (MOWSE) score, which is calculated by

\[
\log_{10}(P) = \frac{1}{2} \log_{10}(m) - \frac{1}{2} \log_{10}(P) + \frac{1}{2} \log_{10}(P)
\]

where \(P\) is the probability that the observed match is a random event. \(P\) is limited by the size of the sequence database being searched (limited by taxonomy), the conditions, and the settings of trypsin digestion. Each calculated value that falls within a given mass tolerance of an experimental value counts as a match. The accepted threshold is that an event is significant if it would be expected to occur at random with a frequency of <5%. In this study, a protein match with a score > 64 was regarded as significant (7,8). The Mascot also generated values of sequence coverage, which is the percentage of trypsin-derived peptides accounting for the whole sequence of the target protein (7). The apparent isoelectric point and relative molecular mass of proteins are calculated according to their position on the gels and confirmed with the searched data.

Western blotting analysis. Western blotting analysis was performed on selected proteins to confirm their proteomic results. Briefly, intestinal protein extract was mixed with sample buffer (62.5 mmol/L Tris, pH 6.8, 25% glycerol, 2% SDS, 350 mmol/L DTT, 0.01%...
bromophenol blue) in a ratio of 1:1 (v:v) and then kept at 37°C for 15 min. Thirty μg protein of each sample was applied for electrophoresis on 12.5% SDS-PAGE gels with constant voltage (125 V) and transferred to polyvinylidene difluoride membrane (GE healthcare). The membranes were incubated for 3 h with rabbit polyclonal antibodies (ABCAM) and antibodies: superoxide dismutase 2 (SOD-2) (1:5000), PARK7/DJ1 (1:2000), with mouse monoclonal antibody against 75-kDa glucose-regulated protein 75 (GRP75; 1:5000 v) and with mouse antibody against β-tubulin (1: 5000) (Zymed). The intensity change of protein bands was estimated quantitatively using Quantity One software (Bio-Rad). The relative molecular weight of each protein band was estimated with molecular markers (Precision Plus Protein Standards Dual Color, Bio-Rad).

**Statistical analysis.** All data for protein expression level in 2-DE and western blotting are expressed as means ± SEM. Significance of the data was determined by 2-tailed student’s t test with Levene’s Test for equality of variances in SPSS 11.5 for Windows with P < 0.05 as significant.

**Results**

**Intestinal morphology and NEC scores.** The Ctrl group showed normal intestinal architecture with preserved villi and no inflammation (NEC score = 0 for all 4 piglets). The FF piglets had disruptive architecture, atrophy of villi, signs of blood congestion, and inflammation (Fig. 1). The NEC scores for the 3 FF piglets were 3, 4, and 5.

**Intestinal proteome profile and protein identification.** On each 2-DE gel, ~450 well-resolved protein spots were detected. However, with a limited database for piglets, only 19 spots with notable changes between Ctrl and FF groups were successfully identified (Fig. 2). We listed the cellular locations as well as the basic information of identified proteins with a lower or higher expression level (P < 0.05) in FF compared with Ctrl (Table 1, the information of peptide sequences used in MS identification (Supplemental Tables 1 and 2)). The proteins with increased expression were ornithine-oxo-acid aminotransferase (OAT; spot 958), SOD-2 (spot 1123), and chain B, structure determination of aquomet porcine hemoglobin at 2.8 angstrom resolution (spots 1241 and 1242). The proteins with a low level were cytoskeleton and associated proteins (tubulin, β 5, spot 894), chain, leucine aminopeptidase (LAP; spot 898), ornithine aminotransferase, mitochondrial precursor (OAP; spot 958), chain A, medium-chain acyl-CoA dehydrogenase with 3-thiaoctanoyl-CoA (spot 988), mitochondrial aldehyde dehydrogenase 2 (spot 903), aldehyde reductase (spot 1045), enolase 1 (spot 942), thyroid hormone binding protein precursor (spot 875), and CRY protein (spot 1080). The protein spot 830 is predicted to be the stress-70 protein, mitochondrial precursor isoform 17 (heat shock protein 70), which is also named GRP75.

**Western blotting confirmation.** Among the proteins analyzed using Western blotting for confirmation, GRP75, DJ1, and β-tubulin were reduced (P < 0.05), whereas SOD-2 tended to be higher (P = 0.10) in FF piglets compared with Ctrl ones (Fig. 3).

**Discussion**

Despite decades of research, there are still no effective means for the detection, prevention, and treatment of NEC owing to the lack of understanding of its underlying mechanisms (1). To establish a database of potential diagnostic or therapeutic protein targets to improve the understanding of NEC, we adapted a gel-based proteomics approach in a piglet model of human NEC. Nineteen differentially expressed proteins with...
### TABLE 1  Differentially expressed intestinal proteins between preterm piglets that were (FF) or were not (Ctrl) formula fed

<table>
<thead>
<tr>
<th>Spot</th>
<th>GenBank Identifier</th>
<th>Protein Description</th>
<th>MOWSE score&lt;sup&gt;3&lt;/sup&gt; (searched/apparent)</th>
<th>Mr&lt;sup&gt;4&lt;/sup&gt; (searched/apparent)&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Expression level (Ctrl)&lt;sup&gt;5&lt;/sup&gt;</th>
<th>Expression level (FF)&lt;sup&gt;5&lt;/sup&gt;</th>
<th>P&lt;sup&gt;6&lt;/sup&gt;</th>
<th>Subcellular location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1123</td>
<td>gi</td>
<td>30841303</td>
<td>Manganese-containing SOD (Homo sapiens)</td>
<td>80</td>
<td>6.90/7.37</td>
<td>23.3/27.3</td>
<td>0.06 ± 0.01</td>
<td>0.18 ± 0.01</td>
</tr>
<tr>
<td>1162</td>
<td>gi</td>
<td>118403904</td>
<td>DJ1 protein (Sus scrofa)</td>
<td>69</td>
<td>6.33/6.63</td>
<td>20.3/23.1</td>
<td>0.22 ± 0.03</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>1241</td>
<td>gi</td>
<td>809283</td>
<td>Chain B, structure determination of aquomet porcine hemoglobin at 2.8 A resolution</td>
<td>139</td>
<td>6.78/7.99</td>
<td>16.1</td>
<td>0.93 ± 0.22</td>
<td>6.65 ± 1.70</td>
</tr>
<tr>
<td>1242</td>
<td>gi</td>
<td>809283</td>
<td>Chain B, structure determination of aquomet porcine hemoglobin at 2.8 A resolution</td>
<td>121</td>
<td>6.78/7.99</td>
<td>16.1</td>
<td>0.10 ± 0.02</td>
<td>1.28 ± 0.20</td>
</tr>
<tr>
<td>830</td>
<td>gi</td>
<td>73970910</td>
<td>Predicted: similar to Stress-70 protein, mitochondrial precursor (GRP75) (Peptide-binding protein 74) (Mortalin) isoform 17 (Canis familiaris)</td>
<td>90</td>
<td>5.73/5.76</td>
<td>73.4/72.8</td>
<td>0.59 ± 0.06</td>
<td>0.36 ± 0.06</td>
</tr>
<tr>
<td>1080</td>
<td>gi</td>
<td>47523096</td>
<td>CRY protein (S. scrofa)</td>
<td>196</td>
<td>6.09/6.34</td>
<td>35.5/33.6</td>
<td>0.13 ± 0.01</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>1024</td>
<td>gi</td>
<td>7395643</td>
<td>Snail homolog 3 (Mus musculus)</td>
<td>72</td>
<td>9.03/8.00</td>
<td>32.6/40.0</td>
<td>0.34 ± 0.07</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>1129</td>
<td>gi</td>
<td>7245833</td>
<td>Chain B, structure of the Rho family GTP-binding protein Cdc42 in complex with the multifunctional regulator RhoGDI (Bos Taurus)</td>
<td>65</td>
<td>5.12/5.33</td>
<td>24.6/25.7</td>
<td>0.26 ± 0.01</td>
<td>0.14 ± 0.02</td>
</tr>
<tr>
<td>1243</td>
<td>gi</td>
<td>2833360</td>
<td>Galectin-2 (S. scrofa)</td>
<td>138</td>
<td>6.90/6.83</td>
<td>13.8/ &lt; 15.0</td>
<td>1.01 ± 0.12</td>
<td>0.43 ± 0.12</td>
</tr>
<tr>
<td>875</td>
<td>gi</td>
<td>339647</td>
<td>Thyroid hormone binding protein precursor (H. sapiens)</td>
<td>75</td>
<td>4.82/4.77</td>
<td>57.5/58.7</td>
<td>0.62 ± 0.10</td>
<td>0.01 ± 0.01</td>
</tr>
<tr>
<td>898</td>
<td>gi</td>
<td>230115</td>
<td>Chain, LAP (E.C.3.4.11.1) (B. Taurus)</td>
<td>281</td>
<td>5.58/6.55</td>
<td>53.3/57.0</td>
<td>0.90 ± 0.14</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td>903</td>
<td>gi</td>
<td>113205888</td>
<td>Mitochondrial aldehyde dehydrogenase 2 (S. scrofa)</td>
<td>122</td>
<td>6.34/6.43</td>
<td>57.3/56.5</td>
<td>0.35 ± 0.05</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>938</td>
<td>gi</td>
<td>4503571</td>
<td>Eno1ase 1 (H. sapiens)</td>
<td>75</td>
<td>7.01/6.96</td>
<td>47.5/50</td>
<td>0.36 ± 0.03</td>
<td>0.18 ± 0.03</td>
</tr>
<tr>
<td>942</td>
<td>gi</td>
<td>4503571</td>
<td>Eno1ase 1 (H. sapiens)</td>
<td>123</td>
<td>7.01/6.96</td>
<td>47.5/50</td>
<td>1.39 ± 0.24</td>
<td>0.31 ± 0.08</td>
</tr>
<tr>
<td>958</td>
<td>gi</td>
<td>57107371</td>
<td>Predicted: similar to ornithine aminotransferase, mitochondrial precursor (OAT) (C. familiaris)</td>
<td>83</td>
<td>5.97/6.66</td>
<td>41.6/46.3</td>
<td>0.12 ± 0.02</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>988</td>
<td>gi</td>
<td>40889734</td>
<td>Chain A, medium-chain Acyl-CoA dehydrogenase with 3-thioacetyl-Coa (S. scrofa)</td>
<td>71</td>
<td>6.46/7.21</td>
<td>43.8/43.8</td>
<td>0.25 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>1021</td>
<td>gi</td>
<td>118625</td>
<td>Sorbitol dehydrogenase (S. scrofa)</td>
<td>74</td>
<td>7.26/7.49</td>
<td>38.4/40.0</td>
<td>0.17 ± 0.01</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>1045</td>
<td>gi</td>
<td>47522702</td>
<td>Alddehyde reductase (S. scrofa)</td>
<td>162</td>
<td>6.51/7.61</td>
<td>36.8/36.1</td>
<td>0.18 ± 0.02</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>894</td>
<td>gi</td>
<td>7106439</td>
<td>Tubulin, β 5 (M. musculus)</td>
<td>160</td>
<td>4.78/4.94</td>
<td>50.1/55.4</td>
<td>0.62 ± 0.11</td>
<td>0.31 ± 0.06</td>
</tr>
</tbody>
</table>

1. As indicated in Figure 2.
2. Sequence identification number generated by GenBank.
3. MOWSE, Molecular weight score generated by Mascot.
4. Isoelectric point (pI) and relative molecular mass (Mr) of proteins.
5. Expression level in % Vol. Data are means ± SEM, n = 8 (Ctrl) or 6 (FF).
6. P is the probability that mean of FF differs from that of Ctrl with P < 0.05 as significant.
primarily roles in the protection of hypoxia, oxidative stress, protein degradation and cell death, proliferation and maturation, signal transduction, energy generation, and metabolism were identified in the NEC piglets. These proteins may have functional and pathological importance in NEC.

The few proteins known to have primarily roles in the protection of hypoxia and oxidative stress are the heat shock proteins (GRP75 and CRY), antioxidant proteins (SOD-2 and DJ1), and the oxygen related protein (chain B, structure determination of aquomet porcine hemoglobin). In the immature infant, NEC is preceded by hypoxia and is characterized by high production of accelerated reactive oxygen species (ROS) (9). Enteral formula feeding may further increase splanchnic blood flow and restricted oxygen delivery to a vulnerable gut (10). GRP75 is a heat shock protein and plays central roles in the regulation of gut flora and protection of the gut from dietary stress (11). GRP75 undertakes several important functions, including stress response, mitochondrial biogenesis, control of cell growth and differentiation, modulation of cytoskeleton, and inhibition of apoptosis (12,13). CRY, a family of the human crystallin λ (CRYL1) (14), represents protective safeguards against low oxygen-induced injuries in the intestine of NEC patients (15). Both GRP75 and CRY exhibit chaperone activity (15), are involved in the protein-folding process, and were recruited for stress protection. Low levels of GRP75 and CRY may affect protein-folding processes within the mucosa, leading to architectural derangement of mucosal damage associated with NEC. With main functions to dismutate superoxide radical into hydrogen peroxide (16,17), the increased level of SOD-2 in NEC plays critical roles in the host-compensatory response to the overproduction of ROS and protection of the host tissue against their damage. In support of our finding, SOD has been shown to increase in a mouse model of colitis (18) and a rabbit model with NEC (19). The DJ1 in PARK7 protein, once thought to be associated with Parkinson disease (20), is either a redox sensor protein or as a direct scavenger of ROS, eliminating hydrogen peroxide through auto-oxidation (21). The reduction of DJ1 in NEC is unclear, but it is evident that the loss of DJ1 may lead to deficits in NADPH quinone-oxidoreductase 1 resulting in loss of the nuclear factor erythroid 2-related factor, which regulates the expression of many antioxidant pathway genes (22). The chain B structure determination of aquomet porcine hemoglobin is a relatively recently discovered protein with less knowledge of its functions. The increase (>12-fold) of this protein may simply result from massive tissue-localized hemorrhage, because hemorrhage is an indicator of NEC (Fig. 1).

An enteral formula diet inhibited some proteins that play critical roles in cell proliferation and cell death, as well as their maturation, growth, and migration. Lowering of the Snail homolog 3 may not protect piglets from necrotic/apoptotic cell death induced by FF. The Snail homolog 3 is a member of snail family of zinc-finger transcription factors (23,24) and contributes to the formation of various tissues during embryonic development (25). It undertakes cell death protection and regulates cell adhesion and migration (24). Galectin 2 is a member of the galectin family with affinities for β galactosides of glycoconjugates (26). It has a central role in cell-cell interactions and formation of clusters (26). The low level of this protein implies that cellular proliferation and maturation of the villous epithelial cells could be affected in NEC tissues, because galectin 2 is mainly expressed in the proliferating zone of crypts (27). The lowering of p55 protein may affect both maturation and growth of the intestine. This newly found protein has multiple functions, such as the capability of binding to thyroid hormone (28) and regulation of folding, assembly, and shedding of many cellular proteins to determine their viability (29). The expression of p55 is generally regulated by thyroid hormones, which are required for growth and maturity of many organs, such as lung, heart, and the central neural system (30). To our knowledge, this is the first report noting the expression of p55 protein in the premature gut. The chain B structure of the Rho family GTP-binding protein Cdc42, in complex with the multifunctional regulator

FIGURE 3 Western blot analysis of GRP75 (A), DJ1 (B), SOD-2 (C), and β-tubulin (D) proteins. Protein levels are means ± SEM, n = 4 (Ctrl) and n = 3 (FF). *Different from Ctrl, P < 0.05.
RhOGDI GTPase and Rho GDI (GDP dissociation inhibitors), regulates the GDP-GTP exchange reaction (31). Cdc42 undertakes several important functions such as actin cytoskeleton rearrangement, gene expression, membrane trafficking (32,33), and epithelial cell migration, which are all essential to the maturation and development of the gut (34). Reduction (46%) of the Cdc42 signaling pathway affects intestinal maturation, possibly inhibiting epithelial cells migration and/or favoring the local inflammatory process.

In the presence of dietary insults and prematurely of the gut, the reduced levels of the following intestinal enzymes with functions in energy generation, metabolism, and detoxification implies imbalance of energy in demand and supply in the NEC-bearing piglets. The 5-fold reduction of enolase 1 may affect energy production via conversion of glucose to pyruvate in the enterocytes in NEC piglets. Because LAP is required for the catalytic breakage of leucine residue from the N terminal of protein (35), the low level of this enzyme implies that amino acid metabolism was affected in NEC piglets. LAP is an exopeptidase and catalyzes the hydrolysis of leucine residues from the N-terminal of proteins, it also serves as a transcription factor involved in protein maturation and protein stability, and interacts with key membrane transporters (35,36). Mitochondrial aldehyde dehydrogenase reduction may lead to alcohol intolerance, because this enzyme catalyzes the oxidation of aldehyde, a detoxification reaction that removes the electrophilic products of alcohol oxidation. OAT is responsible for the synthesis of citrulline and arginine from ornithine. It is a key enzyme concentrated in the crypt cells of the small intestine (37). The ability of neonatal enterocytes to synthesize arginine immediately after birth suggests that the enzymes involved are present parentally (38). Induction of OAT is crucial, as arginine is responsible for ammonia detoxification and the synthesis of molecules, including nitric oxide and polyamines. However, excessive production of nitric oxide, a key inflammatory mediator, may also increase the inflammatory process in NEC.

In conclusion, enteral formula feeding affects protein profiles of the premature small intestine, primarily in a tissue-specific manner. These diverse proteins are known to regulate oxidative defense, signal transduction, protein synthesis and degradation, proliferation and cell death, maturation, and migration, as well as energy metabolism. Thus, the altered expression of the proteome is expected with contribution to inducing oxidative stress, hypoxia, proteolysis, cell death, and delayed protein synthesis and maturation of the gut and therefore compromises health in NEC neonates. This may be the major mechanism responsible for NEC diseases. Further studies of neonatal gut nutrient metabolism involving some of these proteins can advance our understanding of the mechanism responsible for the survival and growth of preterm infants. This new knowledge is important for designing the next generation of enteral and parenteral nutrient cocktails to optimize nutrition and health in this compromised population.

Acknowledgment
The authors thank Ms. Jill Eaton-Evans for her help with the preparation of this manuscript.

Literature Cited