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Basic nutritional investigation

Chronic low intake of protein or vitamins increases the intestinal epithelial cell apoptosis in Wistar/NIN rats

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Abstract

Objective: Malnutrition decreases antioxidant defense and increases oxidative stress in the intestine. We studied the effects of long-term restriction of food, protein, and vitamins on intestinal epithelial cell (IEC) apoptosis and the underlying mechanisms.

Methods: Weanling, Wistar/NIN male rats were fed ad libitum with a control diet, 75% protein-restricted diet, or 50% vitamin-restricted diet for 20 wk. The food-restricted group received 50% of the diet consumed by control rats. IEC apoptosis was monitored by morphometry, Annexin V binding, M30 CytoDeath assay, and DNA fragmentation. Structural and functional integrity of the villus were assessed by the ratio of villus height to crypt depth, and alkaline phosphatase and lys, ala-dipeptidyl aminopeptidase activities, respectively. Oxidative stress parameters, caspase-3 activity, and expression of Bcl-2 and Bax were determined to assess the probable mechanisms of altered apoptosis.

Results: Protein and vitamin restrictions but not food restriction significantly increased IEC apoptosis and only vitamin restriction altered structural and functional integrity of villi. Increased levels of protein carbonyls, thiobarbituric acid reactive substances, and caspase-3 activity along with decreased glutathione levels and Bcl-2 expression were observed in IECs of these rats, whereas food restriction did not affect these parameters.

Conclusions: Protein restriction increased only IEC apoptosis, whereas vitamin restriction also affected the structure and function of villi. Modulation of the pathway mediated by mitochondria through increased oxidative stress appears to be the probable mechanism underlying this effect.

Keywords: Vitamin restriction; Protein restriction; Food restriction; Intestinal apoptosis; Oxidative stress

Introduction

There is abundant clinical evidence that malnutrition associated with low levels of endogenous antioxidant defense and increased oxidant burden contributes to the dysfunction of various tissues, including the intestine [1–3]. The intestine is unique among organs because its constituent cells (enterocytes) turn over within 48 to 72 h [4]. Further, changes in nutritional status including fasting and overfeeding may alter this rate of turnover and affect the intestinal mucosal mass [5]. In addition to luminal nutrients, intestinal mucosa is constantly challenged by the diet-derived oxidants and endogenously generated reactive oxygen species (ROS) [6].

Responses of intestinal epithelial cells (IECs) to these insults depend on the severity of oxidative stress and attendant changes in redox balance [7]. To preserve cellular integrity and tissue homeostasis, the intestine possesses several defense mechanisms: 1) an ability to maintain high antioxidant concentrations, 2) upregulation of antioxidant

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enzyme systems, and 3) induction of cell death by apoptosis to dispose of injured or spent enterocytes [7].

Most forms of apoptosis use ROS as essential intermediate messengers [8–10], but the underlying mechanisms are not clear. Oxidative stress results in the production of several ROS, including O_2^-, H_2O_2, and OH, that induce different intracellular changes: oxidative modification of DNA, increased calcium levels, energy depletion, and oxidation of glutathione, reduced nicotinamide adenine dinucleotide phosphate, proteins, and lipids [11]. In addition, oxidative stress modulates proteins that mediate apoptosis, such as caspases [12], nuclear factor-κB, and Activator Protein 1 (AP1) [13]. Regardless of the mechanisms involved, the fact that antioxidants inhibit apoptosis that is induced by different stimuli indicates a central role for ROS in this process [14,15].

Intestinal cell renewal is decreased in children with malnutrition [16], and consequent changes have been reported in the mucosal mass, integrity and content of DNA and protein, and the activity of brush border enzymes [17–19]. Restricting energy or total diet intake to 40% of ad libitum levels decreases epithelial cell proliferation throughout the intestine, whereas protein restriction increases proliferation in the intestinal epithelium [20]. However, the mechanisms underlying the different responses of intestinal mucosa to food and protein restrictions have not been clarified. Further, data are not available on IEC responses to multiple vitamin deficiencies that are common among developing countries such as India [21]. The present study assessed the effect of long-term restrictions of diet, protein, and vitamins on IEC apoptosis and investigated the probable mechanism underlying the effect.

Materials and methods

Chemicals and reagents

Biotinylated Annexin V plus biotin, M30 CytoDeath, and streptavidin peroxidase were procured from Roche Diagnostics (Mannheim, Germany). Primary antibodies for Bcl-2 and Bax were from Oncogene Research Products (San Diego, CA, USA), and the substrate for caspase-3 (N-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide) was obtained from Calbiochem (San Diego, CA, USA). Biotinylated secondary antibodies, RNase, proteinase K, Nonidet NP-40, agarose, lys-ala-7-amido-4-methyl coumarin, and vitamins used in diets were from Sigma Chemical Company (St. Louis, MO, USA), and other entire analytical grade chemicals were procured from local sources.

Animals and feeding protocol

Animal experimental protocols of the study were approved by the institutional animal ethics committee of the National Institute of Nutrition (Hyderabad, India).

Twenty-four weanling, male Wistar NIN rats that weighed 32 ± 8 g (mean ± standard error of the mean) were obtained from the National Center for Laboratory Animal Sciences at the National Institute of Nutrition. They were housed individually in polypropylene cages with stainless steel wire-mesh floors to prevent coprophagy in a room maintained at 23°C and 60% humidity, with a 12-h light/dark cycle. All rats had free access to deionized, distilled water. Animals were randomized into four groups of six animals each and fed the following diets ad libitum for 20 wk [22]: the control (CON) group received a 28% casein-based, semisynthetic, powder diet (AIN-93); the protein-restriction (PR) group received the CON diet but with 75% restriction of protein (7% instead of 28% of casein and calories were made up with starch); the vitamin-restriction (VR) group received the CON diet but with 50% restriction of all vitamins (0.5% instead of 1% vitamin mixture in the diet); and the food-restriction (FR) group received 50% of the quantity of the CON diet consumed by CON rats. Food intake (daily), body weights (weekly), and hemoglobin and serum protein levels (monthly) were monitored throughout the feeding period. At the end of 20 wk of feeding, blood was collected from all rats after 17 h of fasting, through orbital sinus puncture, into vials containing heparin. Plasma was separated immediately and stored at −20°C for analysis of riboflavin, folic acid, and vitamins A and E. Rats were killed in a CO_2 chamber and between 10:00 AM and 12:00 NOON (in batches of three animals from each group per day) to minimize diurnal variations. A midline incision was made to open the abdominal cavity and a 20-cm segment of jejunum (beginning 12 cm distal to the ligament of Treitz) was removed, placed immediately on ice, and trimmed of excess fat and mesentery.

Processing of the jejunum

Luminal contents of the intestine were flushed out gently with cold phosphate buffered saline. The jejunum was divided randomly into three segments of 5, 5, and 10 cm each as reported previously [23]: one of the two 5-cm segments was filled with 4% buffered paraformaldehyde, placed in 10% neutral buffered formalin for 24 h for fixation, and used for light microscopic observations. The other 5 cm was used for isolation of epithelial cells and DNA extraction. The 10-cm segment of the jejunum was used to prepare the homogenate. For this purpose, jejunal mucosa was gently scraped with a glass slide, and the scrapings were frozen quickly in liquid nitrogen and stored at −80°C for enzyme and biochemical assays.

Histology

The formalin-fixed segment of the jejunum was cut open longitudinally and rolled on itself to make a Swiss roll, which was processed through a graded series of alcohol [24] in a tissue processor (CITADEL 2000, Shandon, Towa,

Taiwan) and then embedded in paraffin at 58°C (HISTOCENTRE2, Shandon). Four-micrometer–thick sections of the processed tissue were cut from the paraffin block in a microtome (GMBH 6380, Leica AG, Mannheim, Germany). Sections were mounted on glass slides coated with chrome alum and gelatin, stained with hematoxylin and eosin, and observed under a light microscope (Nikon, Kawagawa, Japan).

Detection and quantification of apoptosis

For quantification of apoptosis, three serial sections of intestine obtained from each block were processed for morphometry, Annexin V binding, and M30 staining.

Morphometry

Conventional light microscopy of specimens stained with hematoxylin and eosin was used to detect enterocyte apoptosis. This method of identifying apoptotic cells, currently considered the reference standard [25], is very precise if representative morphologic changes are observed [26]. An experienced pathologist who was unaware of groups to which the tissue sections belonged examined jejunal sections for apoptotic enterocytes. Presence of condensed chromatin, nuclear fragmentation, shrunken cytoplasm and formation of apoptotic bodies were considered the hallmarks of apoptotic cells. The number of apoptotic cells in 1000 normal cells was counted under light microscope at 200× magnification. Apoptotic index (percentage) was calculated as: (number of apoptotic cells/total number of epithelial cells counted) × 100.

Annexin V staining

Annexin V binds to phosphatidyl serine, which is externalized to the outer leaflet of the plasma membrane bilayer during initial stages of apoptosis [27]. Thin tissue sections of 4 μm were obtained as mentioned above and cooked at 120°C for 5 min in 0.01 M citrate buffer, and sections were immediately cooled by placing them in deionized water (25°C) for antigen retrieval. Endogenous peroxidase activity was blocked with 3% H_2O_2 in phosphate buffered saline. Subsequently, biotinylated Annexin V (50×) diluted in incubation buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM $CaCl_2$) was layered on tissue sections and incubated at room temperature for 1 h according to the manufacturer's instructions (Roche Diagnostics). To detect Annexin V binding, sections were incubated with streptavidin peroxidase for 30 min and visualized using diaminobenzidine and hydrogen peroxide. Tissue sections were then counterstained with hematoxylin and the brown-colored cells with dense nucleus or apoptotic bodies were identified as positive cells. Tissue sections processed in incubation buffer constituted negative controls.

M30 CytoDeath detection

Caspase-mediated cleavage of cytokeratin-18 is a very early event in apoptosis. A recently developed monoclonal antibody, M30, recognizes a neoepitope in cytokeratin-18 that is produced by caspase cleavage of cytokeratin-18 during apoptosis, which is not present in non-apoptotic cells. M30 CytoDeath has been used successfully for analyzing apoptosis in epithelial cells [28]. An advantage of this method over other methods is the lack of M30 expression in necrotic cells [29].

Formalin-fixed, paraffin-embedded intestinal tissue sections were analyzed for evidence of this cytokeratin-18–specific, apoptotic event according to the method of Leers et al. [30]. Cells with brown-colored cytoplasm and pyknotic (condensed) nuclei were considered to be true M30-positive cells. Tissue sections from at least three different animals in each group were evaluated for M30 immunoreactivity. In addition, 10 samples of gastric adenocarcinoma and three biopsy specimens of normal colorectal mucosa were used as positive and negative controls, respectively, for M30 CytoDeath assay [31].

DNA fragmentation by agarose gel electrophoresis

Epithelial cells were separated from the 5-cm segment of the jejunum, flushed with ice-cold phosphate buffered saline [32], and DNA was extracted from 2×10^6 epithelial cells by using the protocol described by Gong et al. [33]. Briefly, cells were centrifuged at 800*g* for 5 min, and the cell pellet was washed three times and suspended in 40 μL of 0.2 M phosphate citrate buffer (pH 7.8). It was centrifuged at 1000*g* for 5 min after incubation at room temperature for 30 min. Three microliters of Nonidet NP-40 (0.25%) was added to the supernatant followed by 3 μL of RNase (1 mg/mL) and incubated for 30 min at 37°C. Three microliters of proteinase K (1 mg/ml) was added next and incubation continued for another 30 min at 37°C. Twelve microliters of sample loading buffer was added to the supernatant and heated at 65°C for 10 min before loading on an agarose gel (1.5% in Tris EDTA buffer with ethidium bromide). Bands were visualized in an ultraviolet transilluminator (Bio-Rad, Hercules, CA, USA) and images were saved using Quantity One software (Bio-Rad).

Structural integrity of villi

Height of jejunal villi and depth of crypts were measured in formaldehyde-fixed tissue sections as previously described [34] under a light microscope at 100× magnification by using an ocular micrometer (Nikon, Kawagawa, Japan). Only those crypts in which the lumen was present from the base to the mouth of the crypt and only those villi in which the lamina propria core was present from the base to the tip of the villus were considered for this purpose. Heights of 10 villi and crypts were measured per tissue

section, and one slide from six animals of each group was used for this purpose.

Preparation of jejunal mucosal homogenate

A 10% homogenate was prepared in 1.15% KCl containing 0.5 mM Butylated Hydroxy Toluene (BHT) (to prevent ex vivo peroxidation) using the mucosal scrapings frozen previously. The homogenate was subjected to differential centrifugation (RC 5B high-speed centrifuge, DuPont Instruments, Wilmington, DE, USA) at 800*g* for 30 min, 12 000*g* for 30 min, and 100 000*g* (Sorvall Discovery 100SE ultracentrifuge, Sorvall Inc.) for 1 h at 4°C in that order, protein content was determined in the respective supernatants by the modified method of Lowry [35], and supernatants were stored frozen at −20°C until analysis.

Markers of membrane functional integrity

Activities of membrane-bound enzymes alkaline phosphatase and lys-ala-dipeptidyl aminopeptidase are maximum in the upper half and basal side of the villus, respectively [36]. Therefore, activities of these two enzymes were measured in the 12 000*g* supernatant to assess the functional integrity of the jejunal villi. Alkaline phosphatase was determined according to the method of Bodansky [37] using β-glycerophosphate as the substrate, and inorganic phosphate liberated was quantified according to the method of Chen et al. [38]. Lys, ala-dipeptidyl aminopeptidase activity was determined according to the method of Imai et al. [39] using lys-ala-7-amido-4-methyl coumarin as the substrate.

Markers of oxidative stress

First, lipid peroxidation was assessed by quantifying the amount of thiobarbituric acid reactive substances (TBARS) in the 12 000*g* supernatant according to the method of Balasubramanian et al. [40] using malondialdehyde as standard.

Second, protein oxidation was monitored by estimating the protein carbonyls in the 100 000*g* supernatant according to the method of Reznick and Packer [41]. Briefly, 1 mL of 100 000*g* supernatant was incubated with 10 mM dinitrophenylhydrazine for 1 h in the dark followed by protein precipitation with trichloroacetic acid. Dinitrophenylhydrazine and lipid free precipitate was dissolved in 6 M guanidine hydrochloride, and its absorbance was read at 375 nm. The amount of protein in the final pellet was quantified at 280 nm by using bovine serum albumin in guanidine hydrochloride as a standard.

Activity of antioxidant enzymes

Copper/zinc superoxide dismutase.

Activity of copper/zinc superoxide dismutase (Cu/Zn SOD) was determined in the supernatant centrifuged at 100 000*g* according to the method of Marklund and Marklund [42]. This method is based on the competition between pyrogallol oxidation by superoxide radicals and dismutation of superoxide by SOD that is monitored spectrophotometrically and read at 420 nm. One unit of SOD is the amount of enzyme that inhibits the reaction rate by 50% under the given assay conditions.

Catalase.

Activity of catalase was measured according to Aebi [43] in the 12 000 g supernatant (125 μg protein) by following the decomposition of H_2O_2 at 240 nm for 1 min.

Glutathione peroxidase.

Glutathione peroxidase (Gpx) activity was measured according to the method of Paglia and Valentine [44] in 100 000*g* supernatant. When oxidized GSH is reduced in the presence of glutathione reductase, reduced nicotinamide adenine dinucleotide phosphate is oxidized to nicotinamide adenine dinucleotide phosphate, and the optical absorbance of reduced nicotinamide adenine dinucleotide phosphate at 340 nm decreases. After measuring the change in absorbance per minutes at 340 nm, GSH peroxidase activity was calculated by using the molar extinction coefficient of reduced nicotinamide adenine dinucleotide phosphate and is expressed as units per milligram of protein.

Reduced GSH.

Levels of GSH were measured according to the method of Hissin and Hilf [45] in the 12 000*g* supernatant by assessing its reaction with orthophthalaldehyde. The intensity of fluorescence of GSH-orthophthalaldehyde adducts (at pH 8.0) was measured at an excitation of 350 nm and emission of 420 nm (LS-5B fluorescence spectrometer, Perkin-Elmer, Boston, MA, USA).

Caspase-3 Activity

Caspase-3 activity was determined in the 12 000*g* supernatant by using N-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide as the substrate according to the method of Henkels and Turchi [46]. The quantity of *p*-nitroaniline ($\epsilon_{410\ nm}$ 1 mM = 8.8) formed was determined at 410 nm (ultraviolet vis spectrophotometer, Tech Comp, Hitachi, Hong Kong) and expressed as micromoles of *p*-nitroaniline formed per milligram of protein.

Expression of Bcl-2 and Bax

Levels of Bcl-2 and Bax were determined in the 12 000*g* supernatant by immunoprecipitation and western blotting. Briefly, before immunoprecipitation, appropriately diluted supernatant (400 μg protein/mL) was precleared by incubation with preimmune antiserum and protein-G Sepharose. The pre-cleared supernatant was incubated for 1.5 h with monoclonal anti–Bcl-2 or anti-

Table 1
Effect restricted intakes of food, protein, and vitamins for 20 wk on food intake, body weight, hemoglobin, and serum protein levels in Wistar/NIN rats*

Group	Food intake (g/d)	Body weight (g)	Hemoglobin (g/dL)	Serum protein (g/dL)
CON	$10.5^{a} \pm 0.25$	$239^{a} \pm 6.0$	$13.5^{a} \pm 0.17$	$9.06^{a} \pm 0.078$
FR	$5.3^{b} \pm 0.25$	$124^{b} \pm 6.2$	$12.6^{b} \pm 0.15$	$8.33^{b} \pm 0.056$
PR	$10.4^{a} \pm 0.26$	$195^{c} \pm 6.6$	$12.2^{b} \pm 0.22$	$8.04^{c} \pm 0.089$
VR	$10.2^{a} \pm 0.25$	$224^{a} \pm 6.1$	$13.2^{a} \pm 0.20$	$8.91^{a} \pm 0.180$

CON, 28% casein-based diet fed ad libitum; FR, 50% of the quantity of the CON consumed by CON rats fed ad libitum; PR, 7% casein-based diet fed ad libitum; VR, 28% casein based diet but containing 50% of the vitamin mixture present in the CON diet fed ad libitum.

* Values are mean ± standard error of the mean for six rats. Values in a column with different superscripts are significantly different from one another at $P \leq 0.05$ by repeated measures analysis of variance followed by post hoc least significant difference test.

Bax antibodies plus protein-G Sepharose [47]. After washing six times with lysis buffer, the immunoprecipitated material was boiled with sodium dodecylsulfate sample buffer, size-fractionated on 12% sodium dodecylsulfate polyacrylamide gels, and transferred to polyvinyldiflouride membranes by electroblotting. Blots were blocked overnight with Tris buffered saline containing 5% skimmed milk powder, 1% casein, and 0.05% Tween-20. Membranes were then probed appropriately with anti–Bcl-2 or anti-Bax monoclonal antibodies (1 to 2 μg/mL), followed by horseradish peroxidase conjugated anti-rabbit immunoglobulin G. Bcl-2 and Bax protein bands were visualized using diaminobenzidine/H_2O_2 solution and quantified using Quantity One software (Bio-Rad).

Plasma vitamin status

Plasma vitamin A and E levels were analyzed according to the method of Miller et al. [48] in an Agilent 1100 high-performance liquid chromatographic system (Agilent Technologies Inc., Palo Alto, CA, USA) equipped with ultraviolet detector (292 nm for vitamin E and 326 nm for vitamin A) by using a mixture of methanol and water (95:5, v/v) as the mobile phase. Briefly, plasma proteins were precipitated using ethanol and vitamins were extracted from the supernatant into hexane. Hexane extracts were dried under nitrogen gas, reconstituted in 100 μL of ethanol, and analyzed by high-performance liquid chromatography on a reverse phase analytical Shodex C18 column (μ Bondapak, Millipore, Waters, MA, USA). Retention times were 4.2 and 7.6 min for vitamins A and E, respectively, in this system.

Plasma riboflavin was quantitated by high-performance liquid chromatography by the method of Botticher and Botticher [49]. Briefly, plasma samples were treated with clara diastase to liberate protein bound riboflavin and with trichloroacetic acid to precipitate proteins. Samples were centrifuged at 3200*g* for 10 min and supernatants were filtered through 0.2-μm syringe filters and then injected onto a reverse phase, analytical Shodex C18, stainless steel column. Riboflavin was eluted with methanol and water (50:50 v/v) and identified with a fluorescence detector (440/530 nm). The retention time of standard riboflavin was 4.2 min, and the sample peak that corresponded to this retention time was considered to be riboflavin.

Plasma folic acid was determined by a solid-phase, competitive radiobinding assay using a kit from Diagnostic Products Corporation (Los Angeles, CA, USA).

Statistical analysis

All results are expressed as mean ± standard error of the mean. Statistical analysis of data was done by one-way analysis of variance followed by post hoc tests of significance using SPSS 10.0 (SPSS, Inc., Chicago, IL, USA). Because heterogeneity of variance was not observed with any parameter, differences across groups were tested by the parametric least significant difference test. Differences were considered statistically significant at $P \leq 0.05$.

Results

General nutritional status

The effect of 20 wk of food, protein, and vitamin restrictions on food intake, body weight, hemoglobin, and serum protein levels is presented in Table 1. Neither protein nor vitamin restriction decreased the food intake compared with CON rats, but PR rats weighed 82% of CON rats. As expected, rats in the FR group had body weights decreased to 52% of CON rats. Hemoglobin and serum protein levels in FR and PR rats were lower than those in CON rats, whereas VR rats had hemoglobin and serum protein levels comparable to those of CON rats [22].

Compared with CON rats, plasma levels of riboflavin, folic acid, and vitamin A were significantly lower in FR rats, whereas vitamin E levels were unaltered (Table 2). As one would expect, vitamin restriction resulted in significant decreases in the levels of all four vitamins tested (Table 2). Although protein restriction significantly decreased the levels of riboflavin and vitamin A compared with CON rats, it did not alter plasma levels of folate and vitamin E. Plasma levels of these four vitamins were significantly higher in PR than in VR rats.

Table 2
Plasma concentrations of folic acid, riboflavin, and vitamins A and E in Wistar/NIN rats fed FR, PR, or VR diets for 20 wk from weaning*

Group	Folate (μg/dL)	Riboflavin (μg/dL)	Retinol (μg/dL)	Tocopherol (mg/dL)
CON	$4.85^{a} \pm 0.10$	$4.74^{a} \pm 0.06$	$25.84^{a} \pm 0.53$	$76.90^{a} \pm 1.672$
FR	$2.67^{b} \pm 0.08$	$3.10^{b} \pm 0.06$	$21.06^{b} \pm 0.31$	$74.50^{a} \pm 0.764$
PR	$4.60^{a} \pm 0.20$	$4.31^{c} \pm 0.09$	$24.30^{c} \pm 0.45$	$76.33^{a} \pm 0.615$
VR	$2.08^{b} \pm 0.50$	$2.42^{d} \pm 0.11$	$18.84^{d} \pm 0.34$	$52.33^{b} \pm 0.882$

CON, 28% casein-based diet fed ad libitum; FR, 50% of the quantity of the CON consumed by CON rats fed ad libitum; PR, 7% casein-based diet fed ad libitum; VR, 28% casein based diet but containing 50% of the vitamin mixture present in the CON diet fed ad libitum.

* Values are mean ± standard error of the mean for six rats. Values in a column with different superscripts are significantly different from one another at $P \leq 0.05$ by one way analysis of variance followed by post hoc least significant difference test.

Apoptosis in jejunal mucosa

Apoptosis was scored in the villus and crypt regions. Vitamin restriction induced significant apoptosis in the villi as evident from Annexin V (13.4%) and M30 CytoDeath binding (27%; Figure 1). PR rats also showed higher apoptosis than did CON rats as assessed by Annexin V (11.3%) and M30 CytoDeath (23.3%) binding, whereas food restriction had no effect. In line with these findings, increases were observed in the apoptotic index in the villus region of VR (214% of CON) and PR (182% of CON) rats compared with CON rats as computed from the scoring of sections stained with hematoxylin and eosin, and apoptosis in the crypt regions was comparable across groups (Figure 2).

Compared with CON rats, caspase-3 activity was significantly higher in the jejunal mucosa of VR and PR rats, but it was not altered in FR rats. However, the magnitude of increase in caspase-3 activity in PR was lower than that seen in VR rats (Table 3).

The DNA ladder pattern characteristic of internucleosomal DNA cleavage was seen only in PR and VR groups, whereas such a pattern was not detected in the CON and FR groups (not shown).

Structural and functional integrity of intestinal mucosa

Because the PR and VR groups showed significantly higher jejunal apoptotic rates, the effect, if any, of these dietary manipulations on the structural integrity of the jejunal mucosa was assessed by determining the ratio of villus height to crypt depth. Compared with CON rats, the ratio of villus height to crypt depth was significantly lower in VR rats but was comparable in FR and PR rats (Figure 2).

Next, we evaluated the effect of long-term food, protein, and vitamin restrictions on the functional integrity of the intestinal mucosa by monitoring alterations, if any, in the activities of the marker enzymes alkaline phosphatase and lys, ala-dipeptidyl aminopeptidase. Compared with CON rats, vitamin restriction significantly decreased activities of these marker enzymes, whereas food and protein restrictions had no effect (Table 3).

Oxidant status

Alterations in structural and functional integrities are associated with high levels of ROS, which appear to be the key factors leading to tissue injury [50]. Lipid peroxidation and protein oxidation as indicated by TBARS and protein carbonyls, respectively, were lower in FR rats than in other rats (Table 3). Significantly higher levels of TBARS and protein carbonyls were observed in the VR and PR groups compared with the CON group ($P \leq 0.05$). However, levels of TBARS and protein carbonyls in PR rats were significantly lower than those in VR rats (Table 3).

Enzymatic antioxidants

Endogenous protective mechanisms against oxidative stress–induced apoptosis were investigated next. Food restriction did not elicit any change in Cu/Zn SOD activity. Although protein restriction significantly decreased SOD activity compared with CON rats (Figure 3), vitamin restriction increased it. A marked increase in catalase activity was observed in VR rats, whereas PR rats showed no change compared with CON rats. Although catalase activity was significantly higher in FR rats, the value was much lower than that in VR rats (Figure 3). Significantly higher Gpx activity was observed in VR rats compared with other rats. Whereas it decreased marginally but significantly in PR rats, FR rats showed no effect (Figure 3).

GSH levels were decreased by 55% and 42% in VR and PR rats, respectively, compared with CON rats. Interestingly, GSH concentrations were the highest in FR rats (Figure 3, inset) compared with other groups.

Expression of apoptotic regulatory proteins

Changes in GSH levels affect apoptosis by regulating expression of the Bcl-2 family of proteins [51]. Therefore, we checked the expression of Bcl-2 and Bax in IECs. Bcl-2 expression in VR rats was the lowest among the groups. In PR rats Bcl-2 expression was decreased to 67% of CON rats, whereas FR rats showed no effect on Bcl-2 expression

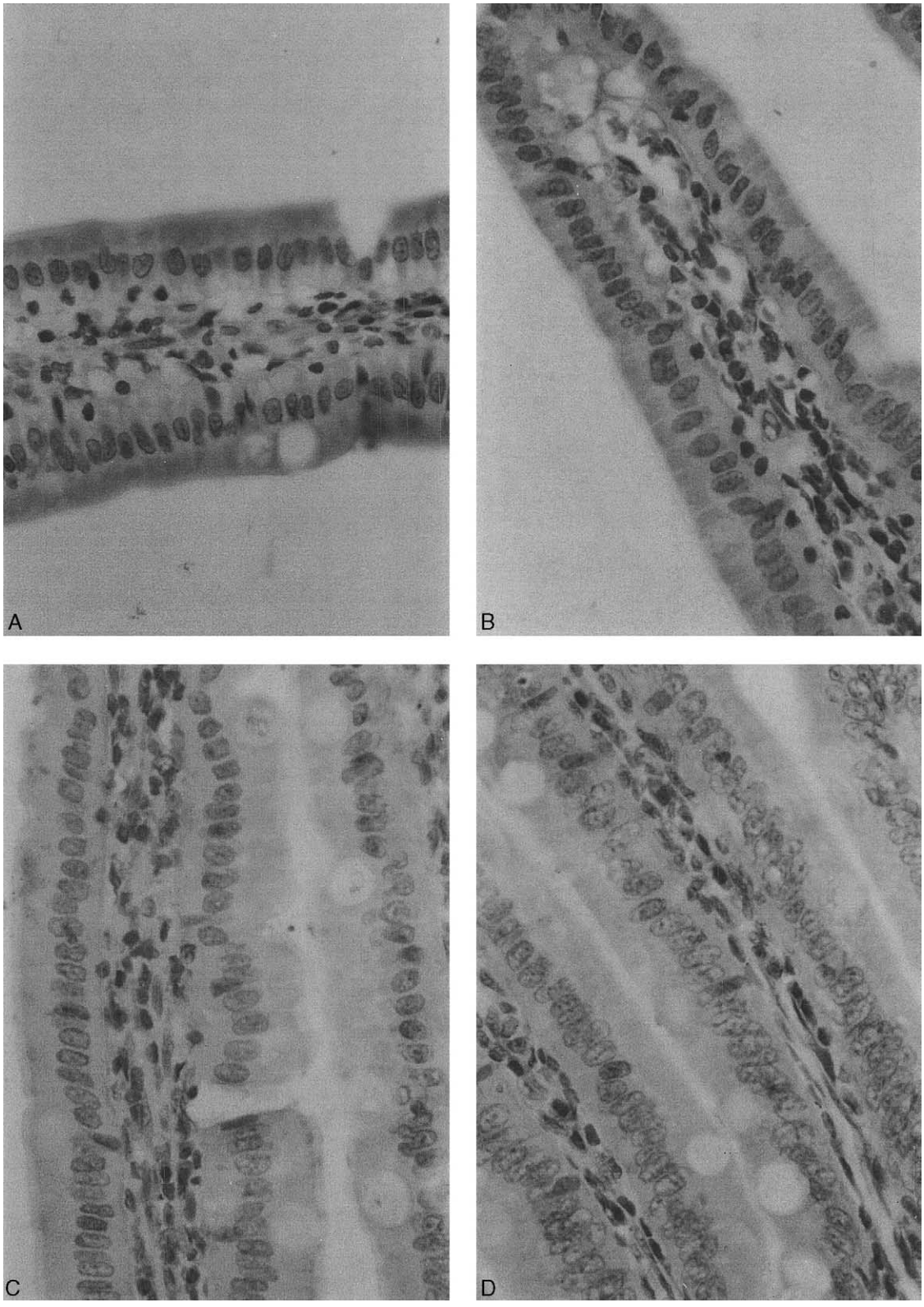

Fig. 1. Immunohistochemical identification of the binding of Annexin V (A, B) and M30 (C, D) monoclonal antibodies in the representative sections of jejuni of rats fed a control diet (A, C) or a vitamin-restricted diet (50% of control; B, D) for 20 wk from weaning. See MATERIALS AND METHODS for details of immunohistochemical processing of tissue sections.

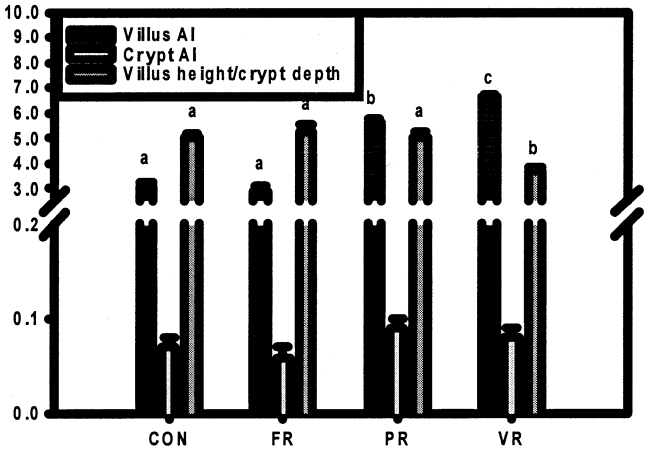

Fig. 2. Effect of restricted intake of food, protein, and vitamins for 20 wk on jejunal epithelial cell apoptotic rates and structural integrity of villi in Wistar/NIN rats. Apoptotic cells were counted in tissue sections stained with hematoxylin and eosin under a light microscope. At least 1000 epithelial cells were counted in one tissue section per animal ($n = 6$/group). Apoptotic cells were scored separately in the villus and crypt regions for each rat. Apoptotic index was determined as: *(number of apoptotic cells/ total number of epithelial cells counted)* × *100*. Villus height and crypt depth were measured in at least 10 villi and crypts in one tissue section per animal by using a calibrated ocular micrometer. Each bar represents the mean value for the group and the line above the bar represents standard error of the mean. Bars of a given parameter in different groups with unlike superscripts are significantly different from one another ($P \leq 0.05$) by one-way analysis of variance followed by post hoc least significant difference test. CON, 28% casein-based diet fed ad libitum (control); FR, 50% of the quantity consumed by CON rats; PR, 7% casein-based diet fed ad libitum; VR, 28% casein-based diet containing 50% of the vitamin mixture in the CON diet.

(Figure 4). Unlike Bcl-2, there was no alteration in Bax expression in any group compared with the CON group.

Discussion

This study investigated the effect of long-term low intake of food, protein, and vitamins on IEC apoptosis in a rat model. We found that frequency of apoptosis in the proliferating region of the jejunum, i.e., the crypt, was not altered with the long-term dietary manipulations tested, but that apoptosis was significantly increased in the villi of PR and VR rats. These observations likely indicated that differentiated cells are more susceptible to this kind of dietary manipulation-induced apoptosis and could be due to their direct contact with these nutrients in the lumen of the intestine. In addition, the VR diet appeared to adversely affect structural and functional integrity of the jejunum, whereas the PR diet had no such effect.

In the intestine, caloric or food restriction has been shown to prevent the growth of intestinal polyps by increasing cell death rates [52]. Forty percent caloric restriction has been reported to increase IEC apoptosis in old (24 to 25 mo) rats but not 4- to 5-mo-old rats [53]. In line with these reports, long-term food restriction for 20 wk in the present study showed no change in the apoptotic index and caspase-3 activity of IECs of rats, which were 6 mo of age at the time of monitoring apoptosis. Accordingly, there were no changes observed in the ratio of villus height to crypt depth in these rats, and activities of the brush border marker enzymes alkaline phosphatase and lys, ala-dipeptidyl aminopeptidase were not altered. Interestingly, there was a significant increase in GSH levels with a concomitant decrease in levels of TBARS and protein carbonyls in FR rats. Food restriction increased the activity of catalase and FR rats showed better antioxidant status and these results are in agreement with those reported previously [54]. Alternatively, decreased oxidative stress in the FR rats could be due to decreased free radical generation because caloric restriction attenuates oxidative stress by decreasing free radical generation [55]. Further, in line with these observations, food restriction did not alter the expression pattern of the pro- and anti-apoptotic regulatory proteins Bcl-2 and Bax in the IECs.

Plasma concentration of lipid-soluble antioxidant vitamin E was not significantly different in CON and FR rats, despite decreased food, including vitamin, intake. Unaltered levels of vitamin E in FR rats can be attributed to the increased absorption of vitamin E during long-term energy restriction and the results are in line with previous observations [56]. However, decreased plasma vitamin status did

Table 3
Indices of lipid and protein oxidation and activities of caspase-3 and marker enzymes for functional integrity of jejunal mucosa in Wistar/NIN rats on different diets for 20 wk*

Group	TBARS (nmol/mg protein)	Protein carbonyls (nmol/mg protein)	Alkaline phosphatase (μmol $P_i \cdot mg^{-1}$ protein $\cdot$ min^{-1})	Lys-ala-dipeptidyl-aminopeptidase (pmol $\cdot$ mg^{-1} protein $\cdot$ min^{-1})	Caspase-3 activity (μmol pNA/mg protein)
CON	$1.06^{a} \pm 0.221$	$1.22^{a} \pm 0.073$	$0.392^{a} \pm 0.0131$	$10.5^{a} \pm 0.88$	$44.2^{a} \pm 0.6$
FR	$0.99^{b} \pm 0.126$	$1.00^{b} \pm 0.079$	$0.373^{a} \pm 0.0092$	$10.7^{a} \pm 0.27$	$47.4^{b} \pm 0.4$
PR	$1.51^{c} \pm 0.138$	$1.60^{c} \pm 0.054$	$0.375^{a} \pm 0.0090$	$10.6^{a} \pm 0.44$	$70.5^{c} \pm 0.6$
VR	$1.98^{d} \pm 0.190$	$2.05^{d} \pm 0.068$	$0.352^{b} \pm 0.0151$	$9.57^{b} \pm 1.72$	$99.1^{d} \pm 0.3$

CON, 28% casein-based diet fed ad libitum; FR, 50% of the quantity consumed by CON rats fed ad libitum; PR, 7% casein-based diet fed ad libitum; TBARS, thiobarbituric acid reactive substances; VR, 28% casein based diet but containing 50% of the vitamin mixture present in the CON diet fed ad libitum.

* Values are mean ± standard error of the mean for six rats. Values in a column with different superscripts are significantly different from one another at $P \leq 0.05$ by one way analysis of variance followed by post hoc least significant difference test.

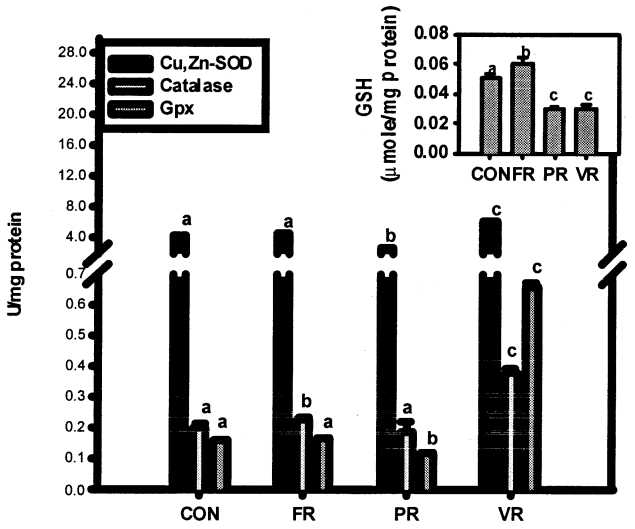

Fig. 3. Antioxidant status of rat intestinal mucosa on long-term restriction of food, protein, and multiple vitamins. Activities of Cu,Zn-SOD, catalase and Gpx were determined as described in MATERIALS AND METHODS section. Inset: As a measure of cellular antioxidant pool, levels of reduced GSH were analyzed in the 12 000 g supernatant ($n = 6$ animals/group). Each bar gives the mean value for the group and the line above the bar is the standard error of the mean. Bars of a given parameter in different groups with unlike superscripts are significantly different from one another ($P \leq 0.05$) by one-way analysis of variance followed by the post hoc least significant difference test. CON, 28% casein-based diet fed ad libitum (control); Cu,Zn-SOD, copper/zinc superoxide dismutase; FR, 50% of the quantity of the 28% casein-based diet consumed by CON rats; GSH, glutathione; Gpx, glutathione peroxidase; PR, 7% casein-based diet fed ad libitum; VR, 28% casein-based diet but containing 50% of the vitamin mixture present in CON diet fed ad libitum.

not result in altered IEC turnover, and this may be due to decreased metabolic rate and hence decreased free radical.

Previous studies have shown that significant adverse effect of a low-protein diet on intestinal function [57] is due to increased formation of TBARS. In keeping with these reports, in the present experiment, feeding a 7% casein diet for 20 wk significantly increased epithelial cell apoptosis and this was associated with increased TBARS and protein carbonyls. Darmon et al. [57] found that feeding a low-protein diet alters epithelial transport due to increased lipid peroxidation, although they did not measure IEC apoptotic rates. Significantly decreased activities of SOD and Gpx in PR rats likely indicated that these rats had a compromised enzymatic antioxidant defense. In addition, PR rats had decreased levels of GSH in the IECs, and this could have contributed to intestinal apoptosis. It is intriguing that the ratio of villus height to crypt depth was not altered in PR rats despite the increased apoptotic rates. Increased proliferation of the IECs has been reported with protein restriction [20]. In the present study, such an increase in IEC proliferation might have compensated for the loss of cells caused by increased apoptosis and may account for the unaltered ratio of villus height to crypt depth. Alternatively, the increase in apoptotic rate may not have been high enough to affect this parameter. That there was no change in the activities of alkaline phosphatase and lys, ala-dipeptidyl aminopeptidase also appears to be in line with this possibility. Nevertheless, decreased expression of Bcl-2 in PR rats may be responsible, at least in part, for the observed loss of IECs.

In the present study, feeding a 50% VR diet for 20 wk significantly increased IEC apoptotic rates and decreased the ratio of villus height to crypt depth. This decrease in the ratio of villus height to crypt depth may be due to the increased apoptotic rates without a compensatory increase in cell proliferation reported previously in PR rats [20]. Previous reports showed that, apart from increasing apoptotic rates, vitamin deficiencies decrease cell proliferation [58,59]. Previous studies on the postweaning development of rat duodenum have shown crypt population to be the target of the earliest effect of riboflavin deficiency, as reflected by a decrease in the number of villi per unit area [59]. In the present experiment, because the intake of all vitamins was restricted to 50% of CON rats, the resultant marginal deficiency of multiple vitamins could be responsible for the changes observed in the intestinal epithelium. These results appear to suggest that vitamins could be one of the key regulators of IEC turnover.

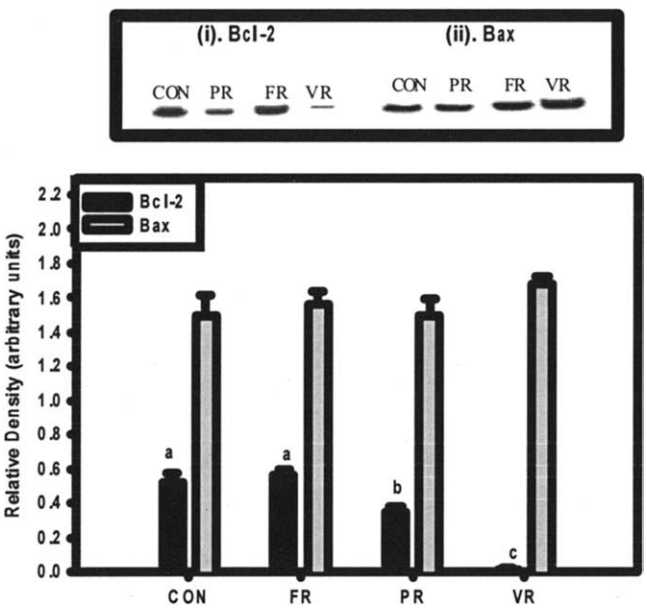

Fig. 4. (Top) Expression of apoptotic regulatory proteins in intestinal epithelial cells of rats fed different diets. Bcl-2 and Bax were immunoprecipitated from supernatant centrifuged at 12 000*g* with appropriate monoclonal antibodies (raised in mouse) and immunoprecipitates resolved on 12% sodium dodecylsulfate polyacrylamide gel electrophoresis were electrophoretically transferred to polyvinyldiflouride membranes and blots (raised in rabbits) were probed with the monoclonal antibodies as described in MATERIALS AND METHODS. (Bottom) Densitometric quantification of Bcl-2 and Bax expression. Each bar represents the mean value for the group and the line above the bar represents standard error of the mean ($n = 3$/group). Bars of a given parameter in different groups with unlike superscripts are significantly different from one another ($P \leq 0.05$) by one-way analysis of variance followed by post hoc least significant difference test. CON, 28% casein-based diet fed ad libitum (control); FR, 50% of the quantity consumed by CON rats; PR, 7% casein-based diet fed ad libitum; VR, 28% casein-based diet containing 50% of the vitamin mixture in the CON diet.

In VR animals, the observed increase in lipid and protein oxidation despite increased activities of antioxidant enzymes appears to suggest increased free radical generation that could not be mitigated by the increased activity of antioxidant enzymes.

Increased oxidative stress alters the functional integrity of intestinal mucosa [60]. A previous study in guinea pigs reported that, under normal circumstances, epithelial cells are removed safely without affecting the permeability of the small intestine [61]. A recent in vitro study in a colon cell line showed that spontaneous and induced apoptoses hollow out the intestinal barrier and may therefore facilitate loss of solutes [62]. Consistent with this report, in the present study, VR-induced apoptosis decreased structural and functional integrity of epithelial cells lining the intestinal lumen as evident from significant decreases in the ratio of villus height to crypt depth and activities of the marker enzymes of the brush border, alkaline phosphatase and lys, ala-dipeptidyl aminopeptidase. Interestingly, these parameters were altered in only the VR rats and not in rats subjected to other dietary manipulations. Similar observations have been reported in iron-induced functional damage of mucosal cells [60]. Despite increased oxidative stress, it was intriguing that the functional integrity of the IECs was not altered in PR rats, probably suggesting that the magnitude of oxidative stress in PR animals was much lower than that in the VR animals.

Increased oxidative stress has been associated with a decreased ratio of Bcl-2 to Bax [63], one of the key determinants of apoptosis in the intestine. Because increased lipid, protein oxidation, and apoptotic rates were observed in PR and VR animals, we tested whether this change was associated with altered expression of apoptotic regulatory proteins such as Bcl-2 and Bax. That the expression of antiapoptotic protein (Bcl-2) was decreased significantly in the VR rats and only marginally in the PR rats but was unaffected in the FR rats is in line with magnitude of changes observed in oxidative stress and apoptotic rates observed in the respective groups of rats. Interestingly, none of the dietary regimes had any effect on the expression of Bax, probably suggesting that the expression of Bax may not be sensitive to the dietary manipulations tested in the present study.

Conclusion

The present results indicated that long-term dietary manipulation can modulate apoptosis of IECs. Long-term restriction of protein or multiple vitamins, but not of calories, enhances intestinal cell death rates. Further, increased cell loss in protein restriction appears to be mitigated to some extent by a compensatory increase in cell proliferation, whereas such a mechanism in vitamin restriction appears inadequate or absent, resulting in altered structural and functional integrity of the villi. Further, the mechanism of the effect appears to be through increased cellular oxidative stress and the consequent compromised functional integrity of the membrane that probably damages mitochondria. This in turn appears to increase caspase-3 activity and result in increased apoptosis of IECs. That the changes were more severe in IECs of VR rats than in PR rats is in line with the magnitude of decrease in plasma concentrations of vitamins and emphasizes that vitamins are probably more important regulators of mucosal cell turnover compared with proteins. Although the correlation between ROS and increased IEC apoptosis is clear, in vivo studies such as these cannot rule out other mechanisms for enhanced apoptosis. Regardless of the mechanism(s), these studies have demonstrated unequivocally the significant effect of long-term low intake of vitamins on IEC apoptosis.

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