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A. A. Teitelbaum, M. G. Gareau, J. Jury, P. C. Yang and M. H. Perdue

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## Chronic psychological stress alters epithelial cell turn-over in rat ileum

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**Boudry G, Jury J, Yang PC, Perdue MH.** Chronic psychological stress alters epithelial cell turn-over in rat ileum. *Am J Physiol Gastrointest Liver Physiol* 292: G1228–G1232, 2007. First published February 1, 2007; doi:10.1152/ajpgi.00358.2006.—Dysregulated epithelial cell kinetics associated with mucosal barrier dysfunction may be involved in certain intestinal disorders. We previously showed that chronic psychological stress, in the form of repetitive sessions of water avoidance stress (WAS), has a major detrimental impact on ileal barrier function. We hypothesized that these changes were related to a disturbance in enterocyte kinetics. Rats were submitted to WAS (1 h/day) for 5 or 10 days. As previously shown, permeability to macromolecules was enhanced in rats stressed for 5 and 10 days compared with controls. WAS induced a decrease in crypt depth at day 5 associated with an increased number of apoptotic cells. Cell proliferation was significantly increased at days 5 and 10. Villus height and the specific activity of sucrase were significantly reduced at day 10. We concluded that WAS induces a disturbance of epithelial cell kinetics, with the pattern depending on the duration of the stress period. These findings help to explain the mechanism underlying altered epithelial barrier function resulting from exposure to chronic psychological stress.

intestine; enterocyte; proliferation; apoptosis

THE ETIOLOGY OF INFLAMMATORY bowel disease (IBD) is complex, involving both genetic and environmental factors. Among environmental factors, psychological stress is recognized as being able to initiate pathophysiology or modulate disease activity (16). In an attempt to understand the relationship between psychological stress and IBD, several groups have demonstrated that psychological stress, either acute or chronic, disturbs intestinal mucosal barrier function in animal models (7, 18, 25, 28). We previously showed that chronic psychological stress [10 days of intermittent water avoidance stress (WAS)] induces an increase in permeability to macromolecules, depletion of mucus, and altered interactions of bacteria with epithelial cells in the terminal ileum of rats (28). The stress-induced reduction of mucosal defense was associated with early signs of inflammation (elevated myeloperoxidase activity and inflammation scores). By increasing bacterial antigen uptake into the mucosa with subsequent T cell activation, stress may initiate or reactivate inflammation, thereby increasing IBD symptoms.

At the cellular level, stress-induced enhanced permeability and bacterial adherence or penetration may arise from abnormal enterocyte turnover resulting in an altered phenotype of epithelial cells being present at the mucosal surface. The constant renewal and differentiation of epithelial cells in the intestine is a tightly regulated process that can lead to

severe dysfunction of the intestine if disturbed. Although spontaneous apoptosis does not seem to impact epithelial permeability under physiological conditions (17), a relation between apoptosis and increased permeability has been shown in cell monolayers and animal models (2, 3, 29). For example, in HT-29/B6 colonic cells, camptothecin, an apoptosis-inducing chemical, time and dose dependently increased both apoptosis and permeability measured in Ussing chambers (2). In rats, increased intestinal permeability (albumin flux and bidirectional clearance of albumin) was documented when the number of apoptotic epithelial cells was increased by doxorubicin; this increase in epithelial permeability was inhibited when the increased apoptosis was prevented by cycloheximide (29). Similarly, the increased colonic permeability induced by intracolonic infusion of PAR1 agonists in rats was abolished by pretreatment with a caspase-3 inhibitor that prevents apoptosis (3). Therefore, the stress-induced increase in intestinal permeability may be related to an increased number of apoptotic cells in the epithelium. A greater proportion of immature enterocytes may also affect permeability since crypt epithelium is twice as permeable as villus epithelium (12). Tight junctions, as shown in freeze-fracture studies, demonstrate different patterns in crypt and villus, with fewer strands in the crypts associated with greater leakiness (15).

The aim of this study was therefore to investigate possible changes in epithelial cell kinetics (proliferation, maturation, and apoptosis) to better understand the mechanism underlying the enhanced intestinal permeability resulting from exposure to chronic psychological stress.

### MATERIALS AND METHODS

**Animals.** Weight (250–350 g)- and age (14–20 wk)-matched Brown Norway background rats obtained from our breeding colony at McMaster University were maintained under pathogen-free conditions on a 12:12-h dark-light cycle. They were provided with food and water ad libitum, except during the last 4 h before death when they had no access to food. These rats have been shown to be sensitive to stress and to exhibit reduced weight gain when exposed to chronic psychological stress (28). All procedures were approved by the Animal Care Committee at McMaster University.

**Stress protocol.** Rats were handled daily for 1 wk and then submitted to WAS for 1 h each day for 5 (WAS 5d) or 10 (WAS 10d) consecutive days. The procedures were performed between 8:00 and 10:00 AM to minimize the effect of circadian rhythm. Rats were weighed and then placed on a platform (8 × 6 cm) in a plastic container (56 × 50 cm) with water (25°C) to 1 cm below the platform, as previously described (28). Control rats were handled and weighed daily but remained in their home cages. Rats were killed by decapi-

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tation, and 15-cm-long segments from the distal ileum (starting 5 cm proximal to the ileocecal valve) were removed for Ussing chamber studies. Additional 1-cm segments were removed and immediately fixed in buffered paraformaldehyde and embedded in paraffin.

**Macromolecular permeability.** Segments of ileum were stripped of external muscle, opened along the mesenteric border, cut into flat sheets and mounted in Ussing chambers (WP Instruments, Narco Scientific, Mississauga, Ontario, Canada), exposing 0.6 cm<sup>2</sup> of serosal tissue area to 8 ml of circulating oxygenated Krebs buffer maintained at 37°C. The buffer consisted of (in mM) 115 NaCl, 1.25 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 2.0 KH<sub>2</sub>PO<sub>4</sub>, and 25 NaHCO<sub>3</sub> at pH 7.35 ± 0.02. Additionally, glucose (10 mM) was added to the serosal buffer as a source of energy, which was balanced by mannitol (10 mM) in the mucosal buffer. Horseradish peroxidase (HRP) (44 kDa) was used as a model protein probe to examine macromolecular permeability (25, 28). Type VI HRP (Sigma Chemical, St. Louis, MO) was added (10<sup>-5</sup> M) to the luminal buffer once equilibrium was reached, 15 min following mounting. Serosal samples (500 µl) were taken at 30-min intervals for 2 h and replaced with fresh buffer to maintain constant volume. Enzymatic activity of HRP was measured by a modified Worthington method as previously described (25) using a kinetic assay consisting of 20-µl samples aliquoted in duplicate in 96-well plates. Phosphate buffer (containing 0.003% hydrogen peroxide and 80 mg/ml o-dianisidine) was added (200 µl), and the enzymatic activity was determined on the basis of the rate of increase in optical density at 460 nm for 15 min. The flux of HRP from the mucosa to serosa was calculated as the average value of two consecutive stable flux periods (60–90 and 90–120 min). HRP flux was expressed as picomoles per centimeter squared per hour.

**Villus and crypt length.** Sections (4 µm thick) were prepared and stained with hematoxylin and eosin. Tissues from all rats were examined under a light microscope. For each rat, a minimum of 15–20 well-oriented crypt-villus units was measured in coded sections by using a calibrated eye piece. All morphological measurements were conducted by one investigator who was unaware of the rat group from which the section was prepared.

**Enterocyte apoptosis.** Sections were processed to identify apoptotic epithelial cells using a modified terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling technique (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, nuclei were permeabilized by incubation with proteinase K (Sigma) for 20 min at room temperature followed by a 2-min incubation with 0.1% Triton X-100 in 0.1% sodium citrate on ice. Sections were then incubated with fluorescein-labeled nucleotides and deoxynucleotidyl transferase for 1 h at room temperature. The incorporated fluorescein was detected by incubating the sections with a solution of HRP-conjugated anti-fluorescein antibody from sheep for 30 min at room temperature. Staining was accomplished by incubation with the peroxidase substrate (0.02% 3-amino-9-ethylcarbazole and 0.02% hydrogen peroxide, made in acetate buffer, pH 5.5). Control sections were prepared by omitting the deoxynucleotidyl transferase. Only well-oriented crypt-villus units were analyzed. For each, the total number of labeled epithelial cells was recorded. For each rat, a minimum of 20 well-oriented crypt-villus units was examined.

**Enterocyte proliferation.** Three hours after the last WAS session, some rats were injected with 200 mg/kg ip of bromo-2'-deoxyuridine (BrdU, Sigma Chemical) to label dividing cells (19).

Sections (4 µm) were prepared and processed for immunoperoxidase staining of BrdU-labeled cells (21). Briefly, sections were first incubated 10 min with 4 N HCl, followed by 20 min with 0.1 mg/ml trypsin (Sigma). Sections were then exposed to the primary antibody (anti-BrdU, Sigma) at a dilution of 1:150 for 1 h at room temperature. They were then exposed to a 1:300 dilution of the secondary antibody (biotinylated mouse anti-mouse IgG, DakoCytomation, Carpinteria, CA) for 1 h and to a 1:300 dilution of HRP-conjugated streptavidin (Dakocytomation) for 30 min. Staining was accomplished by incubation with the peroxidase substrate (0.02% 3-amino-9-ethylcarbazole

and 0.02% hydrogen peroxide, made in acetate buffer, pH 5.5). Control sections were prepared by omitting the primary antibody or by using tissue sections from rats not injected with BrdU. All sections were counterstained with methyl green. Only well-oriented crypt-villus units were analyzed. For each, the total number of labeled epithelial cells was recorded. For each rat, a minimum of 20 well-oriented crypt-villus units was examined.

**Enterocyte maturation.** Enterocyte maturation was assessed by measuring sucrase activity following established techniques (4). Immature crypt cells contain little sucrase which appears as the cells mature and migrate onto the villi (9). Briefly, mucosa scrapings were homogenized in PBS buffer. Homogenates were incubated at 37°C for 60 min with 50 mM sucrose in 0.1 mM sodium citrate buffer pH 6.0 containing 0.1 mM *p*-chloro-mercuribenzoate to inhibit lysosomal β-galactosidase. Released glucose was determined photometrically in a plate reader using the glucose oxidase-peroxidase method (glucose kit, Sigma). Protein was determined by using the Bio-Rad protein assay reagent (Bio-Rad, Hemel, Hempstead, UK), and specific enzyme activity was calculated (nmol hydrolyzed substrate·h<sup>-1</sup>·mg protein<sup>-1</sup>).

**Statistical analysis.** Results are expressed as means ± SE. One-way analysis of variance was performed using the general linear model of SAS (SAS Institute, Cary, NC). A *P* value < 0.05 was considered significant.

## RESULTS

**Macromolecular permeability.** Permeability changes are shown in Fig. 1. HRP flux was significantly increased at both 5 and 10 days, but the defect was more severe at 10 days (increase compared with control: 3.1-fold at 10d vs. 2.4-fold at 5d). This result suggests that a longer period of stress exposure induces greater gut mucosal pathophysiology.

**Villus and crypt length.** Crypt length was reduced by 17% after 5 days of WAS but was back to control values after 10 days (Table 1). Villus height was not altered after 5 days of WAS but was reduced by 16% after 10 days (Table 1). These changes resulted in an increased villus to crypt ratio after 5 days of WAS compared with control values, but a decrease after 10 days (Table 1). These findings suggest that stress-induced changes occur first in crypt epithelium and then spread to the villus with increasing time.

**Enterocyte apoptosis.** WAS for 5 or 10 days had no influence on the total number of apoptotic cells in the villus

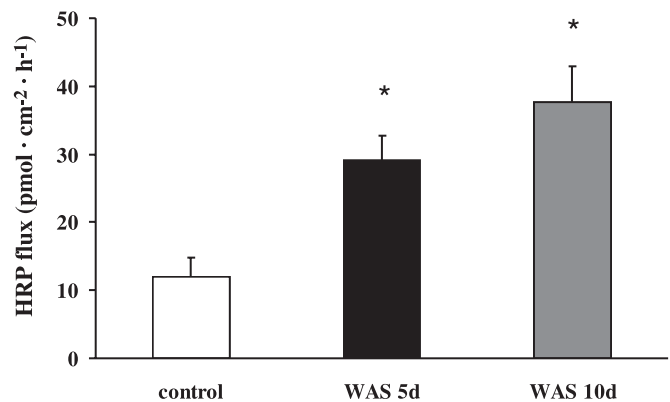


Fig. 1. Effect of psychological stress on permeability to macromolecules [horseradish peroxidase (HRP)] measured in Ussing chambers. \*Significantly different (*P* < 0.05) from control. Water avoidance stress (WAS) induced a 2.4-fold increase of permeability to HRP after 5 days (5d) and a 3.1-fold increase after 10 days (10d), compared with controls.

Table 1. Effect of WAS on villus height and crypt depth in rat ileum

	Control	WAS 5d	WAS 10d
Villus height, $\mu\text{m}$	244.0 $\pm$ 9.9	231.6 $\pm$ 9.0	205.7 $\pm$ 8.4*
Crypt depth, $\mu\text{m}$	101.9 $\pm$ 4.0	84.7 $\pm$ 3.6*	103.3 $\pm$ 3.4
Ratio villus/crypt	2.41 $\pm$ 0.12	2.76 $\pm$ 0.11*	1.99 $\pm$ 0.10*

Values are means  $\pm$  SE of structural measurements at 5 or 10 days of water avoidance stress (WAS 5d or WAS 10d). \*Significantly different ( $P < 0.05$ ) from corresponding control,  $n = 5$ –7 rats per group.

epithelium (Fig. 2A). However, the number of apoptotic cells in the crypt epithelium was increased after 5 days of WAS but had returned to control values after 10 days (Fig. 2B). These data suggest that the initial stress-induced mucosal abnormality is apoptosis of crypt enterocytes.

**Enterocyte proliferation.** The number of BrdU-labeled (newly divided) cells in the crypt was more than double in stressed rats, irrespective of the duration of the stress period, 5 or 10 days (Fig. 3). This result suggests that proliferation may increase to replace the dying and/or damaged cells.

**Enterocyte maturation.** Sucrase activity was not significantly different in stressed rats vs. controls after 5 days of WAS but was decreased to 50% after 10 days (Fig. 4). This finding indicates the presence of a reduced proportion of

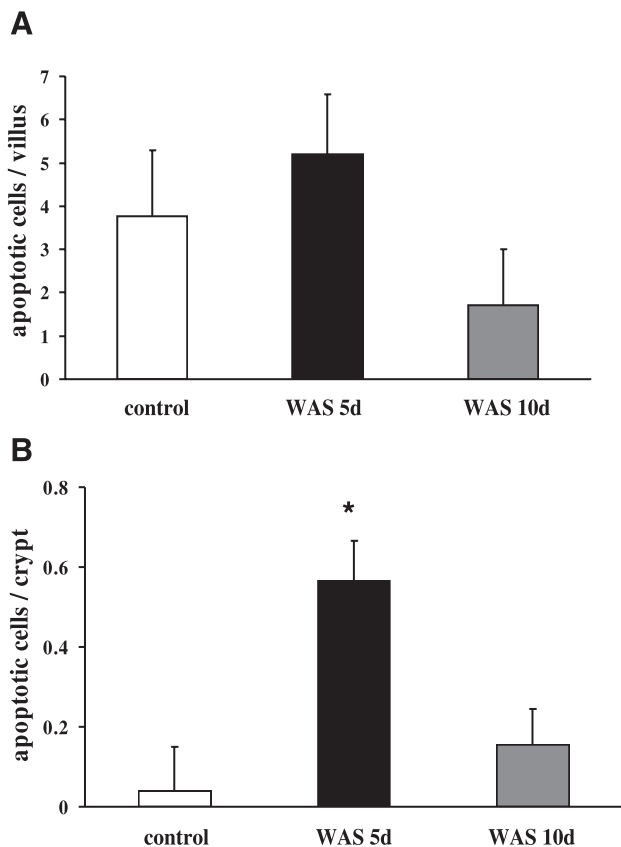


Fig. 2. Effect of psychological stress on apoptosis of epithelial cells in the villus (A) and crypt (B) of rat ileum. Apoptotic cells were localized using the TdT-mediated dUTP nick-end labeling technique. \*Significantly different ( $P < 0.05$ ) from control. WAS, for either 5 or 10 days, did not affect the number of apoptotic cells in the villus epithelium. However, after 5 days of WAS, the number of apoptotic cells in the crypt epithelium was increased.

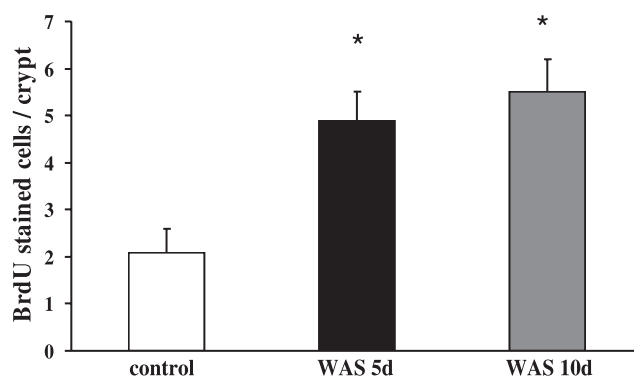


Fig. 3. Effect of psychological stress on epithelial cell proliferation in rat ileum. Proliferation in crypts was measured by the incorporation of bromo-2'-deoxyuridine (BrdU) injected 1 h before death and revealed by immunohistochemistry. \*Significantly different ( $P < 0.05$ ) from control. The number of proliferating cells in the crypt was increased after 5 and 10 days of WAS.

mature epithelial cells on the villi and fits with the reduced value for villus height.

**Summary of the changes.** After 5 days of exposure of rats to WAS, the number of apoptotic enterocytes in the crypts was increased, crypts were shorter, and proliferation was increased. There were no changes in villus cells. Macromolecular permeability was increased more than twofold. After 10 days of exposure of rats to WAS, proliferation was still increased and these immature cells had moved further up the villus; the villus length was shorter. At this stage, the permeability defect had become more severe, being more than threefold greater than control values.

## DISCUSSION

Our study demonstrated that exposure of rats to repetitive sessions of WAS, a model of chronic psychological stress, altered epithelial cell kinetics in the ileal mucosa. Sequential events occurred as the WAS sessions increased. After 5 days, the number of apoptotic cells in the crypts was increased, and the crypts became shorter. Associated with cell death was an increased rate of enterocyte proliferation, as determined by the greater number of cells taking up BrdU. Macromolecular permeability was enhanced, most likely by HRP passing

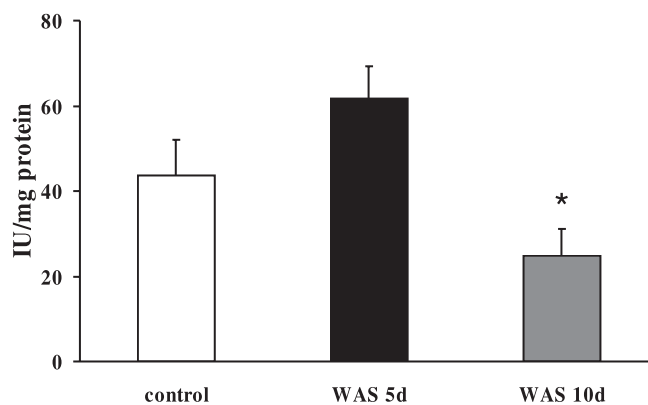


Fig. 4. Effect of psychological stress on the maturation of epithelial cells in rat ileum. The degree of maturation of epithelial cells was assessed by the measurement of sucrase activity (international units, IU) per milligram protein. \*Significantly different ( $P < 0.05$ ) from control. Sucrase activity was not altered after 5 days of WAS but was decreased after 10 days.

through areas of dying epithelium. After 10 days of WAS, the value for the number of apoptotic cells in the crypts was similar to that in control rats, and the crypt length was restored to normal values. However, the enterocyte proliferation rate was still increased and the villi had now become blunted. This suggested that the immature cells may have migrated to the villus surface. Indeed, reduced activity of sucrase confirmed the immature state of the enterocytes. This immature phenotype was associated with a barrier defect that was enhanced to a greater degree compared with the control value and also enhanced compared with the value at *day 5* of WAS. Immature epithelium is known to have greater permeability due to reduced numbers of tight junction strands joining the enterocytes (15).

The effect of stress on mucosal structure and epithelial cell dynamics in the upper gastrointestinal tract has been widely investigated with respect to gastroduodenal ulcers. Although few studies have reported the effects of stress on cell kinetics in the small intestine, where examined, the mucosal architecture has been shown to be altered. Environmental stress (high activity in the room) for several weeks resulted in the appearance of villus edema and detachment of epithelial cells from the basement membrane of the ileum (33), although apoptosis per se was not investigated. Similarly, acute WAS or short-term WAS induced degeneration of the ileal epithelium and immune cell infiltration (6), as we also previously described (28). When proliferation and/or apoptosis were specifically studied, the results were more varied. For example, isolation stress (a very mild stressor) for up to 4 days had no effect on proliferation, apoptosis, or villus length in the midjejunum of rats (32). On the other hand, a 12-h period of cold restraint stress reduced crypt cell proliferation in the duodenum by 56% and in the ileum by 28% (10). Similarly, stress in the form of repeated electric shocks inhibited crypt cell proliferation in rat jejunum (31). In contrast, the number of mitotic figures was significantly increased by 27% in the jejunum (but not in the duodenum) of rats forced to swim for several hours (23). In rats submitted to an acute stress (immersion in water for 90 min) or chronic stress (15 min of restraint stress for 15 consecutive days), apoptosis was enhanced (1). Although it is difficult to come to any definite conclusion from these results, they suggest that short-term acute stress induces epithelial cell damage and/or apoptosis, whereas recovery or possibly adaptation in a chronic stress situation leads to increased proliferation to restore homeostasis. A similar sequence of events was described in nematode-infected rats (22), where initial crypt changes were followed several days later by villus abnormalities; in both stages permeability was increased.

The hallmark of the stress response is the release of corticotropin-releasing hormone (CRH) in the hypothalamus, activating ACTH secretion, which in turn stimulates the secretion of glucocorticoid (corticosterone in rats) from the adrenal glands. In addition, stimulation of the sympathetic nerves results in an increase in circulating catecholamines. Both glucocorticoids and catecholamines have been shown to be involved in the regulation of intestinal epithelial cell turnover (1, 5, 8, 23, 31). However, glucocorticoids are known to enhance maturation during development (11). In addition, adrenalectomy in rats was associated with decreased proliferation (8, 31). We believe that CRH rather than corticosterone is the major effector of stress-induced mucosal abnormalities on the basis of several factors. In response to restraint stress or

WAS, treatment of rats with a CRH antagonist completely prevented the permeability defect, whereas an inhibitor of glucocorticoid synthesis had no effect (24). In addition, CRH injected intraperitoneally into rats reproduced the mucosal pathophysiology. Although corticosterone was elevated after 10 days of exposure to WAS, the concentration was much less than after a single exposure to WAS (28). In contrast, the intestinal abnormalities increased with time. Finally, current studies in progress examining the effect of CRH or analogs delivered to rats chronically in minipumps indicate that CRH acting on its receptors on mast cells in the gastrointestinal tract initiate the mucosal abnormalities. Taken together, previous studies (20, 27, 28) indicate that CRH acts via mast cells to uncouple mitochondrial oxidative phosphorylation in enterocytes. Reduced ATP production can impair physiology (barrier function) and lead to apoptosis (14). In vitro studies are in progress to examine this hypothesis.

The alteration of crypt and villus architecture induced by stress in our rat study was not comparable in term of magnitude and severity to the mucosal structural alterations observed in IBD patients. We observed a reduction in crypt depth at *day 5* and villus height at *day 10* of 17 and 16%, respectively. In IBD patients, severe villus atrophy (erosion or ulceration) is apparent with distorted crypt architecture, inflammatory cell infiltrate, crypt abscesses, and irregular surface appearance with goblet cell depletion (13, 30). Even during the onset of disease, major architectural changes are observed, with often a worsening appearance during the first year (26). It is therefore unlikely that psychological stress is the main event inducing the major mucosal structural damages observed in IBD. However, stress may contribute to and/or enhance the inflammatory process by modifying epithelial cell turnover and increasing permeability with uptake of microbial or other antigens.

In summary, this study showed that psychological stress in rats initially causes apoptosis of crypt epithelial cells leading to increased enterocyte proliferation. Both apoptosis in the crypts and an immature epithelium covering the villus surface can be responsible for a barrier defect. These results may have implications for understanding the pathophysiology of intestinal disorders with a stress-related component.

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