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Luminal bacterial flora determines physiological expression of intestinal epithelial cytoprotective heat shock proteins 25 and 72

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Arvans, Donna L., Stephan R. Vavricka, Hongyu Ren, Mark W. Musch, Lisa Kang, Flavio G. Rocha, Alvaro Lucioni, Jerrold R. Turner, John Alverdy, and Eugene B. Chang. Luminal bacterial flora determines physiological expression of intestinal epithelial cytoprotective heat shock proteins 25 and 72. *Am J Physiol Gastrointest Liver Physiol* 288: G696–G704, 2005. First published November 4, 2004; doi:10.1152/ajpgi.00206.2004.—Heat shock proteins (HSP) 25 and 72 are expressed normally by surface colonocytes but not by small intestinal enterocytes. We hypothesized that luminal commensal microflora maintain the observed colonocyte HSP expression. The ability of the small intestine to respond to bacteria and their products and modulate HSPs has not been determined. The effects of luminal bacterial flora in surgically created midjejunal self-filling (SFL) vs. self-emptying (SEL) small-bowel blind loops on epithelial HSP expression were studied. HSP25 and HSP72 expression were assessed by immunoblot and immunohistochemistry. SFL were chronically colonized, whereas SEL contained levels of bacteria normal for the proximal small intestine. SFL creation significantly increased HSP25 and HSP72 expression relative to corresponding sections from SEL. Metronidazole treatment, which primarily affects anaerobic bacteria as well as a diet lacking fermentable fiber, significantly decreased SFL HSP expression. Small bowel incubation with butyrate ex vivo induced a sustained and significant upregulation of HSP25 and altered HSP72 expression, confirming the role of short-chain fatty acids. To determine whether HSPs induction altered responses to an injury, effects of the oxidant, monochloramine, on epithelial resistance and short-circuit current (I_{sc}) responses to carbachol and glucose were compared. Increased SFL HSP expression was associated with protection against oxidant-induced decreases in transmural resistance and I_{sc} responses to glucose, but not secretory responses to carbachol. In conclusion, luminal microflora and their metabolic byproducts direct expression of HSPs in gut epithelial cells, an effect that contributes to preservation of epithelial cell viability under conditions of stress.

enteric flora; metronidazole; butyrate; short-chain fatty acids; cytoprotection; host defense; intestinal flora; blind loop; stress; mucosal injury

GASTROINTESTINAL EPITHELIAL cells maintain an essential barrier between the underlying tissues and the nonsterile, constantly changing luminal environment of the gastrointestinal tract. At the same time, intestinal epithelial cells play an important role in selective digestion and absorption of nutrients and electrolytes, and, under certain conditions, participate in host defense. Breach or compromise of any of these functions can have catastrophic consequences. When subjected to adverse conditions, however, intestinal epithelial cells have the intrinsic ability to increase their resistance to injury by the rapid induction of endogenous cytoprotective processes. Of these, the expression of cytoprotective HSPs represents a major mechanism for protecting intestinal epithelial cell functions and viability to many forms of stress (27, 31, 40, 54).

Several endogenous physiological or environmental stressors, such as heat, ethanol, heavy metals, caloric restriction, and bacterial infection can trigger gut epithelial cells to increase synthesis of heat shock proteins (HSPs) (7, 27, 31, 40, 54, 58). However, certain HSPs are expressed under nonstress conditions and play an important role in maintaining cell integrity (20, 21, 30, 43). One important function of HSPs is as a cellular molecular chaperone, thereby rescuing intracellular proteins from irreversible denaturation. These chaperone functions include folding polypeptides into mature tertiary structures, refolding and restoring integrity to partially damaged functional proteins and improving protein delivery within the cell (1, 26, 34).

There appears to be cell and organ specificity in the regulation of the stress response that may be essential in fulfilling certain physiological niches. Two HSPs that have been studied and shown to have an important role in the gastrointestinal tract are HSP25 and HSP72. They are selectively expressed in the intestinal mucosa in a region-specific manner (18). Specifically HSP25 and HSP72 are expressed in stomach (17, 19, 22, 48, 57) and colon (19, 24, 33, 40, 48) but not in small intestine (except distal ileum). This distribution specificity could be related to the colon and stomach's physiological microenvironment, being the areas in our bodies that are most consistently exposed to conditions of bacterial and chemical insult. The colonic lumen is densely colonized by a variety of commensal microbial species and areas with bacterial colonization have a predominantly high expression of HSPs (20).

It has been shown by previous studies that bacterial fermentation products, including short-chain fatty acids (SCFA) (40), as well as bacterial cell wall components, such as LPS (21), are potent physiological inducers of HSPs. Contrary to colonic tissue, small intestinal tissue is not exposed to such stimuli and does not show the same HSP distribution. These observations led us to further question whether HSPs expression is tissue specific or directly related to the presence of bacteria and/or their byproducts.

To answer this question, we designed experiments utilizing surgically created self-filling (SFL) or self-emptying (SEL) blind loops in rat small intestine, thereby creating a segment of small intestine with a colon-like luminal environment highly

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populated with bacteria. Our study shows that *1*) the presence of enteric flora can induce HSP25 and HSP72 in tissues that do not normally express these HSPs; *2*) the enteric microflora fermentation byproducts, SCFAs, can provide the necessary stimulus to induce cytoprotective HSPs; and *3*) this intestinal HSP response has significant effects on maintaining tight-junction integrity and cell viability in stress conditions. These findings indicate that HSPs are essential for preservation of epithelial cell viability and function under conditions of stress.

MATERIALS AND METHODS

Animals and organ culture. This project was approved by the Animal Care and Use Committee of the University of Chicago. Male Sprague-Dawley rats (250–300 g), obtained from Taconic (Germantown, NY), were fasted for 12–18 h and given water ad libitum. Rats underwent surgery to create SFL and SEL. Rats were anesthetized [intraperitoneal injection of ketamine (85 mg/kg), and xylazine (12 mg/kg)] and a 7–8 cm section of bowel, distal to the ligament of Treitz, was isolated. In animals with the SFL, the intestine was cut. The proximal end of the cut was sutured closed to prevent luminal spilling; the distal end was reanastomosed to a point 8 cm proximal to the cut, taking great care to maintain mesenteric blood supply. This procedure created an area of small intestine in which peristaltic motion would fill the loop (SFL) and prevent efficient emptying, allowing for a colon-like simulation of commensal bacterial colonization (Fig. 1). As a control, an SEL was created. In these rats, the distal end of the cut was sutured closed and the proximal end was reanastomosed 8 cm distally to the original cut. The peristaltic motion in these control rats keeps the loop free of bacterial colonization and controls for any effect of the surgery (Fig. 1). Rat loop tissue was allowed to adapt for 14–21 days before harvest. Some rats with SFL were treated with metronidazole subsequent to adaptation and before tissue harvest. The metronidazole treatment doses were 0.6 mg/ml po for only 5 days before tissue harvest. To modulate the production of colonic SCFA (derived from bacterial fermentation), rats were fed a specially designed Harlan diet containing no fiber (no fermentable fiber) or 6% (wt/wt) pectin. The diet was obtained from Harlan/Teklad 97201 and 97202 (composition in g/kg: 200 casein, 3 DL-methionine, 335.686 corn starch, 130 maltodextrin, 160 sucrose, 70 soybean oil, TD 94707 vitamin mix, 2.5 choline bitartrate, 0.014 tertbutylhydroquinone (antioxidant), 13.37 Ca-P-deficient TD 79055 mineral mix, 11.43 calcium phosphate dibasic, and 4.0 calcium carbonate with or without 60 g/kg pectin). There was no difference in daily chow intake between the two groups of rats (chow eaten per 3 days 24.8 ± 2.1 g pectin-free chow vs. 25.7 ± 2.9 g nonfermentable fiber chow, $n = 6$). Additionally, weight gain, after a typical initial postsurgery weight loss, in both sets of rat diets was similar (6.2 ± 0.4 vs. 6.4 ± 0.6 g in those rats given pectin-free or nonfermentable fiber chows over 10 days, respectively). To minimize coprophagia, animals were housed in wire-bottom cages. After 5 days, mucosa was harvested from the ileum and the colon and protein lysates were prepared as described previously (31, 40)

For organ cultures, rats were killed and small intestine was harvested. The muocsa was dissected by using glass slides to shear at a plane between the musuclaris propria and the submucosa. The sheared mucosa was into 2- to 3-mm pieces and placed in organ culture dishes (Falcon 3037) in a medium composed of the following: 140 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 0.5 μg/ml $FeSO_4$, 0.08 μg/ml $MnSO_4$·, 0.15 μg/ml $ZnSO_4$·, 0.25 μg/ml $CuSO_4$, the vitamin and essential and nonessential amino acid mixtures for MEM (Life Technologies, Grand Island, NY), insulin-transferrin-selenium mixture (Life Technologies), 20 mM fructose, 5 mM L-glutamine (Life Technologies), 0.8 μg/ml hydrocortisone, 300 μg/ml ascorbate, 15 μg/ml phenol red, 10% (vol/vol) fetal bovine serum (Life Technologies), 100 U/ml penicillin, and 100 μg/ml streptomycin, as described previously (61). Five to six pieces were placed in the middle of organ culture dishes and were perfused with oxygenated Ringer's solution from a reservoir also placed in the 37°C incubator. The medium was continually gassed with 95% O_2-5% CO_2. A peristaltic pump maintained the flow over the jejunal mucosa at ~10 ml/h. The SCFA butyrate was added to the bathing solution of one dish (to 60 mM), and an equal concentration of choline chloride was added to another dish to maintain equal osmolarities of the incubations. One piece of mucosa was removed from each dish at 2, 4, 6, and 8 h and was immediately processed for Western blot analysis of HSPs.

Immunoblotting. Intestinal mucosa, comprised mostly of epithelial cells, was harvested by blunt light scraping. The nature of this scraped

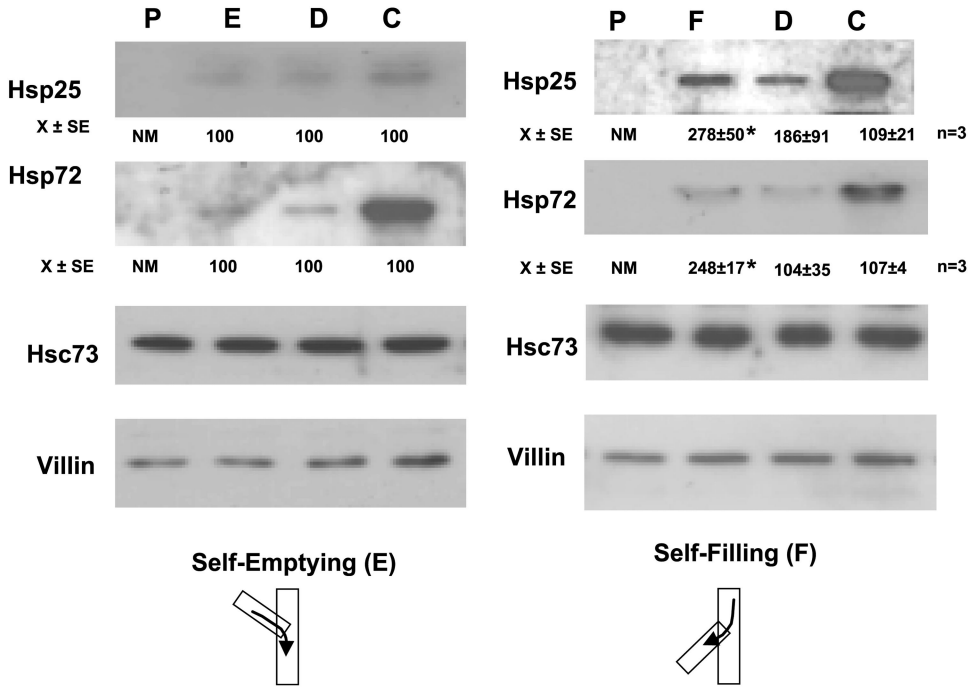

Fig. 1. Creation of a self-filling loop (SFL) induces heat shock protein (HSP)25 and HSP72 expression. HSP expression in jejunum proximal (P) and distal (D) to the anastomosis, as well as colon (C) in rats with SFL (F) and self-emptying (SEL; E) loops were analyzed by Western blotting as described in MATERIALS AND METHODS, using heat shock cognate (HSC73) as well as villin as denominators. Images shown are representative of 4 separate experiments. *$P <$ 0.05 compared with corresponding section of intestine from a rat with an SEL by paired Student's *t*-test. NM, not measured.

mucosa is predominantly epithelial and images of intact vs. scraped mucosa has been presented previously (29). The mucosa is not entirely epithelial but does possess abundant cytokeratin 8 and 18, epithelial cells markers, and little vimentin, a mesenchymal cell intermediate marker, or muscle cell myosin. This analysis supports our belief that the majority of protein in these samples is epithelial. It should also be noted that immunohistochemistry is used to confirm the cellular localization of the response. The scraped mucosa was homogenized and resuspended in lysis buffer (10 mM Tris pH 7.4, 5 mM $MgCl_2$, 50 U/ml each deoxyribonuclease and ribonuclease, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of leupeptin, aprotinin, and pepstatin) as previously described (2). Samples were placed on ice for 10 min, 10 μl of each sample was removed to measure protein concentrations, and one-half volume 3× Laemmli's stop solution was added to the remainder and heated at 70°C for 10 min. Protein concentrations were measured by a modified Lowry method using bicinchoninic acid (13). Twenty micrograms protein were resolved by 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride (Polyscreen; Perkin-Elmer, Boston, MA) membrane using 1× Towbin buffer (25 mM Tris pH 8.8; 192 mM glycine with 15% vol/vol methanol) (19). Nonspecific binding was blocked by using 5% (wt/vol) nonfat dry milk in Tris-buffered saline (TBS) (10 mM Tris pH 7.35, 150 mM NaCl, 5 mM KCl) with 0.05% (vol/vol) Tween 20 (T-TBS) for 1 h at room temperature. Blots were incubated with primary antibodies diluted in blocking buffer overnight at 4°C [anti-HSP25 (SPA-801), anti-HSP72 (SPA-810), anti-HSC73 (anti-heat shock cognate 73; SPA-815) from Stressgen, Victoria, BC, Canada, and anti-villin (V34420) from Transduction Laboratories/Pharmingen, San Diego, CA]. Blots were washed five times (10 min each) at room temperature in T-TBS, incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature in T-TBS, and washed again with T-TBS (4 times, 10 min each at room temperature and a final washing step in TBS for 10 min at room temperature). Blots were visualized by using the Supersignal West Pico enhanced chemiluminescence system (Pierce, Rockford, IL).

Immunohistochemical staining. Sections of colon or ileum from normal colon, normal small intestine as well as SFL and SEL rats were fixed in 10% (vol/vol) neutral buffered formalin for 24 h. Sections were embedded in paraffin and 4 μm cross sections were cut. Sections were stained for HSP25 and HSP72 using the Vectastain Elite ABC kit according to the manufacturer's instructions (Vector Labs, Burlingame, CA). Briefly, sections were heated to 65°C for 30 min and deparafinized with xylene. Slides were hydrated through two washes each with 95, 85, 70, 50% ethanol in water. To expose the antigenic epitopes, slides were microwaved twice for 2 min (half-power) in 100 mM Na citrate pH 6.4. Endogenous peroxidase activity was then blocked by incubation with 0.3% (vol/vol) hydrogen peroxide in water for 5 min. Nonspecific binding to the slides was then blocked by using normal horse serum (10% vol/vol in PBS), then avidin blocking reagent, and finally the biotin blocking reagent. Slides were incubated overnight at 4°C with the same anti-murine HSP25 and HSP72 antibody used for Western blot analysis (diluted 1:50 in PBS). Slides were washed five times with PBS (20 s each) and then incubated with biotinylated horse anti-mouse immunoglobulin IgG (diluted per directions on each lot from Vector). Slides were washed (5 times for 20 s each with PBS) and incubated with freshly prepared Vector ABC Elite reagent for 30 min and washed five times for 20 s each with PBS. Slides were developed by using freshly prepared diaminobenzidine solution provided for 1 min followed by washing in PBS. All slides were counterstained with hematoxylin and mounted by using Distyrene Plasticizer plus Xylene mounting agent (Electron Microscopy Sciences, Ft. Washington, PA). An inverted DMIRB (Leica, Bannockburn, IL) microscope, a model G4 computer (Macintosh, Cupertino, CA), and software (Pixera, Los Gatos, CA) were used to image slides.

Ussing chamber measurements. Sprague-Dawley rats with SFL and SEL were killed, and segments of SFL and SEL were quickly removed, cut along the mesenteric border, and rinsed of luminal contents with Ringer's solution (concentrations in mM: 114 NaCl, 5 KCl, 1.65 Na_2HPO_4, 0.3 NaH_2PO_4, 1.1 $MgCl_2$, 1.25 $CaCl_2$, and 25 $NaHCO_3$, pH 7.4). These segments were then placed in ice-cold Ringer's solution and gassed with 5% CO_2-95% O_2. After being stripped of the muscularis layer by blunt dissection, segments were cut into 2- to 3-cm squares and mounted into Lucite Ussing chambers (exposed surface area 1.12 cm^2). Tissues were maintained at 37°C, bathed on both sides with Ringer's solution with 20 mM fructose and circulated by gas lift with 5% CO_2-95% O_2. Transluminal potential differences and short-circuit current (I_{sc}) were measured under current-clamped and voltage-clamped conditions, respectively (DVC-1000 Dual Voltage Clamp; World Precision Instruments, New Haven, CT).

After a 30-min incubation, during which time the potential difference stabilized, tissues from SFL and SEL were selectively exposed to the physiologically relevant oxidant monochloramine (0.6 mM; mucosal and serosal), and the effects on transluminal mucosal resistance and I_{sc} responses to carbachol (100 μM) and glucose (20 mM) were determined. In each experiment, the selected drug was applied to either the mucosal or serosal surface of the tissue. To offset the electrochemical gradient induced by the selective addition of these drugs, equimolar amounts of mannitol were added to the opposing reservoir. The tissue response was recorded as the difference between *1*) the I_{sc} immediately before the addition of each test drug and *2*) the maximal I_{sc} after each administration. This peak change in I_{sc} was then corrected for exposed mucosal area. At the end of the experiments, glucose was added to achieve final concentrations of 20 mM (serosal), as check of tissue variability. In no case was the change induced in I_{sc} (<20 μA/cm^2).

Statistical analysis. Results are presented as means ± SE of 3–6 experiments. Comparison of means was performed by using Student's *t*-test for both paired and samples for Western blots or one-way ANOVA for transport data of the Ussing chamber. Statistical significance was determined by using InStat software version 3.05 for Macintosh (GraphPad, San Diego, CA).

RESULTS

Expression of HSP25 and HSP72 is region specific in the gastrointestinal tract and induced in SFL. To determine the role of luminal microflora on mucosal epithelial cells, a model was utilized in which stasis of luminal contents results in bacterial colonization. Western blot analyses of HSP25, HSP72, and HSC73 were performed in rats with surgically created SFL vs. SEF (Fig. 1). The SFL (F in Fig. 1) promotes filling by and retention of intestinal contents in the loop, thereby colonizing the SFL with enteric organisms (53). Control SEF (E in Fig. 1) were also created in which the peristalsis directs contents out of the loop, thereby preventing bacterial colonization (46). Intestinal mucosa was harvested from these regions 14–21 days after surgery, a time when bacterial colonization was evident.

To quantify the SFL bacterial colonization, small pieces were cut from all sections of the intestine and weighed. Nonadherent bacteria were washed from the mucosa and aerobic bacteria were quantified by dilutions onto MacConkey agar plates. As anticipated, the colon of rats with both SFL and SEL possessed large numbers of aerobic bacteria (16,710 ± 3,212 and 15,523 ± 3,391 colony forming units/g mucosa, respectively). The proximal section of the small intestine (corresponding to proximal jejunum) possessed 913 ± 221 and 886 ± 170 colony forming units/g mucosa, respectively. The SFL possessed 5,440 ± 758 and the SEL possessed 1,969 ± 154 colony forming bacteria/g mucosa, respectively. The distal

small intestine, corresponding to ileum 5 cm proximal to the ileocecal junction, possessed 4,427 ± 759 and 1,973 ± 69 colony forming units/g mucosa from rats with SFL and SEL, respectively. Bacteria in the SFL were cultured on a number of selection media. The predominant bacteria were *Escherichia coli* and *Bacteroides* with *Klebsiella, Proteus,* and *Morganella* also found routinely. The major species found in the colon were *E. coli* and *Bacteroides*, and it did not appear that new species were occurring in the SFL (11). Predominate forms and relative ratios of those forms were consistent with the commensal flora found in the rat colon (11, 52). Our goal was, at least for the time frame of adaptation studied here, that the flora present in our SFL was similar to the flora of the rat colon.

Mucosa of the SFL (Fig. 1, *right*) showed an increase of HSP25 and HSP72 expression compared with mucosa of SEL (Fig. 1, *left*). HSP expression was also determined in other regions of the intestine. In intestinal regions proximal (P) to the blind loop anastomosis, whether from rats with SFL or SEL, minimal expression of HSP25 and HSP72 were observed. Therefore, densitometric analysis was not performed on these samples and is referred to as not measured (NM) in all figures. In intestinal mucosa immediately distal (D) to the anastomosis of SFL but not SEL increased expression of both HSP25 and HSP72 was found. These results were attributed to spillage of bacteria or bacterial metabolites from the SFL. In the colon, in which chronic colonization with bacterial flora is present, a robust HSP25 and HSP72 expression was observed that was not different between the two groups. In no case was the constitutively expressed chaperone protein HSC73 induced in rats with SFL or SEF in any region. Additionally, villin expression, a marker of villus cell differentiation, was not changed under any of the experimental conditions.

Immunohistochemistry of HSP25 and HSP72 in SFL and SEF. Immunohistochemistry was performed to obtain a better localize expression of intestinal HSPs. HSP25 and HSP72 immunoreactivity was primarily found in villus intestinal epithelial cells of SFL directly exposed to luminal contents (Fig. 2). In contrast, HSP25 and HSP72 were minimally expressed in the crypt or lamina propria regions, either in SFL or SEL. It is notable that the immunohistochemical staining of HSP72 was readily apparent as opposed to the small increase noted by immunoblot analysis (Fig. 1). Because immunohistochemical staining is not quantitative, the actual increases in HSP expression are more accurately reflected by the immunoblot analyses.

Expression of HSP25 and HSP72 in SFL is dependent on the presence of commensal bacteria in the small-intestinal lumen. To elucidate the role of enteric bacteria in promoting expression of inducible HSPs, rats with SFL were treated with metronidazole for 5 days to reduce or alter enteric flora. Animals were allowed to recover from surgery for 8 to 10 days after which one group was treated for 5 days with metronidazole (0.6 mg·kg body wt^{-1}·day^{-1} po), a nitroimidazole-derivate antibiotic. As shown in Fig. 3, metronidazole treatment decreased expression of HSP 25 and HSP 72 in SFL (F) as well as in the distal section of the small intestine (D) and the colon (C). HSPs are normally expressed at only a very low level in the proximal segment of the small intestine (P), and no decrease was noted in this region after metronidazole therapy. Constitutive HSC73 expression was not affected by this treatment and confirms equal loading of protein in all lanes. Interestingly, gram-negative anaerobes and *E. coli* are affected by metronidazole in the rat and these are the bacteria largely responsible for production of SCFAs from dietary fiber. Therefore, the potential role of SCFAs in mediating the increased HSP expression induced by luminal bacteria was investigated.

HSP25 and HSP72 expression are reduced by a nonfermentable fiber diet. Previous reports have documented intraluminal bacterial fermentation of carbohydrates to volatile SCFA in the setting of bacterial overgrowth in rat blind loops (4, 38). To determine the effects of dietary fiber and the potential for

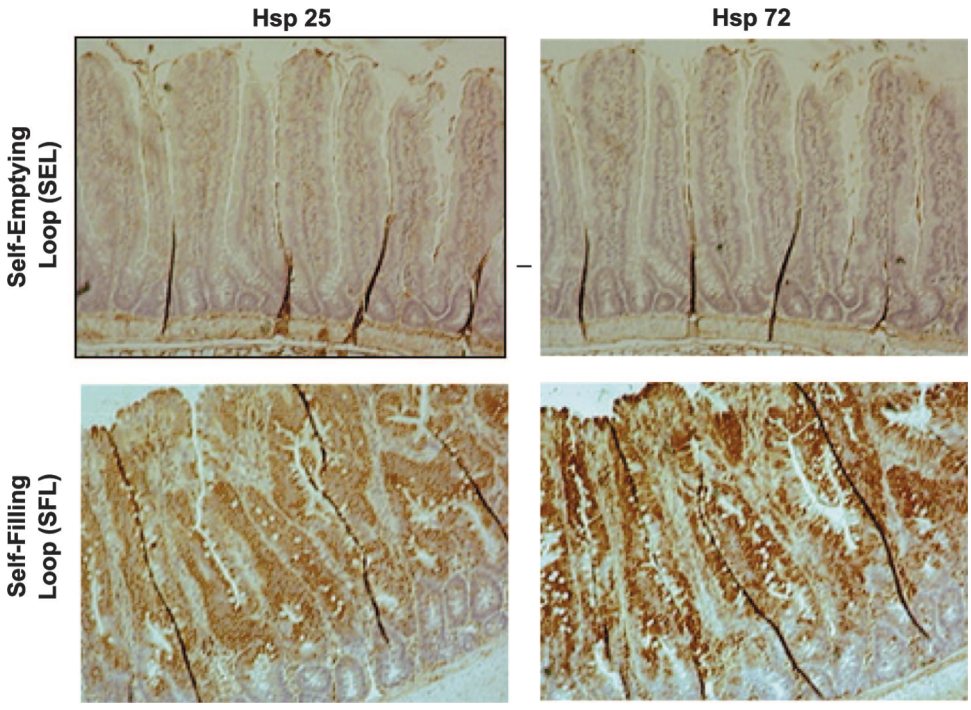

Fig. 2. Villus enterocytes of jejunal SFL express HSP25 and HSP72. Sections of SFL and SEL were stained to localize changes in HSP production. The most abundant distribution of HSPs appears in the luminal mucosal epithelial surface layer in SFL. Images shown are representative of 2 separate experiments.

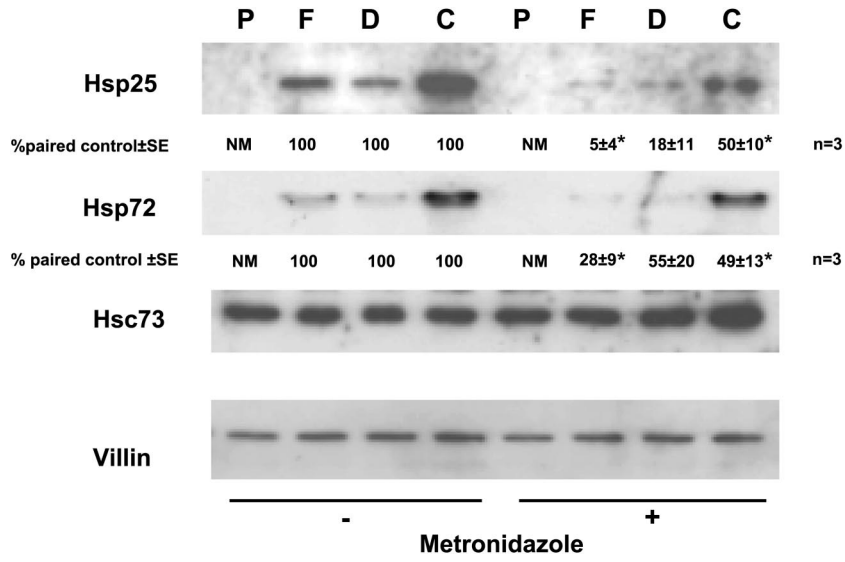

Fig. 3. Metronidazole treatment decreases HSP induction in SFL. Rats with SFL, were treated with metronidazole in their drinking water to eliminate anaerobic bacteria. Jejunal segments proximal or distal to the anastomosis as well as segments of the SFL themselves and the colon were analyzed for HSP25 and HSP72 by Western blotting as described in MATERIALS AND METHODS. Images shown are representative of 4 separate experiments. *$P < 0.05$ compared with the corresponding section of intestine from a rat not treated with metronidazole by paired Student's *t*-test.

SCFA generation on HSP25 and HSP72 expression, rats were fed a specially prepared low-fiber chow (nonfermentable fiber diet) or high-fiber chow (pectin 6% wt/wt) for 10 days. Diets were started 1 wk after surgery. HSP25 and HSP72 expression of small intestine proximal (P) and distal (D) to the surgical loops, as well as in the SFL (F) and colon (C), was assessed by Western blot analysis (Fig. 4). In rats fed the pectin-supplemented chow, increased HSP25 expression within the SFL as well as in the small intestine distal to the surgical loop and colon were observed. No effects on constitutive HSC73 were noted. SCFA are a known byproduct of bacterial fermentation in the gut. Certain studies have implicated their role as cytoprotective agents (18, 49, 50). Thus it appears that SCFA may be an important physiological determinant for epithelial HSP25 and HSP72 expression. These results suggest that the normal colonic expression of intestinal HSP25 and HSP72 may be, in part, related to SCFA bioavailability and not to inherent regional differences.

SCFAs induce HSP25 expression in jejunal organ cultures. We then examined the role of SCFAs in inducing intestinal epithelial HSPs in organ cultures. To determine the role of bacterially derived luminal SCFA in this inductive process, small sections (2–3 mm in size) of jejunal mucosa were incubated in vitro with butyrate (60 mM), a major luminal SCFA (6) for 0, 2, 4, 6, and 8 h. The results show that HSP25 levels are upregulated by incubation with butyrate in small intestinal tissue segments (Fig. 5). The initial induction and subsequent decline of HSP25 and HSP72 in nonbutyrate-treated organs cultures from 2 to 8 h may result from the anoxic, traumatic, or metabolic stress to which organ culture specimens are subjected in their harvest. Consistent with previous results in vitro and in vivo (40), SCFAs produced a modest upregulation of HSP72 expression reflected more by the statistically significant sustained expression from 6–8 h. HSP25 expression in the presence of 60 mM butyrate was also significantly different from the control samples at the 6- to 8-h time points. The greater response to butyrate for HSP25, both in vitro and in vivo and in contrast to that of the thermal response, suggests that activation mechanisms other than the classical heat-shock factor binding to the *cis* heat-shock regulatory element may be involved. The dose of SCFAs necessary to induce HSP25 is within physiological parameters, as SCFAs

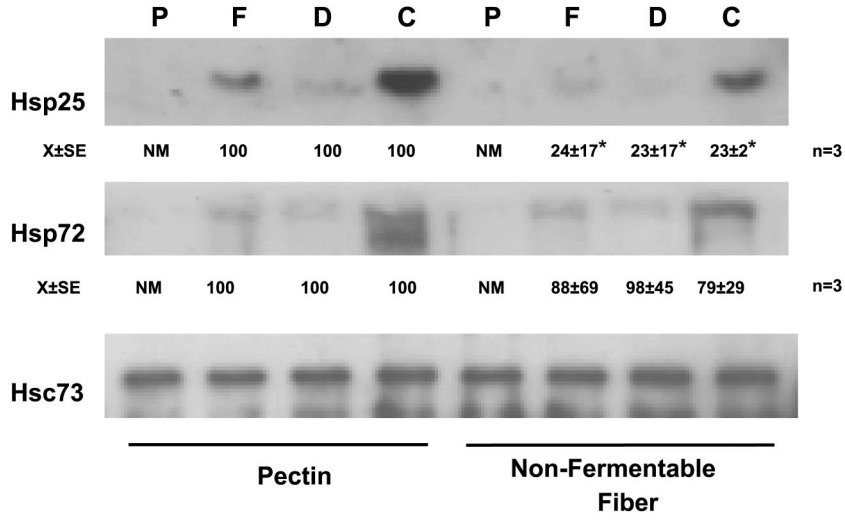

Fig. 4. Pectin-containing diet induces and nonfermentable fiber diet suppresses HSP25 and HSP72 expression in SFL and colon. Rats with SFL were fed either a diet with 6% (wt/wt) added pectin or no fermentable fiber for 7 days. HSP expression in segments proximal or distal to the anastomosis as well as segments of the SFL themselves and the colon, were measured by Western blotting as described in MATERIALS AND METHODS. Images presented are representative of those of 3 separate experiments. *$P < 0.05$ compared with corresponding sections of intestine from a rat with a pectin-containing diet by Student's paired *t*-test.

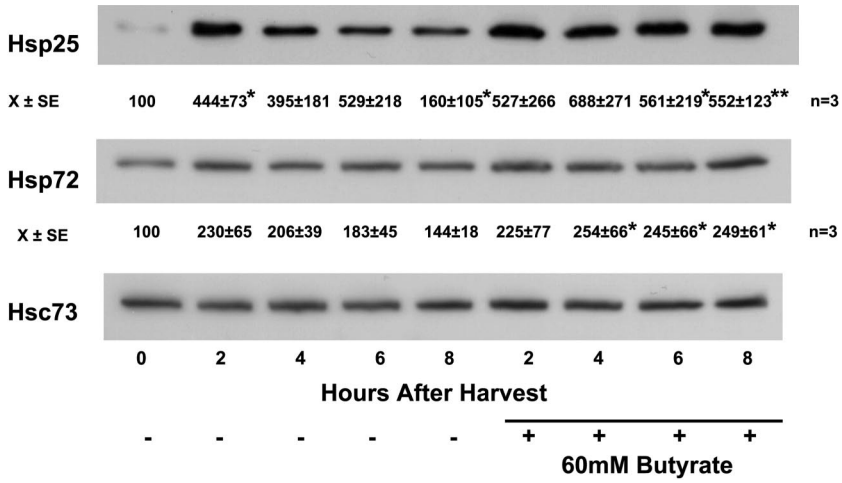

Fig. 5. Butyrate induces HSP levels above nonstimulated samples. Jejunal mucosa from control (not operated on) rats was removed, muscularis externa stripped, and small sections (2 mm) cultured for up to 8 h in media with and without butyrate. At various times, 2 pieces were removed and HSPs were analyzed by Western blotting. Images shown are representative of those of 3 separate experiments. $*P < 0.05$ and $**P < 0.01$ compared with untreated zero time control by paired ANOVA.

are the major luminal anions of colonic fluid, exceeding 100 mM collectively (37).

Barrier and transport functions are protected in SFL. To determine whether the upregulation of HSP25 and HSP72 in rats with SFL conferred survival advantage by rendering the epithelial barrier less sensitive to injurious effects, barrier as well as transport capabilities of mucosa were tested in Ussing chambers. SFL and SEL rat mucosal segments mounted on Ussing chambers were treated with the oxidant monochloramine (0.6 mM, added luminally and serosally) or vehicle. As shown in Fig. 6, *left*, the monochloramine-induced decrease in transmural resistance (ΔR) was blunted in SFL compared with SEL ($P < 0.05$). Values are presented as percent change of paired control tissues, with means ± SE of control values indicated in the figure legends. Responses in I_{sc} changes to mucosal addition of glucose (20 mM) were also protected in SFL treated with monochloramine compared with SEL controls (Fig. 6, *middle*). However, I_{sc} responses to crypt-specific anion secretion induced by serosal addition of carbachol (10^{-5} mM) were impaired in both SFL and SEL. As indicated by our immunohistochemistry sections (Fig. 2), the lack of protection of the secretory response after oxidant injury in the SFL may be explained by the lack of detectable HSP25 and HSP72 in the crypt epithelial cells that do not have direct contact with luminal microflora, thus suggesting that HSP induction is pivotal in maintaining normal epithelial cell integrity and function. Furthermore, these findings support the role of colonic microflora in stimulating HSP25 and HSP72 production by the surface epithelial cells, thereby protecting gut integrity and resisting oxidant injury.

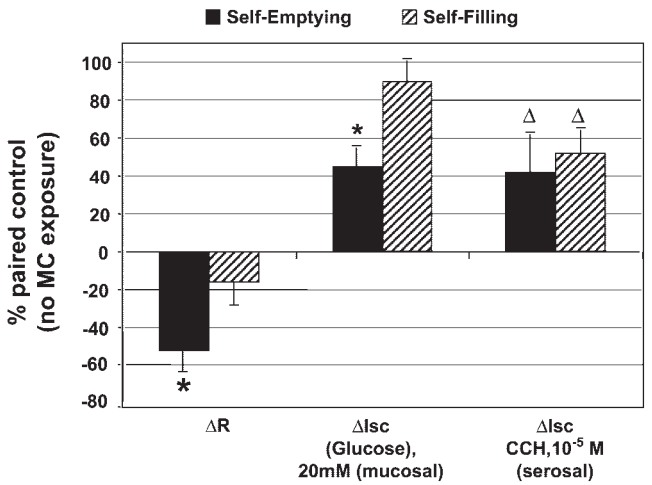

Fig. 6. Effects of the oxidant, monochloramine (MC), on mucosal resistance (ΔR), and short-circuit current (I_{sc}) were stimulated by glucose (mucosal addition) and carbachol (CCH) (serosal addition) in mucosal segments from surgically created SEL and SFL mounted in Ussing chambers. Results are presented as percentage of control resistance and I_{sc} responses of paired, nontreated mucosal sections from the same loop [control baseline $R = 85 \pm 10$ ohms/cm^2; control I_{sc} response to glucose (20 mM), 33 ± 9 μA/cm^2]. Control peak I_{sc} response to CCH (10^{-5}M, serosal), 44 ± 8 μA/cm^2 ($n = 6$ for all). $*P < 0.05$ compared with SFL mucosa; $\Delta P < 0.05$ compared with nontreated control response ($n = 6$ for all) by paired Student's *t*-test.

DISCUSSION

The present study implicates the presence of luminal bacteria and their metabolic byproducts, SCFA, as critical physiological determinants of intestinal epithelial HSP expression. The SCFA butyrate is a major energy source in the colon (6, 51), functioning in colonic intestinal homeostasis and appearing to play a pivotal role in regulating HSPs. The maintenance of HSP25 and HSP72 expression may be important for preserving cell viability and functions, such as barrier integrity and nutrient and electrolyte transport, particularly in the normal colon, which is continuously exposed to commensal bacteria and must be prepared to respond to conditions of stress. The resulting HSPs may protect cells, not only by acting as molecular chaperones, but possibly by downregulating proinflammatory cytokine production to curb noxious processes like heat stress, infection, and inflammation (10, 14, 26, 32, 42, 58). The maintenance of a barrier, impermeant to luminal bacteria, is clearly important.

A better understanding of the physiological role and regulation of intestinal HSPs may be invaluable in elucidating intestinal disease processes. Cytoprotective effects have been shown with induction of HSP25 (35, 36, 40, 45, 55) and specifically with HSP60, HSP72, and HSP90 induction (15, 16, 44, 47) in the intestine. Of note, induction of HSP72 confers protection against acetic acid-induced mucosal damage in rats (33, 44) and rodent models of dextran sulfate colitis as well. Increasing tissue exposure to butyrate either through enemas or

by modulating fiber in diet resulted in significant improvement of colitis. This improvement was seen both in clinical trials (12) and in rat experimental colitis models (3, 18, 41, 56), and appeared, in part, to be mediated by activation of HSP70 and inhibition of NF-κB (56). Another instance of a physiological insult, which appears to be ameliorated through HSPs induction, is the barrier-disrupting effect of toxin A from *Clostridium difficile* (23). Colonic mucosa from mice treated with metronidazole as in the present protocol (600 μg/ml in drinking water for 5 days) demonstrated decreased colonic epithelial HSP25 and HSP72 expression by >80%. When this tissue was mounted in in vitro Ussing chambers, it responded more robustly to toxin A, as determined by changes in electrical resistance of the tissue as well as increased mucosal to serosal fluxes of mannitol, a marker of the paracellular permeability pathway. This data, when taken together, strongly suggest that the physiological expression of the inducible HSPs confer protection against injurious agents to which the intestine may be exposed.

Our study demonstrates that the expression of intestinal HSP25 and HSP72 is closely related to the presence of luminal bacteria and their byproducts, which are potentially regulated by the delivery of fermentable dietary carbohydrates. The importance of SCFA bioavailability in maintaining expression of intestinal epithelial HSP25 and HSP72 is demonstrated. The results, seen in the metronidazole treated loops, strongly supports the indispensable role of the bacterial fermentation byproducts, SCFAs, as being intrinsically important in the induction of HSPs in this animal model. The contribution of SCFAs should be drastically reduced or eliminated in this tissue at harvest (38). The data show significantly attenuated HSP levels in these loops, although the bacteria themselves are still present in the their lumen. This again points to SCFA having a pivotal role in the HSP induction we observe. The pectin diet also implicates SCFA as having a obvious role in HSP induction. Furthermore, the organ culture work demonstrates that butyrate, in particular, is likely to be an important factor in this induction. The epithelial cells of the proximal small intestine, when exposed to bacteria and their products, will constitutively express HSP25 and HSP72 in a fashion similar to the colon and the distal small intestine. Interestingly, these in vivo results demonstrate greater induction of HSP25 compared with HSP72. Although the immunohistochemistry suggested robust induction of HSP72 expression, it should be noted that this assay is not quantitative and that the immunoblot data are more likely to be reflective of true changes in mucosal HSP expression. Preferential induction of HSP25 expression in intestinal epithelial cells has been reported for LPS and SCFA (20, 21). Moreover, physiological expression of intestinal HSP72 appears to be highly dependent on immune cell cues, as expression is nearly eliminated in *Rag1*$^{-/-}$ gene-deficient animals that lack mature T- and B-cell lymphocytes (20).

The present study further demonstrates an expression of HSP25 and HSP72 primarily in the surface epithelium of SFL and the colonic mucosa, i.e., cells closest to the intestinal lumen and region of greatest exposure to bacteria and SCFAs. Little, if any, expression is observed in mesenchymal cells, suggesting a relatively epithelial-specific HSP25 and HSP72 response to luminal bacteria. It remains to be resolved whether this differential effect is due to greater and more direct exposure of surface epithelium with bacteria or bacterial products or whether there is truly a cell-specific (e.g., villus-crypt) response to these stimuli. On the basis of the metronidazole effects, it appears that anaerobic bacteria are particularly important in sustaining physiological expression of HSPs. Anaerobic bacteria are the primary producers of intestinal SCFA and lipopolysacharride, both capable of selectively inducing HSP25 in intestinal epithelial cell (IEC)-18 intestinal epithelial cell cultures and in murine young adult mouse colon colonic cells (20, 40). The potential role of SCFA in determining intestinal mucosal HSP expression is further underscored by the observation that rats on a pectin diet and treatment of rat intestinal cell culture with the SCFA butyrate exhibited increased HSP25, and to a lesser extent HSP72 expression as analyzed by Western blots. The same effect was also observed in mucosa from SFL in rats fed the pectin-containing diet as well as the organ cultures of normal small intestinal mucosa with butyrate. Other stress proteins or additional trophic effects of luminal SCFA could contribute to the protective actions of expressed HSPs. The effects of butyrate and other SCFA may be complex. We have previously shown that butyrate as well as acetate, propionate, and the poorly metabolized isobutyrate all induce HSP25 in IEC-18 rat small intestinal cells (40). The use of isobutyrate is notable, because it is only poorly metabolized. An additional effect of butyrate, which may contribute to its actions is its inhibition of histone deacetylase (39, 60); however, the effects of the other SCFA on this enzyme, a potent transcriptional regulator, have not been determined. One action shared by all of these volatile fatty acids is cellular acidification. It is possible that prolonged cellular acidification may play some role in the induction of HSPs by these fatty acids as well. The relative roles of these pathways in the induction of intestinal HSPs by butyrate and SCFA will be difficult to discern in whole tissue and remain the subject of future experiments.

The increased expression of HSPs in intestinal epithelial cells that are in direct contact with enteric flora (e.g., surface colonocytes or villus epithelial cells of the SFL) likely confers greater protection and resistance to injurious factors. In support of this is the finding that mucosal barrier function, reflected by transmural electrical resistance, and villus cell absorptive function, assessed by glucose-stimulated I_{sc}, were not significantly impaired in mucosa from SFL in contrast to mucosa from SEL after oxidant exposure. These properties are largely inherent to the villus epithelium in which the greatest HSP induction was observed. In contrast, anion secretion stimulated by the muscarinic agent, carbachol, was impaired in both SEL and SFL, possibly because of the absence of induced HSPs in crypt regions, the site in which anion secretion is believed to predominate. Whereas these data are correlative and do not establish cause and effect, they suggest that the induction of intestinal epithelial HSPs by enteric flora plays an important physiological role in rendering the mucosa less sensitive to injury and protecting critical cellular functions. It should be noted that this does not preclude that chronic small intestinal bacterial colonization in conditions, such as pouchitis, may have a deleterious effect on intestinal mucosal integrity. However, the clinical manifestation of pouchitis is present in a minority of patients with ileoanal pouches, and most of these patients have normal mucosa with no histologically evident consequences (25). Therefore, the composition of the enteric flora present may be an important determinant of either the

adverse or advantageous effect of small bowel colonization (9, 25). In support of this theory, studies have indicated that butyrate levels are reduced in patients with pouchitis (5, 8), and both butyrate enemas and probiotics have been shown to alter tissue integrity and inflammation in pouchitis patients (8.12, 59).

In summary, the physiological expression of two important cytoprotective HSPs (25 and 72) in gut mucosa is highly dependent on extracellular cues provided by enteric flora. The epithelial HSP response to enteric flora is not colonocyte-specific, because small intestinal cells directly exposed to enteric flora exhibit a robust HSP response. The absence of normal enteric flora in the small bowel may account for the relatively minimal expression of HSP25 and HSP72 in this region (with the exception of the distal ileum continuously exposed to bacteria in rodent species). This expression of intestinal epithelial HSPs induced, in part, by bacterial fermentation products such as SCFA, plays a role in protecting barrier and transport functions of the intestinal epithelium to injurious agents and conditions.

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