fMLP induces Hsp27 expression, attenuates NF-κB activation, and confers intestinal epithelial cell protection

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N-formylmethionyl-leucyl-phenylalanine (fMLP) is a tripeptide produced by many enteric bacteria, including Escherichia coli, as a by-product of protein synthesis. In addition to being secreted, fMLP is also found in the surface membranes of many types of bacteria. fMLP is a potent chemotactic agent for neutrophils and has been suggested to be proinflammatory (11, 38). It has also been implicated in the pathogenesis of Crohn’s disease, ulcerative colitis, and pouchitis (1, 6, 10, 27, 28, 39) and has experimentally been shown to induce colonic inflammation in animal models (10, 28) and to increase expression of major histocompatibility complex class I molecules in intestinal epithelial cells (34). However, most of these studies were performed with concentrations of fMLP exceeding 500 times the physiological concentration of fMLP found in the colon, ~100 nM (7, 33). In contrast, one study using 10 μM fMLP to perfuse rat colon (~100 times the physiological concentration) reported no inflammatory effects, as judged by myeloperoxidase assay (6). It is therefore uncertain whether physiological concentrations of fMLP produce colonic inflammation. It should be noted that certain probiotic treatments containing bacteria known to express and secrete fMLP have been reported to relieve the symptoms of patients suffering from inflammatory bowel disease, when given orally (25, 46). Taken together, these findings prompt a look at the effects of physiologically relevant concentrations of fMLP on the colonic epithelium.

The intestinal epithelium uses a number of defense mechanisms both to maintain its function and to coexist with normal bacterial flora. These protective mechanisms include secretion of mucus (4), immunoglobulin IgA (31), and defensin peptides (40) from epithelial cells, as well as production of cytoprotective stress proteins such as the heat shock proteins (Hsps) (36, 47). Hsps are a group of evolutionarily conserved proteins with diverse functions, including antiapoptosis (19), mitochondrial protection (5), prevention of Ca2+ disturbances (29), and protection against oxidant-induced injury (36). Hsps have been demonstrated to exert these diverse cytoprotective effects in many tissues, including the colon.

The present studies demonstrate that physiological concentrations of fMLP significantly increase expression of Hsp27, but not of Hsp72, expression in the human colonic epithelial cell line Caco2bbe. The response is specific to fMLP, because related fMLP analogs MRP and MLP were not effective. Hsp27 induction by fMLP was blocked by the fMLP-receptor antagonist BOC-FLFLF and was blocked when the dipeptide transporter PepT1, an entry pathway for fMLP, was silenced. fMLP activated both the p38 and ERK1/2 MAP kinase pathways in Caco2bbe cells, but not the SAPK/JNK pathway. The p38 inhibitor SB203580, but not the MEK-1 inhibitor PD98059, blocked Hsp27 induction by fMLP. fMLP treatment inhibited actin depolymerization and decreased transepithelial resistance caused by the oxidant monochloramine, and this inhibition was reversed by silencing Hsp27 expression. fMLP pretreatment also inhibited activation of proinflammatory transcription factor NF-κB by TNF-α in Caco2bbe cells, reducing induction of NF-κB target genes by TNF-α both in human intestinal biopsies and Caco2bbe cells. In conclusion, fMLP may contribute to the maintenance of intestinal homeostasis by mediating physiological expression of Hsp27, enhancing cellular protection, and negatively regulating the inflammatory response.

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First published December 21, 2006; doi:10.1152/ajpgi.00417.2006.—Sustained expression of cytoprotective intestinal epithelial heat shock proteins (Hsps), particularly Hsp27, depends on stimuli derived from bacterial flora. In this study, we examined the role of the bacterial chemotactic peptide fMLP in stimulating colonic epithelial Hsp expression at concentrations encountered in a physiological milieu. Treatment of the polarized human intestinal epithelial cell line Caco2bbe with physiological concentrations of fMLP (10–100 nM) induced expression of Hsp27, but not Hsp72, in a time- and concentration-dependent manner. Induction of Hsp27 by fMLP was specific since the fMLP analogs MRP and MLP were not effective. Hsp27 induction by fMLP was blocked by the fMLP-receptor antagonist BOC-FLFLF and was blocked when the dipeptide transporter PepT1, an entry pathway for fMLP, was silenced. fMLP activated both the p38 and ERK1/2 MAP kinase pathways in Caco2bbe cells, but not the SAPK/JNK pathway. The p38 inhibitor SB203580, but not the MEK-1 inhibitor PD98059, blocked Hsp27 induction by fMLP. fMLP treatment inhibited actin depolymerization and decreased transepithelial resistance caused by the oxidant monochloramine, and this inhibition was reversed by silencing Hsp27 expression. fMLP pretreatment also inhibited activation of proinflammatory transcription factor NF-κB by TNF-α in Caco2bbe cells, reducing induction of NF-κB target genes by TNF-α both in human intestinal biopsies and Caco2bbe cells. In conclusion, fMLP may contribute to the maintenance of intestinal homeostasis by mediating physiological expression of Hsp27, enhancing cellular protection, and negatively regulating the inflammatory response.

chemotactic peptides; stress proteins; actin; barrier function; NF-κB...
To further investigate the role of fMLP in inflammation, we investigated the ability of fMLP to interfere with transcription initiated by the transcription factor NF-κB. We hypothesize that the protective effects by fMLP could result from its interference with proinflammatory pathways. In support of this, fMLP pretreatment suppresses the activation of NF-κB DNA binding by TNF-α. Furthermore, the induction of known NF-κB target genes by TNF-α is similarly suppressed by fMLP both in human intestinal biopsies and Caco2bbe cells.

MATERIALS AND METHODS

Cell culture. Caco2bbe cells, a derivative of the Caco2 human colonic adenocarcinoma line, was a generous gift from Dr. M. Mooseker (Yale University, New Haven, CT) (43) and was used at passages 52–65. Cells were grown in high glucose DME supplemented with 10% (vol/vol) fetal bovine serum, 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10 μg/ml human transferrin (all media, sera, and supplements from Invitrogen, Grand Island, NY).

Transepithelial resistance measurements. Caco2bbe cells were plated on permeable supports at a density of 10^5/cm^2 and monitored for resistance using an EVOM system (Millipore, Medford, MA) and used when transepithelial resistances were 200–300 Ω/cm^2 (10–12 days after plating). When appropriate, cells were treated with fMLP (100 nM) for 1 day before use. For MAP kinase inhibitor pretreatment, monolayers were pretreated with these agents for 30 min and then treated with fMLP for 2 h, and media were removed and replaced with serum-free, antibiotic-free medium. After 24 h, cells were treated with monochloramine for actin and transepithelial electrical resistance (TER) assays.

Silencing with siRNA. To suppress Hsp27 or PepT1 expression, cells were treated with silencing oligonucleotides. Stealth siRNA oligonucleotides (Invitrogen, Carlsbad, CA) corresponding to nucleotide positions 388–412 of the coding sequence of the human Hsp 27 gene (Genbank NM_001540), nucleotide positions 539–563 of the coding sequence of the mouse Hsp25 gene (NM_013560), and the PepT1 oligonucleotide with four altered bases (negative control) were complexed with siLentFect reagent (Bio-Rad, Hercules, CA). The mouse Hsp25 oligonucleotide contains a sequence not present in the human Hsp 27 sequence and thus served as a negative control. Confluent monolayers were treated with a final concentration of 20 nM oligonucleotides and 0.6 μl of siLentFect per square centimeter. The complexes were added to the cells on both apical and basolateral sides in a minimal volume (500 μl apical, 1,000 μl basolateral) of OptiMEM. After 30 min, complete medium was added and cells were incubated overnight. A second dose of oligonucleotides was applied at 24 h, and cells were used 24 h later. Silencing of Hsp27 or PepT1 expression was monitored by Western blotting.

Western blotting. Cells were incubated with fMLP (all peptides from Sigma, St. Louis, MO) for varying times and concentrations. When appropriate, the fMLP-receptor antagonist BOC-FLFLF or the inactive analogs MRP and MLP (Sigma-Aldrich) were added 10 min before fMLP. As a positive control, cells were heat shocked at 42°C for 23 min and then returned to the CO2 incubator for 2 h to allow the synthesis of heat shock proteins. Cells were rinsed and scraped into cold saline, and cellular lysates were prepared and analyzed for Hsp expression by Western blotting using antibodies to Hsp27 (mouse monoclonal, Affinity BioReagents, Boulder, CO), Hsp72 (mouse monoclonal C92, Stressgen), or Hsc73 (rat monoclonal 1B5, Stressgen). Signals were visualized via an enhanced chemiluminescent detection system (SuperSignal, Pierce Chemical, Rockford, IL).

Analyses of MAP kinase pathways. Three MAP kinase pathways were analyzed: p38, JNK, and ERK1/2 (p44/42 MAP kinases). Cells were treated with anisomycin (10 μg/ml for 30 min, Alexio Biochemicals, San Diego, CA) to obtain a positive control for p38 and JNK activation or with PMA (0.1 μM for 30 min, Alexio Biochemicals) for ERK1/2 activation. Western blot analysis was performed using PhosphoPlus kits for p38, JNK, and p44/42 MAP kinases (Cell Signaling, Beverly, MA). Phosphorylated MAPK isofoms, indicators of kinase activation, were detected by using phosphospecific antibodies as well as antibodies recognizing total amounts of each MAPK. Where indicated, cells were preincubated with inhibitors of specific MAPK pathways, including the p38 inhibitor SB203580 (20 μM, Alexio Biochemicals), the JNK inhibitor SP600125 (30 μM, Calbiochem, San Diego, CA), and the MAP kinase kinase-1 (MEK)-1 inhibitor PD98059 (50 μM, Alexio Biochemicals) for 2 h before treatment with fMLP.

F/G actin assay. Caco2bbe cells grown on permeable supports were treated with fMLP with or without MAP kinase inhibitors or silencing oligonucleotides for hHsp27 or mHsp25. After monochloramine treatment for 1 h (36), cells were rinsed and scraped into saline, pelleted (14,000 g for 20 s at room temperature), and resuspended in 200 μl of 30°C in PBS and lysis buffer (1 mM ATP, 50 mM PIPES pH 6.9, 50 mM NaCl, 5 mM MgCl2, 5 mM EGTA, 5% (vol/vol) glycerol, 0.1% (vol/vol) each Nonidet P-40, Tween 20, Triton X-100, and Complete protease inhibitor cocktail) added and left for 10 min. Samples were centrifuged at 14,000 g for 20 min at 30°C and the supernatants stored for determination of globular (G)-actin. Pellets containing F-actin were resuspended in 200 μl of 4°C distilled water containing 1 μM cytochalasin D and left on ice for 60 min. Protein concentrations of the samples were determined by the bicinchoninic acid procedure (15). Twenty micrograms of each sample was analyzed by Western blots using a polyclonal anti-actin antiserum (Cytoskeleton, Denver, CO). Since the F-actin fraction depolymerizes in the presence of SDS, only the monomeric 45-kDa form is observed on the Western blots.

EMAs. Oligonucleotides used in EMAs (NF-κB consensus, top strand: 5'-GATCAGTTGAGGGACTTCTCCAGCCG-3'; NF-κB consensus, bottom strand: 5'-GATCGCCTGGAAAGTCCCCTACAAT) were designed to have 5'-GATC overhangs when annealed, allowing radioactive labeling by fill-in reactions, as described (48). Five micrograms of protein from nuclear extracts prepared by using the NE-PER extraction kit (Pierce, Lausanne, Switzerland) were mixed with 50,000 cpm (1.0 ng) of the radioactive probe, and protein-DNA complexes were allowed to form. In supershift experiments, 1 μg of the anti-NF-κB p65 antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA) was added to the extracts before binding reactions. The EMASA gels were run (at 200 V in 0.5× Tris-borate-EDTA) and processed as described for autoradiography (48).

RNA isolation, reverse transcription, and real-time PCR. Caco2bbe cells were treated with fMLP or acetate acid (vehicle) for 3 h before treating with 10 ng/ml TNF-α for 1 h. Total RNA was isolated using TRIzol reagent (Invitrogen). RNA (1 μg) was reverse transcribed by random priming (Reverse Transcription System, Promega Catalys) and one-tenth was used for real-time PCR performed on an ABI PRISM 7700 sequence detection system (Applied Biosystems; Rotkreuz, Switzerland) using TaqMan Gene Expression Assays (Applied Biosystems) for human TNF-α (HS00174128) and IL-8 (HS00174103). Constitutively expressed 18S rRNA was measured as an internal standard for normalization (Applied Biosystems). Relative mRNA levels were calculated by the comparative threshold cycle (ΔΔCT) method. Each PCR was performed in triplicate, and all experiments were repeated three times. The mRNA levels obtained in control conditions are set to one, and other tests are shown relative to this.

Short-term culture of human intestinal biopsies. The study was approved by the local ethics committee of the University Hospital Zurich. Written, informed consent was obtained from patients undergoing diagnostic ileocolonoscopy. The patients showed no evidence of ileal disease and only biopsies from patients with a C-reactive protein level <5 ng/l were used. Three patients (two women, mean

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were calculated using the TaqMan Gene Expression Assay Hs00200229 (Applied Biosystems). Constitute-
ected villin mRNA was measured as an internal standard for sample normalization using the 
performed as above. Constitutively expressed villin mRNA was mea-
trituration by repeated forceful passage through 21-gauge needles and then reverse transcribed and analyzed by real-time PCR for TNA-α performed as above. Constitutively expressed villin mRNA was measured as an internal standard for sample normalization using the TaqMan Gene Expression Assay Hs00200229 (Applied Biosystems). Each PCR was performed as a triplicate and relative mRNA levels were calculated using the ΔΔCT method. The TNA-α mRNA level in biopsies treated with TNA-α alone from each patient was set to 100%, and the results from the other treatments were calculated relative to this. Data shown represents mean values from the three eligible patients ± standard deviations.

**IL-8 release studies.** Caco2bbe cells were pretreated with 400 nM fMLP or the vehicle (acetic acid) for 3 h, before stimulation with 10 ng/ml TNA-α for 1 h. Media were harvested from the cells 24 h later and IL-8 release measured by ELISA (Endogen, Rockford, IL).

**Data presentation and analyses.** Numerical data are presented as means ± SE and were compared with Student’s t-test or for multiple comparisons, an analysis of variance using a Bonferroni correction. Statistical calculations were done by using InStat version 3.05 (GraphPad, San Diego, CA). Data obtained from real-time PCR experiments were analyzed with one-way ANOVA, followed by post hoc Tukey’s test.

**RESULTS**

**fMLP induces expression of Hsp27, but not of Hsp72 or Hsc73, in Caco2bbe cells.** To determine whether fMLP treatment influences expression of Hsps in intestinal cells, Caco2bbe cells were treated with 100 nM fMLP for varying time periods. The concentration of fMLP selected approximates the level found in human stool and colonic lumen (7, 33, 34). Treatment of Caco2bbe cells with 100 nM fMLP induced Hsp27 expression already within 1 h, and the induction peaked at 12–24 h (Fig. 1A). In contrast, no changes in the expression of constitutive heat shock cognate 73 (Hsc73) protein or another heat shock protein, Hsp72, were observed. It should be noted that basal expression of Hsp72 is elevated in Caco2bbe cells (35), possibly obscuring any further stimulation by fMLP.

Changes in Hsp27 expression were quantitated by Western blotting (Fig. 2A). Basal expression of Hsp27 by fMLP, implying that an fMLP binding site is involved (Fig. 2A). The requirement for fMLP uptake into the cells was investigated by silencing PepT1 expression with siRNA. PepT1 is known to transport not only di- and tripeptides, but also fMLP and muramyl dipeptide (52). Effective silencing PepT1 expression was confirmed by Western blotting (Fig. 2B), but PepT1 expression was not altered by a related control siRNA oligonucleotide with four bases altered. Furthermore, the PepT1-specific siRNA led to decreased apical glycyl-sarcosine uptake (52) (from 98 ± 17 to 23 ± 9 pmol per Transwell per 10 min) whereas the control siRNA (96 ± 14 pmol/Transwell per 10 min) did not. As shown in Fig. 2C, reduced PepT1 expression decreased fMLP-mediated induction of Hsp27, suggesting that uptake of fMLP into the cells is involved in Hsp27 induction (Fig. 2C).

**fMLP activates specific MAPK signaling in Caco2bbe cells.** In other cell types, fMLP is known to stimulate multiple members of the MAP kinase family (8, 42). Therefore, we investigated the potential involvement of three MAP kinase pathways in mediating the induction of Hsp27 expression by fMLP in Caco2bbe cells. First, we determined whether the ratios of active (phosphorylated) to inactive (nonphosphorylated) p38 and ERK1/2 kinases are affected by fMLP treatment
in Caco2bbe cells, using antibodies that specifically recognize phosphorylated kinases and those that do not differentiate between the phosphorylation states. As shown in Western blots in Fig. 3A, treatment of cells with 100 nM fMLP rapidly and transiently induced the phosphorylation of both the p38 kinase and the ERK1/2 kinases. The activation of SAPK/JNK pathway was not stimulated by fMLP treatment in Caco2bbe cells, although anisomycin caused a robust SAPK/JNK activation (data not shown). No significant changes were observed in the total expression levels of the p38 and ERK1/2 kinases upon fMLP treatment (Fig. 3A). To further assess the role of MAPK activation in the induction of Hsp27 by fMLP, Caco2bbe cells were pretreated with specific inhibitors of MAPK pathways, before incubation with fMLP and preparation of cell extracts for Western blotting. As shown in Fig. 3B, an inhibitor of the SAPK/JNK pathway, SP600125, had no effect on fMLP-induced expression of Hsp27 in Caco2bbe cells, in accordance with the lack of fMLP-induced phosphorylation of SAPK/JNK in Western blot analysis (data not shown). On the other hand, pretreating the cells with the p38 inhibitor SB203580 for 2 h significantly reduced the Hsp27 induction in response to fMLP (Fig. 3B). However, pretreatment of cells with the MEK-1 inhibitor PD98059 did not affect the degree of Hsp27 induction by fMLP (Fig. 3B). None of the MAPK inhibitors had any effect on the constitutive expression of Hsc73.

fMLP protects against actin depolymerization induced by the oxidant monochloramine. One function of Hsp25 in murine intestinal epithelial cells is protection against actin depolymerization in response to treatment with the oxidant monochloramine (NH₂Cl) (24). To determine whether fMLP-induced Hsp27 would exert a similar protective effect on human intestinal epithelial cells, RNA silencing was used. A 25-mer silencing RNA oligonucleotide that corresponds to bases 388–412 of the coding sequence of the human Hsp27 gene and a negative control RNA oligonucleotide specific for the mouse Hsp25 gene (bases 539–563) were synthesized. Hsp27 silencing reduced both basal and fMLP-induced Hsp27 protein expression whereas the oligonucleotide to the mouse Hsp25 had no effect (Fig. 4A). To determine functional consequences of silencing Hsp27 expression, the state of actin polymerization as well as TER were measured in the context of injury
caused by the oxidant monochloramine. In untreated Caco2bbe cells, actin is present predominantly in the filamentous F form, and only 20–25% of the total actin exists in the globular G form. This distribution of actin in untreated cells was not affected by Hsp27 silencing (data not shown). Also, treatment of cells with fMLP did not affect the relative proportions of the two actin forms (Fig. 4B). However, treatment of Caco2bbe cells with NH2Cl resulted in actin redistribution, strongly increasing the ratio of G to F actin. Pretreatment with fMLP for 24 h inhibited the NH2Cl-induced redistribution of actin (Fig. 4B). The ability of fMLP to prevent oxidant-induced actin depolymerization was prevented by decreasing Hsp27 expression by siRNA oligonucleotides specific to the human Hsp27 gene, but was not affected by transfecting the siRNA oligonucleotides derived from the mouse Hsp25 gene (Fig. 4B). To further establish the role of fMLP-induced Hsp27 in protection against NH2Cl-induced actin depolymerization, cells were treated with various MAP kinase inhibitors before treatment with fMLP. In agreement with the results shown in Fig. 3B, the p38 inhibitor SB203580, which blocked the fMLP induction of Hsp27 expression, also blocked the ability of fMLP to protect against oxidant-induced actin depolymerization (Fig. 4C).

Treatment of cells with either the MEK-1 inhibitor PD98059 or the SAPK/JNK inhibitor SP600125 did not affect fMLP-induced protection against actin depolymerization upon oxidant challenge. Images for F and G actin were analyzed by scanning densitometry using NIH Image 1.54 software, and for each condition the total actin was set to 100% and F and G were calculated as percentages. Means ± SE are presented for each condition.

To determine whether fMLP treatment affects paracellular permeability, TER, a marker for tight junction integrity, was measured in confluent Caco2bbe cells pretreated with fMLP or vehicle and subjected to oxidative stress via NH2Cl treatment. Additionally, the human Hsp27 silencing oligonucleotides or the mouse Hsp25 oligonucleotides were used to determine the importance of Hsp27. Reduction in basal Hsp27 expression via silencing did not significantly alter basal TER (259 ± 46 Ω·cm2) in untreated cells; 238 ± 42 Ω·cm2 in mouse Hsp25 oligonucleotide treated cells, and 235 ± 49 Ω·cm2 in human Hsp27 oligonucleotide treated cells, n = 4). Treatment of cells with fMLP did not affect TER (248 ± 37 Ω·cm2) in untreated cells; 226 ± 35 Ω·cm2 in scrambled oligonucleotide-treated cells and 231 ± 39 Ω·cm2 in Hsp27 silenced cells, n = 4). As shown previously (37), treatment with NH2Cl rapidly decreases TER in Caco2bbe cells (Fig. 5A). Pretreatment of Caco2bbe cells with fMLP significantly protected against the oxidant-induced TER decreases (Fig. 5A), an effect that was prevented by the human Hsp27 siRNA but not by the mouse Hsp25 siRNA (Fig. 5B). Further support for the role of Hsp27 was obtained by blocking fMLP induction of Hsp27 with the p38 inhibitor SB203580, which prevented fMLP-mediated protection against TER changes caused by monochloramine. For the sake of clarity, the data using the p38 inhibitor SB203580 to inhibit fMLP induction of Hsp27 and subsequent protection of oxidant-induced TER changes are not presented in Fig. 5; however, at both 40 and 60 min, the ability of fMLP to decrease NH2Cl-induced changes in TER were inhibited by nearly 80%. Neither SAPK/JNK inhibition (SP600125) nor MEK-1 inhibition (PD98059) blocked fMLP-mediated protec-
NH$_2$Cl-induced TER decreases. Caco2bbe monolayers were treated with basal TER calculated as a percentage of the TER before treatments; fMLP did not alter oligonucleotides was for a total of 48 h. fMLP was added 24 h before NH$_2$Cl. Changes in TER were measured for 1 h after NH$_2$Cl. TER values were when basal resistances had stabilized at 200–300 cm$^2$/V/s.

Caco2bbe monolayers were grown on permeable supports for 10–12 days, transepithelial electrical resistance (TER) through induction of Hsp27. It is becoming increasingly apparent that, in many cells, silencing with fMLP, in conditions known to induce Hsp27 expression, with fMLP, would affect NF-κB activation by TNF-α, a pivotal transcription factor for the inflammatory response. Having shown the suppressive effect on NF-κB DNA-binding by fMLP, we next studied whether induction of known NF-κB target genes is consequently affected by a similar treatment. To this end, we measured mRNA levels of two such NF-κB target genes, namely TNF-α and IL-8, by real-time PCR. In agreement with the inhibition of NF-κB DNA-binding activity, the induction of both TNF-α (Fig. 7A) and IL-8 gene (Fig. 7B) expression was moderately but statistically significantly, reduced by pretreatment of cells with fMLP. Furthermore, fMLP pretreatment led to a mild suppression of IL-8 release from the Caco2bbe cells in response to exposure to TNF-α (Fig. 7C).

fMLP suppresses TNF-α gene induction in response to TNF-α treatment in short-term tissue culture of human intestinal biopsies. We next investigated whether fMLP pretreatment could also suppress the induction of the NF-κB target gene TNF-α ex vivo using human tissue samples. Ileal and cecal biopsies were obtained from three healthy patients (exhibiting a C-reactive protein level <5 mg/l in the serum). These biopsies were then pretreated with fMLP for 3 h, before incubating them in the presence of the proinflammatory cytokine TNF-α for an hour. Similarly to the Caco2bbe cells, the cytokine-induced level of TNF-α mRNA expression was significantly suppressed in both ileum (Fig. 8A) and cecum (Fig. 8B) samples from the three patients upon pretreatment with fMLP.

**DISCUSSION**

Enteric flora can be potentially harmful to the host if mucosal barrier integrity is breached, resulting in systemic seeding by 10.220.33.3 on May 1, 2017 http://ajpgi.physiology.org/ Downloaded from

Fig. 5. Treatment with fMLP protects against NH$_2$Cl-induced decrease in transepithelial electrical resistance (TER) through induction of Hsp27. Caco2bbe monolayers were grown on permeable supports for 10–12 days, when basal resistances had stabilized at 200–300 Ω·cm$^2$. A: cells were treated for 24 h with fMLP before addition of NH$_2$Cl (0.6 mM) where indicated. Changes in TER were measured for 1 h after NH$_2$Cl. TER values were calculated as a percentage of the TER before treatments; fMLP did not alter basal TER. B: inhibition of fMLP-induced Hsp27 blocks protection against NH$_2$Cl-induced TER decreases. Caco2bbe monolayers were treated with silencing for human Hsp27 or mouse Hsp25 as designated. Treatment with oligonucleotides was for a total of 48 h. fMLP was added 24 h before NH$_2$Cl treatment as in A. Neither silencing oligonucleotide altered basal TER. TER data were calculated as percentage of value prior to NH$_2$Cl as in A. All values at each point in each experiment were taken at 3 points in the well and averaged. Values presented are means ± SE from 4 independent experiments. For A, NH$_2$Cl and fMLP + NH$_2$Cl conditions were both different from control at P < 0.01 after 10 min, and *indicates difference between these 2 conditions by analysis of variance. For B, *indicates P < 0.05 of either fMLP + NH$_2$Cl with or without mHsp25 oligonucleotide compared with NH$_2$Cl, fMLP + NH$_2$Cl + hHsp27 oligonucleotide was not different from NH$_2$Cl alone at any time point.

**fMLP suppresses NF-κB activation by TNF-α in Caco2bbe cells.** It is becoming increasingly apparent that, in many cellular models, the heat shock response (HSR) can attenuate NF-κB-mediated activation of inflammatory response (32). Thus we investigated whether pretreatment of Caco2bbe cells with fMLP, in conditions known to induce Hsp27 expression, would affect NF-κB activation upon subsequent challenge of cells with a known NF-κB inducer, the proinflammatory cytokine TNF-α. As assayed by EMSA using a consensus NF-κB binding motif as the radiolabeled probe, pretreatment of Caco2bbe cells with fMLP decreased the TNF-α-induced nuclear NF-κB DNA-binding activity (Fig. 6). Therefore, physiological concentrations of fMLP may decrease the ability of subsequent stimuli to activate NF-κB, a pivotal transcription factor for the inflammatory response.

Fig. 6. Pretreatment of Caco2bbe cells with fMLP attenuates induction of NF-κB by TNF-α. Confluent Caco2bbe cells were incubated in the presence of 400 nM fMLP or the vehicle (acetic acid), for 3 h. After this, the cells were treated with 10 ng/ml TNF-α, or with the vehicle (water), for 1 h longer. Nuclear extracts were then prepared and subjected to EMSA analysis using a consensus NF-κB binding element as a radiolabeled probe. The identity of the protein-DNA complex was confirmed by using an antibody raised against the NF-κB subunit p65 (lane 5). The image shown is representative of 5 independent experiments.
of microorganisms or bacterially derived substances that can cause inflammation and septic shock. However, the enteric flora also have many trophic and protective effects that protect the mucosa against pathogens and other injurious agents (13, 17, 18, 22). For instance, bacterial-derived factors like fMLP, staphylococcal enterotoxin B, AMP, flagellin, and LPS modulate intestinal epithelial function and behavior under normal and pathophysiological circumstances, contributing to maintenance or restoration of mucosal homeostasis (3, 12, 24).

In the present study, we demonstrate that physiological concentrations of fMLP, a bacterially derived product present in the normal colon, induce Hsp27 expression in Caco2bbe cells. Caco2bbe cells treated with fMLP are protected against oxidant-induced depolymerization of actin and decreases in TER. The protective role of fMLP on both the actin skeleton and TER were absent in cells where Hsp27 expression was silenced. The response to fMLP is specific as equivalent concentrations of related tripeptides MLP fail to induce Hsp27 expression. The response appears to involve the G1-coupled formyl peptide receptor, as fMLP-induction of Hsp27 is largely blocked by pretreatment of cells with the formyl peptide receptor antagonist BOC-FLFLF. Hsp27 induction by fMLP involves the p38 MAP kinase pathway; however, fMLP also stimulates ERK1/2 in Caco2bbe cells. Although expression of PepT1, the cellular uptake system for fMLP, is induced in chronically inflamed colon, it remains unclear whether this contributes to inflammation or whether it is an unique innate host adaptive mechanism, possibly for detection of and response to the presence of bacteria in the lumen. Previous efforts to characterize colonic effects of fMLP have implied that fMLP induces inflammation instead of cytoprotection; however, these studies often used supraphysi-

Fig. 7. fMLP pretreatment suppresses TNF-α-stimulated TNF-α and IL-8 expression as well as IL-8 release in Caco2bbe cells. Confluent Caco2bbe-cells were pretreated with 400 mM fMLP (columns 2 and 3) or the vehicle acetic acid (columns 1 and 4) for 3 h, before adding 10 ng/ml TNF-α (columns 3 and 4) or the vehicle water (columns 1 and 2), and the incubation continued for 1 further hour. Con, vehicle-treated control cells. The effects of the treatments on the mRNA expression of the TNF-α (A) and IL-8 (B) genes were measured by real-time PCR. The relative mRNA expression levels were calculated by comparative threshold cycle method, using eukaryotic 18S rRNA for sample normalization. C, pretreatment of Caco2bbe cells with fMLP decreases TNF-α-stimulated IL-8 release. Cells were treated similarly as A and B. Media were harvested 24 h after the treatments for determination of the IL-8 levels. Data shown are means ± SD and are representative of 3 independent experiments.

Fig. 8. Pretreatment with fMLP suppresses TNF-α-stimulated TNF-α expression in human intestinal biopsies. Ileal and cecal biopsies from 3 healthy patients were treated in short-term tissue culture, similarly to Caco2bbe cells (Fig. 7). The relative mRNA expression levels were calculated by comparative threshold cycle method, using villin mRNA expression for sample normalization. Relative mRNA levels obtained for the TNF-α-treated biopsies are set to 100%. The data shown represents mean values of the 3 patients ± SD. The average TNF-α threshold cycles for the control biopsies treated with vehicles only were 32.57 ± 2.01 in ileum and 36.40 ± 2.56 in cecum.
ological concentrations of fMLP. Notably in the present studies, supraphysiological fMLP concentrations failed to induce Hsp27 in Caco2bbe cells. It is possible that, when subjected to supraphysiological concentrations of fMLP, colonic epithelial cells in situ also do not undergo a Hsp response, rendering them most susceptible to injury by the inflammatory response.

Much is known about HSR and its cross-communication with the chief transcriptional mediator of the inflammatory pathways, NF-κB. HSR has been suggested to decrease activation of NF-κB via several mechanisms: 1) heat shock activates the promoter of the human gene encoding the inhibitor of NF-κB, IκBo (14, 45, 51, 53, 54); 2) heat shock inhibits the phosphorylation and degradation of IκBo (2, 45, 50, 54); 3) HSR increases IκBo-phosphatase activity in the cell (21, 23); and 4) heat shock stabilizes the IκBo mRNA and increases its half-life. The association between HSR and the NF-κB pathway has been more extensively reviewed elsewhere (32). The present studies using silencing RNA demonstrate a specific role for Hsp27 in protecting the actin cytoskeleton from NH2Cl-induced injury and Hsp27 can also contribute to the modulation of NF-κB. Both Hsp27 and Hsp72 have been specifically implicated in the regulation of NF-κB. Hsp72 has been found to coimmunoprecipitate with the NF-κB subunit p65 or a different antibody to IκBo, suggesting that Hsp72 forms a complex with NF-κB/IκKB (9). Binding to this complex, Hsp72 inhibits nuclear translocation (9). Similarly, Hsp27 has been demonstrated to associate with the IKK kinase complex following stimulation by TNF-α (41). The association of Hsp27 with the IKK-β decreases IKK kinase activity, thus acting to inhibit NF-κB activation. Additionally, in the intestine, as in other tissues, heat shock induces other protective proteins such as heme-oxygenase 1 (Hsp32) and manganese superoxide dismutase, which can augment the protective effects of heat shock (20, 49).

The present experiments demonstrate that physiological levels of bacterially derived fMLP produce an Hsp27 response that is capable of protecting the epithelium against oxidative damage. We additionally investigated whether treatment of Caco2bbe cells with fMLP, at a physiological concentration shown to induce Hsp27 expression, affects the degree of NF-κB activation by its known inducer, the proinflammatory cytokine TNF-α. Indeed, preincubation with fMLP attenuated NF-κB signaling in Caco2bbe cells, as measured by both NF-κB DNA-binding activity and induction of NF-κB target genes. Importantly, the fMLP-mediated suppression of NF-κB target gene activation by TNF-α could be reproduced in ileal and cecal biopsies derived from healthy human subjects. Interestingly, it has been recently suggested that Hsp27 associates with the IκB kinase complex, hence resulting in reduced NF-κB activation by TNF-α (41). Thus we speculate that induction of Hsp27 expression by fMLP is a potential mechanism by which fMLP treatment of Caco2bbe cells negatively interferes with NF-κB activation. The effects of fMLP on TNF-α-stimulated NF-κB signaling are particularly interesting, given the recent demonstration that NF-κB plays an essential role in TNF-α-induced increase in tight junction permeability in intestinal epithelial cells (30).

Taken together, the experiments presented here demonstrate that physiological levels of bacterially derived peptide fMLP elicit a Hsp27 response that is capable of protecting the epithelial cells of the colon against oxidative damage. Furthermore, fMLP may suppress inflammatory pathways in the epithelial cells by attenuating NF-κB signaling. Therefore, although the exact mechanisms responsible for the partial success of probiotic therapies in treatment of chronically inflamed colonic cells have yet to be fully explained, the induction of cytoprotective heat shock and anti-inflammatory responses by the bacterial peptide fMLP may contribute to this process.

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