Escherichia coli LPS induces heat shock protein 25 in intestinal epithelial cells through MAP kinase activation

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Kojima, Keishi, Mark W. Musch, Mark J. Ropeleski, David L. Boone, Averil Ma, and Eugene B. Chang. Escherichia coli LPS induces heat shock protein 25 in intestinal epithelial cells through MAP kinase activation. Am J Physiol Gastrointest Liver Physiol 286: G645–G652, 2004. First published November 20, 2003; 10.1152/ajpgi.00080.2003. —Protection of colonic epithelial integrity and function is critical, because compromises in mucosal functions can lead to adverse and potentially life-threatening effects. The gut flora may contribute to this protection, in part, through the sustained induction of cytoprotective heat shock proteins (HSPs) in surface colonicocytes. In this study, we investigated whether Escherichia coli LPS mediates bacteria-induced HSP by using cultured young adult mouse colon (YAMC) cells, an in vitro model of the colonic epithelium. E. coli LPS led to an epithelial cell-type specific induction of HSP25 in a time- and concentration-dependent manner, an effect that did not involve changes in HSP72. YAMC cells expressed the toll-like receptors (TLR)2 and TLR4 but not the costimulatory CD14 molecule. Whereas LPS stimulated both the p38 and ERK1/2 but not the stress-activated protein kinase/c-Jun NH2-terminal kinase, signaling pathways in the YAMC cells, all three were stimulated in RAW macrophage cells (in which no LPS-induced HSP25 expression was observed). The p38 inhibitor SB-203580 and the MAP kinase inhibitor PD-98059 inhibited HSP25 induction by LPS. LPS treatment also conferred protection against actin depolymerization induced by the oxidant monochloramine. The HSP25 dependence of the LPS protective effect was outlined in inhibitor studies and through adenovirus-mediated overexpression of HSP25. In conclusion, LPS may be an important mediator of enteric bacteria-induced expression of intestinal epithelial HSP25, an effect that may contribute to filamentous actin stabilization under physiological as well as pathophysiologic conditions and thus protection of colonic epithelial integrity.

actin; barrier function; cytoprotection; stress kinases; toll-like receptors

THE EPITHELIAL LINING OF THE intestine is a complex and dynamic tissue that is responsible for maintaining the barrier and absorptive functions of the gastrointestinal tract. In addition, the epithelium plays an important role in host defense. In areas such as the colon, maintenance of these functions is critical, because breaches in the mucosal barrier can result in systemic seeding of bacteria and/or bacterial-derived substances that have adverse and potentially life-threatening effects. Yet, despite the inherent harshness of the luminal environment of the colon, there are many proposed beneficial effects of gut flora that are both trophic and protective against potential enteric pathogens (21, 22, 24, 25). Among their many functions, luminal bacteria have been implicated in the regulation of mucosal growth, development and maturation (22), apoptosis (47), and cytokine secretion (17, 40) through either direct or indirect interactions with epithelial, gut immune, and other mucosal mesenchymal cells. Colonic flora also provide the epithelium with utilizable metabolic substrates, particularly short-chain fatty acids (SCFAs) that are readily absorbed and metabolized by the colonic epithelial cells (43). Among their putative actions, SCFAs sustain the physiological expression of colonic epithelial cytoprotective heat shock proteins (HSPs), particularly HSP25 (41), which may render surface lining cells more resistant to potential injurious effectors within the colonic lumen. When SCFA bioavailability is compromised by colonic diversion, colitis ensues (19). In addition, this resultant inflammation may also be a consequence of reduced mucosal levels of other enteric flora-derived factors needed to sustain mucosal health. Alterations in enteric flora associated with antibiotic treatment and Clostridium difficile colitis represent yet other examples of the physiological significance of normal commensal flora (23). Whereas this condition has been attributed to the cytotoxic and pathogenic toxins made by C. difficile, it is also possible that the increased susceptibility of gut mucosa to injury results from diminished trophic effects of commensal flora.

Several bacteria-derived factors have recently been identified that can modulate the function and behavior of the intestinal epithelial cells under normal and pathophysiologic circumstances. These include pathogenic toxins such as Staphylococcal enterotoxin B, adenosine-5′-monophosphate, chemotactic peptides such as N-formyl-methionyl-leucyl-phenylalanine, flagellin, and LPS (2, 15, 34, 35). The latter is a glycolipid derived from the outer membrane of gram-negative bacteria that is capable of eliciting a robust proinflammatory response in a wide variety of mammalian cells, including those of the intestine (1, 6, 8, 11). Innate immune cells are particularly reactive to LPS and express a variety of inflammatory cytokines in response to this agent. Accordingly, the free entry of LPS into the systemic circulation would be catastrophic and could result in a generalized proinflammatory state with resultant physiological shock, thus underscoring the importance of endogenous mechanisms responsible for the maintenance of normal barrier and defense functions.

The intestinal epithelium utilizes a number of defense mechanisms to maintain its functions and to coexist with normal bacterial flora. These include secretion of mucins, IgA immunoglobulins, and defense peptides (3, 33, 39). The production of stress proteins, particularly cytoprotective HSPs, may also...
represent one of these protective measures (28, 38, 42, 46). HSPs comprise a large, highly conserved family of proteins that demonstrate important molecular chaperone activities (20, 37). More recent reports have implicated HSPs in the regulation of proinflammatory signaling cascades including regulation of NF-κB and stress-activated protein kinase/c-Jun NH₂-terminal kinase (SAPK/JNK) (5, 14). Some HSPs also have anti-apoptotic activity (7). Some are constitutively expressed with little physiological variation in mammalian cells [e.g., heat shock cognate (HSC73)], whereas others are induced under conditions of cell stress (e.g., HSP72). Inducible HSPs, however, can also be variably expressed under basal physiological conditions by specific cells and tissues of the body, including the lung, heart, and intestine (27, 41, 46). In the gastrointestinal tract, the colon, but not the small intestine, exhibits physiological expression of HSP25 and HSP72. This expression appears predominantly confined to the surface epithelial cells that are in closest proximity to the luminal contents (27, 41). This observation raises the possibility that colonic bacteria and/or bacterial-derived factors may provide important physiological cues and play an important role in the persistent induction of colonic epithelial HSP expression. Conversely, the absence of substantial bacterial flora in the small bowel would account for the minimal expression of inducible HSPs in the intestinal epithelium.

In this study, we explore the hypothesis that Escherichia coli LPS, which exists in high concentrations in the colonic lumen, is a determinant of the physiological expression of inducible HSPs characteristic of the colonic epithelium. Using an in vitro model of the colonic epithelium, we report that LPS stimulates the specific expression of the small-molecular-weight HSP25, an effect associated with stabilization of cytoskeletal filamentosous (F)-actin during oxidant stress. In addition, we have examined potential cellular signaling pathways mediating this effect. These include members of the MAPK family, namely p38, JNK, and ERK1/2 (for reviews see Refs. 9 and 32). Although not reported in intestinal epithelial cells, MAPKs mediate some of the activating effects of LPS in innate immune cells (16, 18, 31). In this study, we report that p38 and ERK1/2 but not JNK mediate LPS-induced HSP25 expression in the cultured murine colonic intestinal epithelial cell line young adult mouse colon (YAMC). This effect is cell specific, because MAPK activation by LPS in the murine macrophage cell line RAW264.7 is not associated with the induction of HSP25.

MATERIALS AND METHODS

Cell culture. The YAMC cell line was used as well as its small intestinal counterpart, mouse small intestinal epithelial (MSIE) cell. These cell lines are derived from the Immortomouse that express a temperature-sensitive SV40 large T antigen transgene under control of the IFN-γ-sensitive segment of the class MHC II promoter (45). The cells were a generous gift of Dr. R. Whitehead (Vanderbilt University, Nashville, TN). YAMC and MSIE cells were grown at the permissive temperature of 33°C in RPMI 1640 medium with 5% (vol/vol) FBS, 5 U/ml murine IFN-γ, 2 mM glutamine, 50 µg/ml streptomycin, and 50 U/ml penicillin (FBS, penicillin/streptomycin, and glutamine were all from Invitrogen, Grand Island, NY) and also supplemented with 6.25 mg/l insulin, 6.25 mg/l transferrin, 6.25 µg/l selenious acid, 1.25 g/l BSA, and 5.35 mg/l linoleic acid (ITS+ Premix; Collaborative Biomedical Products, Bedford, MA). Stock cultures were maintained at 33°C and were split at a ratio of 1:6 every 4 days. For experiments, cells were split at the same ratio and plated onto 35-mm tissue culture dishes. At the 1:6 ratio used to split cells, cells became confluent in ~3 days. Before all experiments, cells were cultured in IFN-γ-free media under nonpermissive conditions at 37°C for 24 h to destabilize the temperature-dependent SV40 large T antigen and to cease cell proliferation.

The mouse macrophage cell line, RAW264.7 (cat. no. TIB-71; American Type Culture Collection, Manassas, VA) was grown in DMEM supplemented with 4 mM l-glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, and 10% (vol/vol) FBS.

To specifically induce HSP25, an inducible adenoviral system was used. The rat full-length HSP25 cDNA was subcloned into the tetracycline (Tet)-Off adenovirus (BD Clontech, Palo Alto, CA), in which the production of HSP25 is regulated by a promoter containing seven copies of the tetracycline regulation element and a short section of the cytomegaloviral (CMV) promoter min-CMV. The HSP25 adenovirus was packaged by using the HEK-293 cell line supplied by the manufacturer, which is stably transfected so that the replication-defective Tet-Off adenovirus used (an adenovirus 5 derivative) is correctly packaged. Packaged HSP25 adenovirus, along with adenovirus expressing the tetracycline operator protein (under control of the constitutively strong CMV promoter), were coinfected and cultured for 2 days. Tetracycline (1 µg/ml) was added to the tissue culture medium. After 2 days, cells were fed with YAMC medium with or without tetracycline and allowed to sit for 2 days. At this point, cells were stimulated with the oxidant monochloramine (NH₂ Cl) where indicated and harvested for actin polymerization studies as described below.

HSP expression by Western blot analysis. Cells were treated with LPS (from E. coli serotype 055:B5; Sigma, St. Louis, MO) in IFN-γ-free RPMI 1640 medium under nonpermissive conditions. The concentrations and incubation times with LPS are denoted with each set of experiments. Heat shock was performed by taking an untreated dish of cells, wrapping it in paraffin, and immersing it in a 42°C water bath for 25 min. Cells were placed back in the 37°C incubators for 2 h for analysis of protein (by Western blots) or mRNA production (by Northern blots). For Western blot analysis, cells were harvested by scraping in ice-cold PBS (composition in mM: 137 NaCl, 2.7 KCl, 4.3 NaH₂PO₄, and 1.4 K₂HPO₄, pH 7.4) and centrifuged (14,000 g for 10 s at room temperature). Cell pellets were resuspended in lysis buffer (10 mM Tris pH 7.4, 5 mM MgCl₂, 50 U/ml DNase, and RNase, containing a complete protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN)). An aliquot was removed for protein determination by the bicinchoninic acid procedure (44), and to the remainder ¼ volume 3X Laemmli stop solution [composition 3% (wt/vol) SDS, 32.5 mM Tris, pH 6.8, 50% (vol/vol) glycerol, 10% (vol/vol) 2-mercaptoethanol, and 10 mg/ml bromophenol blue] was added. Ten micrograms of protein per sample were separated by SDS-PAGE [12.5% (wt/vol) polyacrylamide gels were used in all cases] and immediately transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 5% (wt/vol) Carnation nonfat milk (Nestle, Solon, OH) in Tris-buffered saline with 0.05% (vol/vol) Tween-20 (TTBS, composition in mM: 10 Tris, pH 7.4, 140 NaCl, and 5 KCl) at room temperature for 1 h and then incubated overnight at 4°C with a specific anti-HSP25 antibody (SPA 801; Stressgen, Victoria, BC, Canada), anti-HSP72 antibody (SPA 810; Stressgen) or anti-HSC73 antibody (SPA 815; Stressgen). Blots were washed five times for 10 min each in TTBS and then incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. After five 10-min washes in TTBS and a single wash in TBS, blots were developed by using an enhanced chemiluminescence system (Super-signal; Pierce, Rockford, IL).

Images were quantitated by scanning densitometry using NIH Image 1.54 software. Within each experiment, the control value at time 0 was set to 100%, to which all samples were calculated as a
percentage. The values from all appropriate experiments were then used to calculate a mean and standard errors.

Northern blot analysis of HSP25 mRNA. Total cellular RNA was extracted from LPS or heat-shocked cells by using the TRizol reagent (Invitrogen, Gaithersburg, MD) according to the manufacturer’s instructions. Northern blot analysis was performed as described previously (4, 42). Denatured total RNA (20 μg/lane) was separated through a 1% agarose-formaldehyde gel (1% w/vol; SeaKem LE agarose; FMC, Rockland, ME) in a buffer containing 6% w/vol formaldehyde, 10 mM MOPS, 5 mM Na acetate, and 1 mM EDTA, pH 7.0 (all from Fisher Chemical, Pittsburgh, PA) and transferred to a nylon membrane (Hybond-N; Amersham-Pharamacia, Piscataway, NJ). After prehybridization, the membranes were hybridized to [α-32P]dCTP (Perkin Elmer New England Nuclear Life Sciences, Boston, MA) random prime-labeled (DECAprime II; Ambion, Austin, TX) cDNA probes for murine HSP25 and GAPDH at 42°C overnight in XOTCH solution (7% w/vol SDS, 1% w/vol BSA, 200 mM NaCl, 20 mM EDTA, 15% vol/vol deionized formamide). Blots were washed twice with 2× SSC/0.1% SDS at 42°C for 10 min followed by one wash in 0.5× SSC/0.1% SDS at 55°C for 15 min and one wash in 0.1× SSC/0.1% SDS at 65°C for 15 min. Blots were exposed to film overnight at −80°C.

Detection of toll-like receptor 2, toll-like receptor 4, and CD14 mRNA expression by RT-PCR. Total cellular RNA was extracted by using the TRizol reagent (Invitrogen), and contaminating DNA was removed by treatment with RQ1 RNase-free DNase (Promega, Madison, WI). RT-PCR was performed by using the Access RT-PCR system kit (Promega) following the manufacturer’s instructions. The PCR primers, amplicon sizes, and reaction conditions for mouse toll-like receptor (TLR)2 (349 bp), TLR4 (540 bp), and CD14 (1,326 bp) have previously been described (30, 36). PCR products were resolved on 1.2% agarose gels stained with ethidium bromide.

MAPK pathways. Three MAPK pathways were analyzed: p38, JNK, and ERK1/2 (also called p44/42 MAP kinases). To determine activation, protein samples were harvested at varying times after LPS treatment. Anisomycin (10 μg/ml for 30 min) was used as a positive control for p38 and JNK activation, and PMA (0.1 μg/ml for 30 min) for ERK1/2 activation. Western blot analysis was performed by using Phospho Plus kits for p38, JNK, and p44/42 MAP kinases (Cell Signaling, Beverly, MA). Phosphorylated MAPK isoforms, indicators of pathway activation, were detected by using phosphospecific antibodies. Total amounts of each MAPK (using antibodies that recognize both phosphorylated and nonphosphorylated forms) were also examined to control for differences in protein loading. Where indicated, cells were preincubated with inhibitors of specific MAPK pathways including the p38 inhibitor SB-203580 (20 μM/0.1% DMSO; Alexis, San Diego, CA), the JNK inhibitor curcumin (20 μM/0.1% ethanol; Alexis) and the MAP kinase kinase-1 (MEK)-1 inhibitor PD-98059 (50 μM/0.1% DMSO; Alexis) for 2 h before LPS.

Globular and F-actin assay. Cells were grown to confluence, switched to 37°C with IFN-γ omitted from the media, and treated with LPS (10 μg/ml for 24 h) where indicated. Immediately before the assay, some cells were treated with phalloidin (30 μg/ml) for 2 h, cytochalasin D (10 μg/ml) for 15 min, or the oxidant NHCl2 (0.6 mM) from a freshly prepared stock as previously described (38) for 30 min. Cells were washed on the petri dish once with room-temperature PBS, scraped into PBS, and pelleted (14,000 g for 20 s at room temperature). Cell pellets were resuspended in 200 μl of 30°C lysis buffer (in mM: 1 ATP, 50 PIPEs, pH 6.9, 50 NaCl, 5 MgCl2, and 5 mM EGTA, with 5% vol/vol glycerol, 0.1% vol/vol Nonidet P-40, Tween 20, and Triton X-100, and the complete protease inhibitor cocktail). Cells were homogenized by gently pipetting up and down 10 times and incubated at 30°C for 10 min. Samples were spun at 100,000 g for 60 min at 30°C and the supernatants were removed for determination of globular (G)-actin. Pellets containing F-actin were resuspended in 200 μl of 4°C water with 1 μM cytochalasin D and left on ice for 60 min. Twenty microliters of each fraction was removed, to which 6 μl of 3× Laemmlı stop solution were added followed by heating to 65°C for 10 min. Samples were resolved by 12.5% SDS-PAGE and immediately transferred to PVDF membranes. After transfer, analysis of G-actin was performed by using a polyclonal anti-actin antiserum (Cytoskelton, Denver, CO). Washes and signal detection were carried out as described above.

RESULTS

LPS induces HSP25, but not HSP72 and HSC73, in YAMC cells. To determine whether LPS induces HSPs, YAMC cells were treated with 5 μg/ml LPS for varying times. LPS increased HSP25 expression as early as 12 h, which later peaked at 24 h and declined thereafter (Fig. 1A). By contrast, no changes in the constitutively expressed HSC73 and inducible HSP72 were observed. Changes in HSP25 were quantitated by using scanning densitometry and are presented in Fig. 1A, bottom. Within each experiment, the time 0 control was set to 100%, to which all LPS-treated values were calculated as a percentage. Means and standard errors were then calculated from the percentages from all separate experiments. Similarly, densitometric analysis was also performed for the concentration dependence in Fig. 1B and the Northern blot data of Fig. 1D. To determine the concentration dependence of this effect, cells were harvested after 24-h treatment with varying concentrations of LPS. As shown in Fig. 1B, LPS stimulated a concentration-dependent HSP25 increase, occurring at concentrations as low as 10 ng/ml with a maximal effect at 5 μg/ml. Even at the highest concentration used here, this is likely below the concentration of LPS in colonic fluid. A sample of proteins from heat-shocked cells is presented for the Western blots in Fig. 1, A and B. To determine that the switch from 33 to 37°C did not alter the heat responsiveness or represent a heat stress by itself, the heat-shock response was determined at both temperatures. For both sets of cells, IFN-γ was omitted from the medium for 1 day before heat shock. The omission of IFN-γ from the medium and the temperature shift from 33 to 37°C had little effect on either the basal unstimulated expression of HSP25 and HSP72 or their ability to respond to heat (Fig. 1C).

The effect of LPS on HSP25 mRNA was subsequently examined by Northern blot analysis. Total RNA was extracted from YAMC cells at various times after incubation with LPS (5 μg/ml). As shown in Fig. 1D, HSP25 mRNA expression increased as early as 6 h after LPS exposure and declined by 24 h. It is notable that the magnitude of LPS-induced HSP25 mRNA and protein expression was much less that induced by thermal stress (42°C for 23 min).

LPS induction of HSP25 occurs in intestinal epithelial cells but not in a macrophage cell line. To determine whether LPS induction of HSP25 is specific for cultured, conditionally immortalized YAMC colonic intestinal epithelial cells, conditionally immortalized MSIE small intestinal cells as well as the murine macrophage cell line RAW264.7 were examined. As shown in Fig. 2, LPS induced HSP25 expression in the MSIE cells, suggesting that the ability to respond is not limited to colonic epithelial cells. In contrast, LPS failed to induce HSP25 in nonepithelial, macrophage/macrophage-like RAW264.7 cells. To determine whether these differences could be explained by cell-specific differences in LPS receptor expression, CD14, TLR2, and TLR4 mRNA levels were assessed by RT-PCR. No PCR product for CD14 was detected in either MSIE or YAMC.
cells, whereas CD14 was expressed in RAW264.7 cells. By
contrast, both TLR4 and TLR2 mRNA were detected in all
three cell lines (Fig. 3).

LPS induces specific MAPK activation in YAMC cells. To
evaluate the MAPKs in the LPS induction of HSP25, the state
of activation of three MAPK pathways was examined. Each
MAPK is activated via phosphorylation by specific “upstream”
kineses. Therefore, activation is assessed by the appearance
of phosphorylated forms of these MAPKs using antibodies spe-
cific to these phosphorylated, activated forms. LPS (10 ng/ml)
stimulated all three MAPK pathways in RAW264.7 cells (Fig.
4A), although the time course for activation of these various
pathways differed. In contrast, LPS (5 μg/ml) activated the p38
and ERK1/2 pathways in YAMC cells but not the SAPK/JNK
pathway (Fig. 4B). To determine that the same amount of
protein was loaded per sample and to assess whether the
expression of the MAPK was altered, total MAPKs were
quantified by antibodies that recognize both phosphorylated
and nonphosphorylated forms. In all cases, no changes in the
total levels of any of the MAPK pathways were observed in
RAW or YAMC cells (Fig. 4).

p38 and ERK1/2 contribute to LPS-induced HSP25 expres-
sion in YAMC cells. To further assess the role of MAPK
activation in LPS induction of HSP25, selective inhibitors of
MAPK pathways were used, including SB-203580 (an inhibi-
tor of p38 MAPK), PD-98059 (an inhibitor of MEK-1 required
for the activation of ERK1/2), and curcumin (an inhibitor of
SAPK/JNK). Curcumin had no effect on LPS-induced expres-
sion of HSP25 in YAMC cells. This effect was in accordance
with the lack of LPS-induced phosphorylation of SAPK/JNK
detected by Western blot analysis. In contrast, both SB-203580

Fig. 1. LPS induces heat shock protein (HSP)25 expression in young adult mouse colon (YAMC) cells. For A, B, and D, densitometric analyses are shown below the HSP25 Western blots. In each experiment, values for the untreated cells at time 0
was set to 100%, to which all values were calculated within each experiment. Means and standard errors were determined for 4 separate
experiments in A and B and 3 for D. A: time course of LPS induction of YAMC HSPs. YAMC cells were treated with 5 μg/ml LPS for varying times or left untreated as controls (Con) and, along with paired control samples, proteins were harvested and analyzed for HSP production at 0, 6, 12, 24, and 48 h by Western blot analysis. Heat shocked (HS) cells (42°C for 23 min followed
by a 2-h recovery at 37°C) were used as positive controls. Images shown are representative of those of 4 separate experiments. B: concentration dependence of LPS induction of HSPs. YAMC cells were treated with varying concentrations of LPS for 24 h or heat
shocked, and proteins were harvested and analyzed for HSP production by Western blotting. Images shown are representative of four separate experiments. C: heat shock response at 33 and 37°C. YAMC cells were maintained at 33°C or switched to 37°C (in IFN-γ-deficient medium), and cells were heat shocked at 42°C for 23 min or left in their respective incubators. Heat-shocked cells
were returned to their appropriate incubators and allowed to recover for 2 h, at which time both control and heat-shocked cells were
harvested. The image shown is representative of 3 separate experiments. D: LPS induced HSP25 mRNA in YAMC cells. YAMC
cells were treated with 5 μg/ml LPS for varying times. HSP25 mRNA was measured by Northern blot analysis as described in
MATERIALS AND METHODS. Images shown are representative of those of 3 separate experiments. Hsc, heat shock cognate.

Fig. 2. LPS induces HSP25 in intestinal epithelial cells but not macrophages. All cell lines were treated with 5 μg/ml LPS for 24 h, and proteins were
harvested and analyzed for HSP production by Western blot analysis. Images shown are representative of 3 separate experiments. MSIE, mouse small
intestinal epithelial cells.
CD14, TLR2, and TLR4 should be 1326 bp, 349 bp, and 540 bp, respectively. Images shown are representative of those of 3 separate experiments.

A sample from YAMC cell RNA in the absence of avian myeloblastosis virus reverse transcriptase was used as a negative control (–). Expected PCR products for transcriptase was used as a negative control (–). Expected PCR products for CD14, TLR2, and TLR4 should be 1326 bp, 349 bp, and 540 bp, respectively. Images shown are representative of those of 3 separate experiments.

and PD-98059 inhibited LPS induction of HSP25 protein (Fig. 5, A and B) and mRNA expression (Fig. 5C), and also affected basal expression of HSP25 protein and mRNA (Figs. 5, B and C). Controls for the DMSO and ethanol demonstrated no induction of HSPs at the amounts used (Fig. 5, A and B). MAPK inhibitors had no effect on the constitutive expression of the HSC73. As previously described, the changes in HSP25 were quantitated by using scanning densitometry. As before, within each experiment, the time 0 control was set to 100%, to which all treated values were calculated as a percentage. Means and standard errors were then calculated from the percentages from all separate experiments.

G/F-actin distribution. Because HSP25 has been described as an actin-binding and F-actin-stabilizing protein in vivo, we determined whether LPS induction of HSP25 is associated with protection of F-actin during subsequent exposure to the oxidant NH2Cl. Previous studies have shown that NH2Cl rapidly dissociates F-actin (38). Cells treated with LPS were subjected to treatment with vehicle or NH2Cl. By itself, LPS had little effect on the G/F-actin distribution in YAMC cells (Fig. 6A). Phallolidin, which binds and stabilizes the barbed ends of F-actin filaments, increased the amount of F-actin, whereas cytochalasin D, an F-actin-disrupting agent, greatly increased the amount of G-actin (Fig. 6A). NH2Cl (0.6 mM) stimulated a disruption of F-actin filaments as demonstrated by a decrease in the F-actin and increase in the G-actin (Fig. 6A). YAMC cells pretreated with LPS and then treated with NH2Cl demonstrated significantly less change in the G/F-actin distribution, indicating a protective effect possibly mediated by induction of HSP25.

To determine the importance of HSP25 in the LPS-induced protection of actin depolymerization after NH2Cl, two sets of experiments were performed. First, the pharmacological inhibitors of the stress kinases, PD-98059 and SB-203580, both of which block the LPS induction of HSP25, were tested for their ability to inhibit the LPS-induced protection against actin depolymerization. In addition, an inducible adenoviral system was used to increase cellular HSP25 independent of other treatments. Inhibition of either the p38, ERK1/2, or both kinase pathways prevents the ability of LPS to protect against actin depolymerization caused by NH2Cl (Fig. 6B). The requirement for HSP25 was specifically demonstrated by using the adenoviral system. The viral system used is the Tet-Off approach, such that only when tetracycline is removed, HSP25 expression increases. As demonstrated in Fig. 6C, adenoviral infection by itself (with both the HSP25-Tet-Off and the Tet operator virus) does not alter the basal distribution of G/F-actin. When tetracycline is maintained, NH2Cl causes a depolymerization of actin. When tetracycline is removed and HSP25 is induced (demonstrated in Western blots not shown), NH2Cl stimulates actin depolymerization to a much lesser degree, demonstrating a protective role of HSP25.

DISCUSSION

Our studies propose an important role of certain bacterial LPS in stimulating and maintaining the physiological expression of HSP25 in intestinal epithelial cells, particularly in the colon in which luminal LPS concentration is high. We speculate that the sustained and specific expression of HSP25 in surface colonocytes is important in protecting and preserving critical functions of the mucosa under physiological as well as
pathophysiological states. Notably, HSP25 is a putative actin-associated protein (13, 29) that binds and stabilizes the F-actin cytoskeleton during periods of stress. Its ability to stabilize the F-actin associated protein (13, 29) that binds and stabilizes the F-actin pathophysiological states. Notably, HSP25 is a putative actin-associated protein (13, 29) that binds and stabilizes the F-actin cytoskeleton during periods of stress. Its ability to stabilize the F-actin associated protein (13, 29) that binds and stabilizes the F-actin

The present data would also suggest that LPS induction of intestinal epithelial HSP25 may be mediated by TLR4. TLRs act as transmembrane receptors for a number of bacterial components such as lipoteichoic acid (lipid A), peptidoglycans, flagellin, and single-stranded DNA (10, 12, 26). Of the TLRs that may bind E. coli LPS and be potentially involved in the present studies, TLR2 and TLR4 are the best candidates. TLR2 and TLR4 (but not the costimulatory membrane protein CD14) are both expressed in the YAMC and MSIE intestinal epithelial cells, similar to previous reports (6) in other cultured and primary intestinal epithelial cells. It is notable that intestinal epithelial cells, both in vitro and in vivo, express little CD14 (6), a finding that may impart a relatively desensitized state to LPS. Teleologically, this may be important in reducing inappropriate and potentially catastrophic activation of TLR-mediated pathways in intestinal epithelial cells that are continuously exposed to high concentrations of LPS in the colonic fluid.

Taken together, our data suggest that LPS may maintain the physiological expression of intestinal epithelial HSP25 in vivo, an effect that appears to be associated with increased F-actin stability during oxidant-induced stress. This effect may be, in part, mediated by LPS-induced increases in HSP25 mRNA, which may either result from mRNA stabilization or increased gene transcription. In the latter case, the role of HSF-1 appears to be minimal and the possibility of other promoter elements of the HSP25 gene in this regard must be considered. Our data also show that ERK1/2 and p38 MAPKs mediate LPS-induced expression of HSP25. These effects are cell-type specific because LPS does not induce HSP25 in the RAW macrophage cell line despite

Fig. 5. Inhibition of p38 or ERK1/2 kinases blocks LPS-induced HSP25 expression in YAMC cells. A: YAMC cells were treated with SB-203580 (SB) (20 μM/0.1% DMSO), curcumin (20 μM/0.1% ethanol), and PD-98059 (PD) (50 μM/0.1% DMSO) for 2 h and were followed by stimulation with 5 μg/ml LPS for 24 h. Proteins were harvested and analyzed for HSP production by Western blotting. DMSO (0.1%) or ethanol (0.1%) was used as vehicle control. Images shown are representative of those of 3 separate experiments. B: p38 and/or ERK1/2 kinase signaling pathways mediate not only LPS-induced HSP25 expression but also maintain basal HSP25 protein expression. Cells were pretreated with 20 μM SB-203580 or 50 μM PD-98059 for 2 h before LPS (5 μg/ml) for an additional 24 h. Proteins were harvested and analyzed for HSP production. In the absence of LPS, treatment with 20 μM SB-203580 or 50 μM PD-98059 led to reduced basal expression of HSP25. C: activation of p38 and/or ERK1/2 kinase pathways mediates not only LPS-induced HSP25 mRNA but also maintain basal HSP25 mRNA expression. Cells were pretreated with 20 μM SB-203580 or 50 μM PD-98059 for 2 h before LPS (5 μg/ml) for an additional 24 h. RNA was then extracted, and HSP25 mRNA was assessed by Northern blotting as described in MATERIALS AND METHODS. Images shown are representative of those of 3 separate experiments.

Fig. 6. LPS prevents oxidant-induced actin depolymerization. YAMC cells were treated with LPS (10 μg/ml, 24 h) in A and B, phalloidin (phal) [a filamentous (F)-actin stabilizer as positive control (cont)] (10 μg/ml, 2 h) in A, cytochalasin D (cyto-D) [an F-actin destabilizer yielding globular (G)-actin] (1 μg/ml, 15 min) in A, or monochloramine (NH2Cl; 0.6 mM, 30 min) in A and B. Cells were processed for G- and F-actin as described in MATERIALS AND METHODS. Images shown are representative of 3 separate experiments. C: distribution of G/F-actin is shown for noninfected cells with and without NH2Cl. In all lanes, Adeno HSP25, cells were infected with the Tet-Off adenovirus containing the rat HSP25 under the control of the Tet operator. Tetracycline (Tet) was maintained in some cultures (+) whereas it was omitted from some cultures 2 days after infection (−). At this point, the G/F-actin distribution was determined in untreated cells (−NH2Cl) as well as in cells treated with NH2Cl (+NH2Cl; 0.6 mM, 30 min).
stimulating many stress kinases. In conclusion, commensal enteric flora may contribute to physiological maintenance of the actin cytoskeleton of the colonic mucosa, an effect mediated, in part, by LPS.

GRANTS

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