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Intracellular Heat Shock Protein-70 Negatively Regulates TLR4 Signaling in the Newborn Intestinal Epithelium

Amin Afrazi,*† Chhinder Sodhi,*† Misty Good,‡§ Hongpeng Jia,*† Richard Siggers,*† Ibrahim Yazji,*† Congrong Ma,*† Matthew D. Neal,*† Thomas Prindle,*† Zachary S. Grant,*† Maria F. Branca,*† John Ozolek,*†‖ Eugene B. Chang,# and David J. Hackam*†

Necrotizing enterocolitis (NEC) is the leading cause of gastrointestinal-related mortality in premature infants, and it develops under conditions of exaggerated TLR4 signaling in the newborn intestinal epithelium. Because NEC does not develop spontaneously, despite the presence of seemingly tonic stimulation of intestinal TLR4, we hypothesized that mechanisms must exist to constrain TLR4 signaling that become diminished during NEC pathogenesis and focused on the intracellular stress response protein and chaperone heat shock protein-70 (Hsp70). We demonstrate that the induction of intracellular Hsp70 in enterocytes dramatically reduced TLR4 signaling, as assessed by LPS-induced NF-κB translocation, cytokine expression, and apoptosis. These findings were confirmed in vivo, using mice that either globally lacked Hsp70 or overexpressed Hsp70 within the intestinal epithelium. TLR4 activation itself significantly increased Hsp70 expression in enterocytes, which provided a mechanism of auto-inhibition of TLR4 signaling in enterocytes. In seeking to define the mechanisms involved, intracellular Hsp70-mediated inhibition of TLR4 signaling required both its substrate-binding EEVD domain and association with the cochaperone CHIP, resulting in ubiquitination and proteasomal degradation of TLR4. The expression of Hsp70 in the intestinal epithelium was significantly decreased in murine and human NEC compared with healthy controls, suggesting that loss of Hsp70 protection from TLR4 could lead to NEC. In support of this, intestinal Hsp70 overexpression in mice and pharmacologic upregulation of Hsp70 reversed TLR4-induced cytokines and enterocyte apoptosis, as well as prevented and treated experimental NEC. Thus, a novel TLR4 regulatory pathway exists within the newborn gut involving Hsp70 that may be pharmacologically activated to limit NEC severity. The Journal of Immunology, 2012, 188: 4543–4557.

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Abbreviations used in this article: Hsp70, heat shock protein-70; iNOS, inducible NO synthase; NEC, necrotizing enterocolitis; siRNA, small interfering RNA.

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modulation of apoptosis after various forms of stress (9–12). Through its combined roles of both clearing proteins and modulating cell death, the net effect of Hsp70 induction within cells is to restore the host to a nonstressed environment (13–15). Although cytoplasmic Hsp70 has not been linked to the regulation of TLR4 signaling inside the enterocyte, Hsp70 was shown to serve a protective role in the intestine, as demonstrated by Chang and colleagues (16, 17), although upstream regulatory pathways within the gut were not identified. Taken together, these findings in the literature now raise the exciting possibility that intracellular Hsp70 could represent a novel regulator of TLR4 signaling at baseline and in the development of NEC.

In support of this hypothesis, using enterocytes that either lack or are induced to express Hsp70, as well as by examining mice that either lack Hsp70 or that overexpress Hsp70 within the intestinal epithelium, we now determine that intracellular Hsp70 limits TLR4 signaling in enterocytes and, moreover, that Hsp70 plays a central role in the pathogenesis of NEC. The mechanism by which Hsp70 limits TLR4 signaling in the gut involves an increase in CHIP-mediated ubiquitination and degradation of TLR4 via the ubiquitin–proteasomal pathway. Importantly, pharmacologic upregulation of Hsp70 within the intestinal mucosa led to a reduction in TLR4 signaling and a decrease in enterocyte apoptosis, leading to an attenuation in NEC severity. Taken together, these findings illustrate a novel pathway linking the regulation of Hsp70 with the negative control of TLR4 signaling within the gut and provide evidence that the development of NEC results, in part, from exaggerated TLR4-induced enterocyte apoptosis that is due, in part, to reduced Hsp70 activity. Moreover, these results suggest that pharmacologic upregulation of Hsp70 could provide a novel approach to the prevention and/or treatment of NEC through the inhibition of TLR4 signaling in the newborn intestine.

Materials and Methods

Cell culture and reagents

IEC-6–enterocytes were obtained from the American Type Culture Collection (Manassas, VA). LPS (Escherichia coli 0111:B4 purified by gel-filtration chromatography, >99% pure) was from Sigma-Aldrich. The TLR2 ligand Pam3 and the TLR5 ligand flagellin were from InvivoGen. Abs were as follows: p65 subunit of NF-κB (Santa Cruz Biotechnology); TLR4 (IMAGEGENEX, Santa Cruz Biotechnology [LI14]); cleaved-caspase 3 (Cell Signaling); the inducible isoform of Hsp70 (StressGen SPA-810 and Santa Cruz Biotechnology [inducible, K20]); the constitutive (control) isoform of Hsp70, i.e., Hs70 (StressGen SPA-815); Ubiquitin (Millipore); V5 (GenScript).

Where indicated, cells were pretreated with the proteasome inhibitor MG-132 (10 μM; Calbiochem) 2 h prior to the indicated experimental condition. IEC-6 enterocytes were treated with LPS at concentrations that we have shown to be present in mice and humans with NEC (i.e., 50 μg/ml) (3).

Preparation of lentiviruses and cell transfection

Lentiviruses expressing V5-tagged Hsp70–dominant–negative C-terminal deletion mutant (ΔEVEV), wild-type Hsp70, small interfering RNA (siRNA) to Hsp70, CHIP Hsp70-docking mutants (K30A), CHIP U-Box (H260Q) mutants, and LacZ were generated using a combination of ViralPower HiPerform Lentivarial and Lentiviral pLent6.3/V5-DEST Gateway expression system (Invitrogen). In brief, the recombinant V5-tagged pLent6.3/V5-DEST expression plasmids expressing wild-type and dominant-negative C-terminal deletion mutant (ΔEVEV) Hsp70, CHIP Hsp70-docking mutants (K30A), CHIP U-Box (H260Q) mutants, and LacZ were first generated using Gateway directional TOPO cloning systems (Invitrogen). V5-tagged pLent6.3/V5-DEST expression plasmids were end sequenced to verify the correct directional cloning. High-expression lentiviral particles were next generated by cotransfection of recombinant V5–tagged pLent6.3/V5-DEST plasmids and ViralPower Packaging Mix in reverse 293FT cells and used for transduction in destination IEC-6 cells for expression of recombinant proteins. mRNA and protein expression of recombinant proteins were verified by quantitative RT-PCR, Western blot, and immunofluorescence staining. Cells were transduced with virus particles 48 h prior to additional treatment.

IEC-6 cells were pretreated with either Hsp70 siRNA (100 nM; Dharmacon) via Lipofectamine LTX (Invitrogen), according to the manufacturer’s control siRNA direction, or a non–targeted siRNA that was directed at no known target for 48 h in antibiotic-free media at 37°C in a humidified chamber with 5% CO2. The media were changed, and cells were treated with LPS and assessed for apoptosis, NF-κB translocation, and/or cytokine expression.

The NF-κB promoter GFP–reporter system was generated via the pPACKFl1 Lentivector Packaging Kit (System Biosciences) using the pSIFI-H1-siLuc-copGFP positive-control expression plasmid and delivered via lentiviral transduction of IEC-6 cells 48 h prior to stimulation with LPS and/or heat treatment, as described above.

Hsp70 induction and knockdown

For the induction of Hsp70, IEC-6 cells were placed in an incubator at 42°C (5% CO2 and 23% O2) for 45 min, followed by either a 4-h (LPS-mediated apoptosis analysis) or 8-h (NF-κB translocation assay) recovery period at 37°C under otherwise the same ambient conditions, after which they were exposed to the indicated treatment. In parallel, IEC-6 cells were treated with Celastrol (3 μM in DMSO) for 30 min at 37°C and allowed an 8-h recovery period at 37°C prior to treatment with LPS.

Targeted knockdown of Hsp70 was accomplished via transfection with siRNA to Hsp70 or siRNA against no known target. Chemical inhibition of Hsp70 was performed via 2-h pretreatment with quercetin (20 μM; Sigma) or DMSO.

Induction of Hsp70 within cells was accomplished in vivo through the administration of Celastrol (1 mg/kg), as described below. To confirm that the effects of this compound on TLR4 signaling occurred through upregulation of Hsp70, all studies were performed in Hsp70-deficient mice after injection of Celastrol for comparison, as described below.

Experimental endotoxemia and NEC

All experiments were approved by the Children’s Hospital of Pittsburgh Animal Care Committee and the Institutional Review Board of the University of Pittsburgh, C57BL-6, Swiss Webster, Hsp70+/−, and mice overexpressing Hsp70 on the villain promoter (Hsp70−/−), as well as their appropriate wild-type controls, were generated as described (16, 17) or obtained from The Jackson Laboratory. All animals were age and sex matched prior to use. Experimental NEC was induced in 10-d-old mice, as we described (4, 7, 18), using formula gavage (Similac Advanced infant formula; Ross Pediatr Nutritional, a division of Mead Johnson) (2.5 times/d) and hypoxia (5% O2, 95% N2) for 10 min in a hypoxic chamber (Billups-Rothenberg) twice daily for 4 d. Where indicated, mice were administered Celastrol (1 mg/kg) via i.p. injections 1 d prior to the model and on day 3 of the model. Volume-matched DMSO was administered as a vehicle control in all Celastrol-treatment models. The severity of disease was determined on histologic sections of the terminal ileum by a pediatric gastroenterologist, who was blinded to the study condition, according to the work of Radulescu et al. (19), as follows: 0: normal intestine; 1: epithelial lifting or separation; 2: sloughing of epithelial cells to the mid-villus level; and 3: necrosis of the entire villous. This protocol results in the development of patchy necrosis involving the small intestine similar to human NEC, with an increase in circulating cytokines that mimics that seen in human NEC (18).

Preparation and processing of human intestinal tissue from infants with and without NEC

Intestinal samples were obtained from human neonates undergoing intestinal resection for NEC, for unrelated indications (control), or at the time of stoma closure. All human tissue was obtained and processed as discarded tissue via waiver of consent with approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue-procurement guidelines.

Immunohistochemistry, immunoprecipitation, and SDS-PAGE

The immunofluorescence microscopy of IEC-6 enterocytes, as well as mouse and human intestine, was performed as previously described (20), and evaluated using an Olympus FluoView 1000 confocal microscope under oil-immersion objectives. Images were assembled using Adobe Photoshop CS2 software (Adobe Systems). In parallel, Cryo-Gel (Cancer Diagnostics)
frozen sections of terminal ileum were sectioned (6 μm), rehydrated with PBS, and fixed with 2% paraformaldehyde. Non-specific binding was blocked with 5% BSA.

For immunoprecipitation, IEC-6 lysates were collected, and 500 μg total protein was pre-cleared with 20 μl/sample Agarose Protein A/G beads (Santa Cruz Biotechnology) for 30 min at 4°C. Samples were centrifuged, and supernatants were collected and treated with Abs to Hsp70 (K20) or ubiquitin, as indicated, or an isotype IgG control Ab and then incubated with Agarose Protein A/G beads overnight at 4°C. Samples were then centrifuged, supernatants were discarded, and beads were washed three times in PBS and resuspended in equal volumes of 2× gel-loading buffer and boiled at 95°C for 3 min. Lysates were then subjected to SDS-PAGE and immunoblotted with various Abs (1:1000), as indicated. Blots were developed using ECL reagent (ECL-Super Signal; Pierce) and developed on radiographic film.

**Quantitative real-time PCR**

Quantitative real-time PCR was performed, as previously described, using the Bio-Rad CFX96 Real-Time System (3) and the primers listed in Table I. Where indicated, gene expression was assessed on 2.5% agarose gels using ethidium bromide staining. Images were obtained with a Kodak Gel Logic 100 Imaging System using Kodak Molecular Imaging software. The expression of the following genes by quantitative RT-PCR was measured relative to the housekeeping genes β-actin and GAPDH.

**Determination of enterocyte signaling and mucosal injury in response to TLR4 activation**

For the determination of NF-κB translocation, IEC-6 cells were treated with LPS (50 mg/ml, 45 min). The extent of NF-κB translocation was determined as described (18), using an adaptation of the methodology of Ding and Li (22). In brief, a threshold limit was set based upon the emission signal for the nuclear stain DAPI in serial micrographs of cells that were costained for the p65 subunit of NF-κB, whereas a corresponding cytoplasmic region of interest was defined by stenciling a circular region, 12 pixels beyond the nucleus, upon each cell. The average integrated pixel intensity pertaining to the corresponding NF-κB emission within the cytoplasmic and nuclear regions was then determined for >200 cells/treatment group, in at least four experiments per group, using MetaMorph software version 6.1 (Molecular Devices).

The release of IL-6 from IEC-6 cells was determined by an ELISA kit (R&D Systems), according to the manufacturer’s instructions.

Enterocyte apoptosis was determined in IEC-6 cells after 16 h of treatment with LPS under concentrations that we measured in NEC (3) (50 mg/ml) by immunostaining with Abs to cleaved-caspase 3 and performing confocal immunofluorescent analysis. The number of cleaved-caspase 3+ cells was identified in a blinded fashion using MetaMorph software (Molecular Devices) and expressed as a percentage of cleaved-caspase 3+ cells/high-power field, with >100 fields/experiment studied and >100 cells/field.

Enterocyte apoptosis in vivo was determined by measuring the percentage of enterocytes positive for cleaved-caspase 3 by confocal microscopy per high-power field. More than 50 fields/sample were evaluated, as described (7).

**Statistical analysis**

All experiments were repeated at least in triplicate, with >100 cells/high-power field. For mouse experiments of endotoxemia, >4 mice/group were assessed; for experiments of NEC, >10 mouse pups/group were included, and litter-matched controls were included in all cases. Statistical analysis was performed using SPSS 13.0 software. ANOVA was used for comparisons for experiments involving more than two experimental groups. Two-tailed Student’s t test was used for comparison for experiments consisting of two experimental groups. For analysis of the severity of NEC, χ² analysis was performed. In all cases, statistical significance was accepted at p < 0.05 between groups.

**Results**

**Hsp70 induction limits TLR4 signaling in enterocytes**

To determine whether the induction of Hsp70 could limit TLR4 signaling, we first briefly exposed cultured enterocytes (IEC-6 cells) to conditions known to increase the expression of Hsp70 (42°C, 45 min Fig. 1A) and then treated cells with the TLR4 agonist LPS at concentrations that we previously measured from the stool of humans and mice with NEC (3). The extent of TLR4 signaling was determined by assessing the extent of NF-κB activation, as reflected by the degree of translocation of the p65 subunit of NF-κB from the cytoplasm to the nucleus as described in Materials and Methods, the degree of induction of the pro-inflammatory cytokine IL-6, and the degree of enterocyte apoptosis. As shown in Fig. 1, LPS treatment caused a marked increase in NF-κB activation (Fig. 1Di, 1Dii) that was significantly reduced following heat treatment (Fig. 1C, 1Diii). Heat treatment also prevented the increase in IL-6 expression that occurred in LPS-treated IEC-6 cells (Fig. 1B) (primer sequences for quantitative RT-PCR are listed in Table I), as well as a reduction in the extent of LPS-mediated apoptosis, both down to levels similar to untreated control cells (Fig. 1Fi–iii). We used the expression of cleaved-caspase 3 as an estimate of apoptosis in these studies, because its increased expression was shown to be a terminal event in the apoptosis cascade in enterocytes, and it provides a reliable and reproducible estimate of enterocyte apoptosis in both human and experimental NEC (7, 23–25). To determine whether a heat-induced increase in Hsp70 was required for the observed attenuation in TLR4 signaling, cells were exposed to heat after siRNA-mediated knockdown of Hsp70 in IEC-6 cells (Fig. 1E), which abrogated the protection in TLR4 signaling previously observed after heat treatment with regard to the induction of IL-6 expression (Fig. 1B), NF-κB translocation (Fig. 1C), and enterocyte apoptosis (Fig. 1F). Taken together, these findings indicate that Hsp70 can inhibit TLR4 signaling in enterocytes. We next sought to investigate the mechanisms mediating this effect and focused initially on determining whether an association between Hsp70 and TLR4 was required for the negative effects of Hsp70 on TLR4 signaling.

An EEVD-mediated association between TLR4 and Hsp70 is required for the inhibition of TLR4 signaling in enterocytes by Hsp70

The effects of Hsp70 on target proteins are largely influenced by interactions with accessory proteins called cochaperones (26, 27). In a variety of cell types, Hsp70 regulates the intracellular function and fate of proteins through the formation of direct protein–protein interactions that occur largely through an EEVD-binding domain in its C terminus (28–30). To investigate whether an association between TLR4 and Hsp70 was required for the negative effects on TLR4 signaling, IEC-6 cells were stably transduced with lentivirus expressing either LacZ as a vector control or with Hsp70 bearing a truncation mutation in the EEVD domain while sparing the N-terminal ATPase domain, as described (31) (Fig. 2Ai). After LPS treatment, cells were subjected to immunoprecipitation using Abs to Hsp70 in the absence or presence of heat exposure. As shown in Fig. 2Ai, TLR4 was detected in immunoprecipitates obtained from IEC-6 lysates that had been transduced with control vector, and this association was increased after heat exposure. By contrast, the stable delivery via lentiviral transduction of a mutant Hsp70 lacking the C terminus EEVD-binding domain markedly reduced the ability to detect TLR4 in the immunoprecipitates after LPS treatment, which did not vary after heat exposure, indicating that the EEVD mutation resulted in a reduction in the extent of association between Hsp70 and TLR4 (Fig. 2Aii). Although heat treatment of IEC-6 cells that had been transduced with LacZ vector conferred significant protection from both LPS-induced NF-κB translocation (Fig. 2Bi–iii) and enterocyte apoptosis (Fig. 2Ci–iii), this heat-mediated reduction in LPS signaling was lost in IEC-6 cells that had been transduced with the C terminus EEVD-binding mutant (Fig. 2Biv–vii, 2Civ–vii). As expected, LPS caused a significantly greater degree of IL-6 ex-
pression after transduction of ∆EEVD Hsp70 compared with nontransfected cells (Fig. 2Bxi), consistent with the reduced effect of Hsp70 in inhibiting TLR4 signaling in these cells. To further investigate the link between Hsp70 expression and TLR4 signaling, IEC-6 cells were transduced with lentiviruses expressing wild-type Hsp70, which resulted in a significant increase in the degree of Hsp70 expression compared with nontransfected cells (Fig. 2Aii). Importantly, as shown in Fig. 2Bvii–x, compared with LacZ-transfected control cells, the addition of LPS led to a minimal degree of NF-κB translocation in IEC-6 cells that overexpress Hsp70, consistent with the notion that these cells are less responsive to TLR4 signaling due to the effects of Hsp70 on TLR4. Moreover, the degree of LPS-induced NF-κB translocation in Hsp70–IEC-6 cells was minimally affected by heat exposure, consistent with the observation that these cells already overexpress Hsp70. Taken together, these findings demonstrate that the induction of Hsp70 leads to a reduction in TLR4 signaling and that the effects of Hsp70 occur through an association between TLR4 and Hsp70 dependent upon EEVD domain in its C terminus.

Given the role of Hsp70 in regulating the ubiquitin-mediated degradation of target proteins, we, therefore, next sought to investigate whether Hsp70 associations with TLR4 could subsequently alter the ubiquitination state of TLR4 in enterocytes. The induction of Hsp70 leads to the ubiquitination and degradation of TLR4 via the cochaperone CHIP

In various cell types, Hsp70 regulates the fate of target proteins, in part, by critically influencing their ubiquitination state and sub-

FIGURE 1. Hsp70 induction limits TLR4 signaling in enterocytes. (A) Confocal photomicrographs showing the expression of Hsp70 (green), β-actin (red), and DAPI (blue) in IEC-6 enterocytes that were either untreated (i) or exposed to 42°C for 45 min (ii). Quantitative RT-PCR showing the expression of IL-6 (B) or quantification of the extent of NF-κB translocation (C) in IEC-6 cells that were either untreated (white bars) or exposed to heat (black bars) and were either untransfected or were transfected with Hsp70 siRNA. Quantification in (C) is based upon >50 cells/field and >50 fields examined in four separate experiments. *p < 0.05, versus untreated control, **p < 0.01, versus heat control, ***p < 0.001, versus control cells transfected with Hsp70 siRNA. (D) Confocal photomicrographs of IEC-6 enterocytes that were untreated (i), treated with LPS (50 μg/ml, 45 min) (ii), or treated with LPS after pretreatment with heat (iii). (E) Representative SDS-PAGE showing lysates of IEC-6 that were untreated (control), incubated with PBS alone (vehicle), or transfected with either control siRNA against no known substrate (nontargeted siRNA) or siRNA to Hsp70 (Hsp70 siRNA). Blot was stripped then and reprobed with Abs to β-actin. (F) Confocal photomicrographs (i–iv) and quantification (v) of IEC-6 enterocytes that were untreated (i), treated with LPS in the absence (ii) or presence of pre-exposure to heat as above (iii), or pretreated 48 h prior with siRNA to Hsp70, as in (iv). Representative images are taken of >50 fields examined with >50 cells/field in four separate experiments. Scale bar, 10 μm. Representative apoptotic cells are indicated by arrows. *p < 0.05, versus control, **p < 0.01, versus LPS control, ***p < 0.001, versus heat+Hsp70 siRNA saline.
sequent degradation through the proteasomal system (32). The degree of ubiquitination effected by Hsp70 is directly influenced by the activity of a cochaperone molecule CHIP, an E3-ligase bearing a ubiquitin ligase U-box domain and a Hsp70-docking domain (33, 34). We now postulate that Hsp70 induction limits TLR4 signaling by promoting its ubiquitination state via CHIP. To determine whether Hsp70 induction affected TLR4 expression via effects on the extent of ubiquitination of TLR4, we first immunoprecipitated lysates of IEC-6 cells using anti-ubiquitin Abs in the absence or presence of heat and then performed SDS-PAGE using Abs to TLR4. As shown in Fig. 3Ai, heat exposure increased the degree of TLR4 ubiquitination, which was associated with a reduction in TLR4 expression (Fig. 3Aii). Further evidence of the importance of Hsp70 in regulating TLR4 expression after heat treatment is shown in Fig. 3Aiii. After the administration of siRNA for Hsp70, the expression of TLR4 was significantly reducible NO synthase (iNOS) was assessed by RT-PCR over time in IEC-6 cells (Fig. 4A–C), which peaked at the time at which TLR4 signaling decreased (Fig. 4B, 4C). As shown in Fig. 4A (red staining) and Fig. 4B (solid bars), LPS caused a time-dependent signaling response in IEC-6 cells using two separate techniques. IEC-6 cells were transiently transduced with an NF-kB–promoter driven GFP-reporter construct as a readout of TLR4 activation, treated with LPS, and assessed for the expression of GFP by confocal microscopy over time. Cells were also stained for Hsp70 to assess its induction in response to LPS. Second, the induction of the proinflammatory molecule inducible NO synthase (iNOS) was assessed by RT-PCR over time after treatment with LPS. As shown in Fig. 4A (red staining) and Fig. 4B (solid bars), LPS caused a time-dependent signaling response in IEC-6 cells, which reached a maximum at 4–6 and then decreased by 16 h. LPS also caused a time-dependent increase in the expression of Hsp70 in IEC-6 cells (Fig. 4A–C), which peaked at the time at which TLR4 signaling decreased (Fig. 4B, 4C). As a positive control for the effects of Hsp70 on the confocal-based assay, heat exposure resulted in a marked inhibition of TLR4 signaling, as revealed by reduced GFP expression and an increase in Hsp70 expression (Fig. 4Aiv–vi). Of note, the effects of LPS and heat on the intracellular induction of Hsp70 in IEC-6 cells is also shown in Fig. 4D.

Several lines of evidence indicate that the intracellular increase in Hsp70 expression that was observed to occur in response to LPS could limit TLR4 signaling. First, treatment of IEC-6 with the Hsp70 inhibitor Quercetin resulted in an increase in the extent of TLR4-induced IL-6 expression (Fig. 4Fi). Second, the increased expression of Hsp70 that was noted in response to LPS resulted in an increase in the degree to which TLR4 could be detected in lysates of IEC-6 cells that had been immunoprecipitated with Abs to Hsp70 (Fig. 4E), indicating that the increased Hsp70 expression in response to TLR4 activation also resulted in increased

### Table I. Primer sequences for quantitative RT-PCR

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During the inflammatory response, the activation of TLR4 in response to LPS must be carefully controlled; the failure to negatively regulate a TLR4 response would necessarily result in a proinflammatory cytokine storm each time a TLR4 signal was initiated. Having shown that Hsp70 induction can inhibit TLR4 signaling in enterocytes, we next considered the possibility that TLR4 activation itself could lead to an induction of Hsp70, which could then serve to negatively regulate (and therefore to self-limit) TLR4 responsiveness. To do so, we first sought to evaluate the time dependency of the signaling response of TLR4 in IEC-6 cells using two separate techniques. IEC-6 cells were transiently transduced with an NF-κB–promoter driven GFP-reporter construct as a readout of TLR4 activation, treated with LPS, and assessed for the expression of GFP by confocal microscopy over time. Cells were also stained for Hsp70 to assess its induction in response to LPS. Second, the induction of the proinflammatory molecule inducible NO synthase (iNOS) was assessed by RT-PCR over time after treatment with LPS. As shown in Fig. 4A (red staining) and Fig. 4B (solid bars), LPS caused a time-dependent signaling response in IEC-6 cells, which reached a maximum at 4–6 and then decreased by 16 h. LPS also caused a time-dependent increase in the expression of Hsp70 in IEC-6 cells (Fig. 4A–C), which peaked at the time at which TLR4 signaling decreased (Fig. 4B, 4C). As a positive control for the effects of Hsp70 on the confocal-based assay, heat exposure resulted in a marked inhibition of TLR4 signaling, as revealed by reduced GFP expression and an increase in Hsp70 expression (Fig. 4Aiv–vi). Of note, the effects of LPS and heat on the intracellular induction of Hsp70 in IEC-6 cells is also shown in Fig. 4D.
association of Hsp70 with TLR4, consistent with the mechanism of action of Hsp70 in reducing TLR4 signaling shown in Figs. 2 and 3. Third, inhibition of Hsp70 using siRNA resulted in a marked exaggeration in the extent of TLR4 signaling, as measured by an increase in both LPS-induced iNOS expression (Fig. 4Fii) and LPS-induced enterocyte apoptosis (Fig. 4Fiii) compared with
control cells that had been transfected with control siRNA or no siRNA. To further define a link between TLR4 and Hsp70 signaling, we performed flow cytometry on either wild-type IEC-6 cells or Hsp70-deficient IEC-6 cells that we had generated through lentiviral transduction of Hsp70 siRNA, in the presence of heat and LPS stimulation. As shown in Fig. 4G, LPS caused an increase in TLR4 expression that was reduced upon heat exposure in wild-type cells. In contrast, TLR4 expression was increased in Hsp70-deficient IEC-6 cells, which was not reduced further upon heat exposure. Importantly, heat exposure did not prevent NF-κB translocation in IEC-6 cells that were treated with the TLR2 ligand Pam(3)CSK (4) and the TLR5 ligand flagellin, and these findings were not affected by the presence of Quercetin, suggesting that the effect of heat-induced TLR4 suppression is specific for TLR4 signaling (data not shown). Taken together, these findings reveal that TLR4 can induce the expression of Hsp70, which inhibits LPS signaling in enterocytes, and by extension, that Hsp70 can exert a physiological role in constraining the effect of TLR4 signaling that occurs. Therefore, we next sought to investigate the role of Hsp70 on LPS-induced TLR4 signaling in vivo.

FIGURE 3. The induction of Hsp70 leads to the ubiquitination and degradation of TLR4 via the cochaperone CHIP. (Ai) Representative immunoblots in which LacZ- and H260Q-transfected IEC-6 enterocytes were exposed to heat or were maintained at 37˚C and then immunoprecipitated with anti-ubiquitin Abs and immunoblotted with anti-TLR4 Abs, displaying polyubiquitinated species (pUB-TLR4). The location of TLR4 on the gel is shown. (Aii) Representative SDS-PAGE of IEC-6 cells probed with anti-TLR4 Abs that were either nontransfected or transfected with H260Q and then maintained at 37˚C or exposed to heat, in which heat exposure leads to a reduction in TLR4 expression that is not seen in H260Q-transfected cells. (Aiii) SDS-PAGE showing expression of TLR4 and loading protein control in either wild-type (WT) IEC-6 cells or IEC-6 cells treated with siRNA to Hsp70 (siHSP70) that were either untreated (Ctrl) or treated with heat as in Materials and Methods (Heat). (B) Percentage apoptosis per high-power field > 50 fields with >50 cells/field. Representative confocal photomicrographs of IEC-6 enterocytes treated with MG-132 (C), or transduced with LacZ (D), H260Q-CHIP (E), or K30A-CHIP (F), treated as indicated, and immunostained with cleaved-caspase 3 (green), β-actin (red), and DAPI (blue). Arrows point to apoptotic cells. Scale bar, 10 μm. *p < 0.05, control (all bars) versus LPS (all bars), **p < 0.01, LPS (open bar) versus LPS+Heat (open bar) versus LPS open bar, ***p < 0.001, Control versus LPS + Heat (black, red, and blue bars) in three separate experiments.
**Hsp70 negatively regulates TLR4 signaling in the intestinal epithelium**

The systemic administration of LPS to newborn mice is known to cause a significant inflammatory response in the small intestine that includes an increase in the expression of iNOS and an induction in enterocyte apoptosis (36–38). Based upon the above findings, in which Hsp70 was found to inhibit enterocyte TLR4 in vitro, we next sought to evaluate the effects of Hsp70 on enterocyte TLR4 signaling in vivo. To do so, we injected saline or LPS into wild-type mice or into two additional mouse strains: mice that were globally deficient in Hsp70 (Hsp70<sup>−/−</sup>) and mice that selectively overexpress Hsp70 within the intestinal epithelium (Hsp70<sup>vlillin</sup>-

As shown in Fig. 5A, the injection of LPS into wild-type mice caused a time-dependent increase in the mucosal expression of iNOS, as well as a significant induction in enterocyte apoptosis (Fig. 5Bi, 5Bii, 5C). In contrast, injection of LPS into mice that selectively overexpress Hsp70 within the intestinal epithelium resulted in a marked reduction in LPS-mediated iNOS expression within the intestinal epithelium compared with wild-type mice (Fig. 5A) and a reduction in enterocyte apoptosis (Fig. 5Bi, 5Biv, 5C), demonstrating that Hsp70 could negatively regulate TLR4 signaling in vivo and supporting the in vitro data shown in Fig. 4. Moreover, the injection of LPS into Hsp70<sup>−/−</sup> mice resulted in a significantly increased degree of apoptosis compared with LPS-
Hsp70 signaling negatively regulates the development of NEC

We and other investigators demonstrated that NEC is a disease characterized by TLR4-mediated apoptosis within the newborn small intestine (3, 5, 24). Having now shown that Hsp70 can limit TLR4 signaling in enterocytes both in vivo and in vitro, we next sought to evaluate whether a lack of Hsp70 may lead to an increase in the severity of NEC. As shown in Fig. 6A, the expression of Hsp70 in both mice (Fig. 6Ai, 6Aii) and humans (Fig. 6Aiii, 6Aiv) with NEC was significantly decreased compared with control bowel, indicating the possibility that a lack of Hsp70 may play a role in NEC development. To investigate directly whether Hsp70 could regulate the development of this disease, NEC was induced in wild-type, Hsp70−/−, and Hsp70−/− mice using a combination of formula gavage and intermittent hypoxia. As shown in Fig. 6B, the induction of NEC in wild-type mice resulted in enterocyte apoptosis (Fig. 6B, 6Bii, 6Ei), mucosal disruption (Fig. 6Biii, 6Biv), and an increase in the expression of iNOS in the intestinal mucosa (Fig. 6Eii). Importantly, the induction of NEC in Hsp70−/− mice showed a significant increase in the extent of enterocyte apoptosis (Fig. 6Ci, 6Cii, 6Ei), mucosal disruption (Fig. 6Ciii, 6Civ), and iNOS expression (Fig. 6Eii), as well as increased disease severity (Fig. 6Eiii), whereas the induction of NEC in the Hsp70−/− mouse that overexpresses Hsp70 in the intestinal epithelium resulted in a marked reduction in each of these measures (Fig. 6Di, 6E). Taken together, these findings illustrate that Hsp70 plays a key role in the regulation of NEC. We next sought to evaluate whether pharmacologic induction of Hsp70 could inhibit TLR4 signaling and affect NEC severity.

Pharmacologic induction of Hsp70 limits TLR4 signaling in enterocytes in vitro and in vivo and attenuates the severity of experimental NEC

In the final series of studies, we sought to evaluate whether the pharmacologic induction of Hsp70 could inhibit TLR4 signaling in vitro and in vivo and, thus, attenuate the severity of NEC. To do so, we used the small molecule Celastrol (39, 40), a novel cell-permeable triterpenoid antioxidant that was shown to induce Hsp70 expression and activity in a variety of cells (41, 42). As shown in Fig. 7Ai, treatment of IEC-6 cells with Celastrol led to a rapid induction of Hsp70, as determined by SDS-PAGE. In parallel, the exposure of IEC-6 cells to Celastrol resulted in a marked increase in cytoplasmic Hsp70 expression (red staining in Fig. 7Bi–iv), as well as a significant reduction in the extent of TLR4 signaling, as measured by a reduction in the extent of LPS-induced apoptosis (Fig. 7Biv versus Fig. 7Bii, 7Ei), a reduction in the extent of LPS-induced NF-kB translocation (Fig. 7C, 7Eii), and a significant reduction in the extent of LPS-induced IL-6 expression (Fig. 7Eiii). There were no effects of Celastrol treatment alone on enterocyte apoptosis, NF-kB translocation, or IL-6 expression (Fig. 7Biii, 7Ciii, 7Eiii).

Having shown that the injection of Celastrol into mice can induce Hsp70 expression within the intestinal mucosa (Fig. 7Aii), we next sought to determine whether Celastrol could inhibit TLR4 signaling in the intestinal epithelium via effects on Hsp70 induction. To do so, wild-type and Hsp70−/− mice were injected with Celastrol 24 h prior to LPS and then assessed for the extent of enterocyte apoptosis and iNOS expression in the intestinal mucosa. As shown in Fig. 7Di–iv and 7Fi, the administration of Celastrol to wild-type mice led to a significant reduction in the extent of LPS-induced enterocyte apoptosis compared with the effects of LPS in wild-type mice that did not receive Celastrol, as well as to a significant reduction in the extent of LPS-induced...
expression of iNOS in the intestinal mucosa compared with wild-type mice (Fig. 7Fii). Importantly, there was no protective benefit of Celastrol when it was administered to Hsp70−/− mice, confirming that its protective effects required Hsp70 induction (Fig. 7Fv–viii). The specificity of Celastrol for TLR4-mediated enterocyte apoptosis was confirmed in vitro. Although LPS caused a significant increase in enterocyte apoptosis in Hsp70-deficient IEC-6 cells, the addition of Celastrol did not confer protection; in fact, these Hsp70-deficient enterocytes were significantly more susceptible to apoptosis than were their non-transfected counterparts (Supplemental Fig. 1).

Based upon the above findings, we next sought to evaluate whether the induction of Hsp70 that occurs with the administration of Celastrol could attenuate the severity of experimental NEC. To test this directly, we first administered either DMSO or Celastrol by i.p. injection to newborn pups on days 0 and 1 of the experimental model and assessed the effects on extent of mucosal disruption, enterocyte apoptosis, induction of iNOS, and disease severity. As shown in Fig. 8, the administration of Celastrol markedly reduced the degree of mucosal disruption (Fig. 8Ai–iii), enterocyte apoptosis (Fig. 8Av–vii, 8Ci), mucosal iNOS expression (Fig. 8Ci), and disease severity (Fig. 8Cii) compared with mice that received DMSO alone, consistent with the mechanism of action for Celastrol shown in Fig. 7.

Having shown that the induction of Hsp70 through the administration of Celastrol could prevent the development of NEC when administered prior to the start of the experimental model, we next sought to determine whether the administration of Celastrol could reduce the severity of NEC once the disease had been established in mice. To do so, we injected mice with Celastrol on the last 2 d of the 4-d model, at a time in which significant inflammation is already established. Strikingly, as shown in Fig. 8, mice with NEC that received Celastrol after disease induction showed restoration of mucosal architecture (Fig. 8Av), a significant reduction in enterocyte apoptosis (Fig. 8Aviii, 8Ci), and a reduction in NEC severity (Fig. 8Cii), all to levels that were similar to that of mice without NEC and that were comparable to levels observed in mice receiving Celastrol as a prevention strategy (compare checkered and solid bars in Fig. 8C). Taken together, these findings suggest that the pharmaceutical induction of Hsp70 with compounds such as Celastrol may be used as a novel approach to the prevention or treatment of NEC through effects on the inhibition of TLR4 signaling in the newborn small intestine, as summarized in Fig. 9.

**Discussion**

We now define a novel mechanism by which TLR4 is regulated in the newborn intestinal epithelium that has important implications in the pathogenesis of NEC, a disease that is characterized by exaggerated TLR4 signaling within the intestinal mucosa (3–5). Specifically, we identify that the induction of Hsp70 leads to
a reduction in TLR4-induced signaling in enterocytes, as measured by a reduction in NF-κB activation, cytokine induction, and apoptosis, and that induction of Hsp70 either pharmacologically or genetically leads to a reduction in TLR4 signaling and a marked inhibition in the severity of NEC. The current results identify a novel pathway that links cytoplasmic Hsp70 induction with TLR4 regulation and demonstrate that impaired Hsp70 expression or function may, in part, underlie the causes of this devastating disease. These findings represent a novel departure from current thinking in the field by revealing that future treatments for NEC may involve nonspecific immunological approaches, such as the elimination of microbial pathogens or the administration of particular feeding regimens (43), as well as the pharmacologic induction of an intracellular chaperone, such as Hsp70, to limit disease progression through inhibitory effects on the innate immune receptor TLR4.
One of the most important findings of the current study involves the proposed mechanism of action of Hsp70 in limiting TLR4 signaling within enterocytes. As a molecular chaperone, Hsp70 can associate with cochaperone proteins through an EEVD motif in its C terminus (28, 29). As shown in Fig. 2, we now demonstrate that the inhibition of TLR4 signaling in response to Hsp70 induction required this EEVD-binding motif (30), because the introduction of a mutant lacking this domain prevented the association between TLR4 and Hsp70, as well as reversed the protection of Hsp70 induction on TLR4 signaling (Fig. 2). This mechanism of action for Hsp70 is in agreement with recent work of Chow et al. (44), who showed that Hsp70 mutants with a functional EEVD motif but lacking N-terminus ATPase activity were still capable of protecting L929 fibroblasts from apoptosis induced by proinflammatory cytokines. We also determined that the association between Hsp70 and TLR4 results in enhanced ubiquitination and degradation of TLR4, a process that we have now determined to require the cochaperone CHIP (Fig. 3). CHIP has not previously been linked to TLR4 signaling, and we further reveal that mutations in both its docking (K30A) and ubiquitination domains (H260Q) prevented the protective effects of Hsp70 induction on TLR4. These findings define the mechanism by which CHIP acts to mediate the inhibitory effects of Hsp70 on TLR4 signaling and are in agreement with the known function of CHIP in regulating the activity of other Hsp70 targets through ubiquitination (45, 46), yet to our knowledge, they represent the first direct link of CHIP to an intestinal inflammatory disease. It is noteworthy that in the original description of the CHIP-deficient mouse, attention was drawn to the intestinal phenotype that was observed when mice were subjected to a brief hyperthermic stress, characterized by friability of the small intestine with marked apoptosis of the intestinal epithelium (47), although potential CHIP targets that could mediate this effect on the small intestine during stress were not identified. It is tempting to now speculate that CHIP may play a central role in the maintenance of intestinal homeostasis, in part by preventing the unbridled activation of immune targets of CHIP, such as TLR4.

It should be noted that the current findings in which cytoplasmic Hsp70 serves to curtail the signaling of TLR4 within the intestinal epithelium lie in distinction to a growing and somewhat controversial body of work concerning the extracellular role of Hsp70 and other heat shock proteins in activating the innate immune system via TLR4 (48–53). In this regard, Retzlaff et al. (54) showed that the exogenous administration of Hsp70 could increase IL-1, IL-6, and TNF in cultured macrophages, whereas Wheeler et al. (55) showed that the extracellular exposure of Hsp70 to neutrophils from wild-type mice leads to the release of IL-8, yet this effect is not observed in neutrophils from C3H/HeJ mice that have inhibitory mutations in TLR4. Although very exciting, such studies were recently called into question by concerns that the observed effects might actually result not from the heat shock proteins themselves, but rather from contaminants, such as LPS, which could inadvertently be present within the protein preparations or be bound specifically to the heat shock proteins (56). For example, Wallin et al. (57), Bausinger et al. (58), and Gao and Tsan (59)
FIGURE 9. Proposed model: Hsp70 regulates TLR4 signaling in enterocytes in the pathogenesis of NEC. As described in the text, under healthy conditions (left panel), TLR4 is activated by host microbes. The degree of activation is limited by Hsp70 through effects on TLR4 degradation through proteasomal pathways via CHIP. In contrast, under the conditions of stress that favor the development of NEC (right panel), the reduction in Hsp70 expression accompanied by the increase in TLR4 expression leads to exaggerated TLR4 activation and the development of increased enterocyte apoptosis and pro-inflammatory cytokine expression in the newborn intestine. This leads to the development of NEC. Moreover, pharmacologic induction of Hsp70 can curtail TLR4 signaling and both prevent and treat experimental NEC.

showed that the activation of immune cells previously attributed to Hsp70 were lost when highly purified (i.e., contaminant-free) recombinant proteins were used, although these results were recently and convincingly rebutted in two review articles on this topic (60, 61). In contrast to studies in the field of extracellular Hsp70 biology, the novelty and importance of the current findings lie in the newly discovered link between TLR4 and Hsp70 within the enterocyte both in vitro and in vivo, as well as the potential etiological relevance to the development of NEC. And although they represent an extension of the classic role of Hsp70 in modulating the fate of cytoplasmic proteins, the relevance, if any, to the body of literature surrounding the fate of Hsp70 outside of the cells is unknown.

The current findings in support of a role for Hsp70 in the protection from the development of NEC through the inhibition of TLR4 signaling in the small intestine may or may not apply to other diseases of intestinal inflammation, including ulcerative colitis and Crohn’s disease, in which TLR4 signaling may play a lesser, or perhaps even opposite, role. Although we (3, 4) and other investigators (5, 6) showed that the development of NEC requires TLR4 activation, it was shown that TLR4 plays a protective role in experimental colitis (62, 63). Several reasons may account for this apparent discrepancy that have relevance to the current study. TLR4 activation leads to intestinal injury in a well-defined and physiologically relevant context (i.e., the newborn small intestine).

In support of this concept, we recently demonstrated that TLR4 activation with LPS leads to increased enterocyte apoptosis in the terminal ileum of newborn mice but not adult mice, as well as in the small intestine but not the newborn colon (7). Further, reports that demonstrate a protective role for TLR4 in models of colitis have typically been based upon the use of global TLR4 knockout mice, in which TLR4 signaling is disrupted in enterocytes as well as T cells and myeloid cells. We recently showed that TLR4 signaling within the enterocyte itself is important for the induction of intestinal injury leading to NEC, using enterally administered adenoviral constructs that bear inhibitory mutations in TLR4 whose expression is largely favored within the small bowel mucosa (4, 18). Therefore, it is reasonable to conclude that the protective effects attributed to TLR4 signaling in the gut by previous investigators may reflect, in part, the mitigating effects of TLR4 signaling on other cells. In support of this possibility, we note that Fukata et al. (64) recently showed, in an elegant study using chimeric mice, that TLR4 signaling in colonic epithelial cells worsened intestinal inflammation. These findings argue that the effects of TLR4 in the development of intestinal inflammation are strongly influenced by a variety of factors, including the effector cells involved, developmental factors, and involved region of the intestine. The precise effects of Hsp70 at these varying stages of development and within these different cell types remain to be explored in further detail, but they are likely to provide important clues to the underlying causes of these diseases.

Based upon the current findings, we now propose a model by which Hsp70 limits TLR4 signaling and plays a key role in influencing the development of enterocyte apoptosis and the development of NEC (Fig. 9). Under healthy conditions, the relationship between the indigenous flora of the host and the baseline activation of TLR4 exists in homeostatic balance, which we now attribute, in part, to a constitutive role of Hsp70 in limiting the extent of TLR4 signaling by controlling its degradation through proteasomal pathways. The interaction between TLR4 and Hsp70 may occur within intracellular compartments, such as the Golgi apparatus, where TLR4 signaling was shown to occur within the enterocyte (65). In contrast, under the conditions of stress that favor the development of NEC (increased LPS, hypoxia, and prematurity), the exhaustion of Hsp70 signaling, accompanied by the relative increase in TLR4 expression, leads to exaggerated TLR4 activation and the development of the increased enterocyte apoptosis and proinflammatory cytokine expression in the newborn intestine that leads to NEC. It is also notable that the pharmacologic induction of Hsp70 can curtail TLR4 signaling and both prevent and treat experimental NEC.

In summary, we identified a novel pathway by which Hsp70 serves to limit TLR4 signaling in the intestinal epithelium, and moreover, showed that factors that increase Hsp70 signaling can attenuate NEC severity through inhibition of TLR4. We believe that such findings offer new insights into the molecular requirements that lead to NEC development, as well as offer novel therapeutic approaches for this devastating disease.

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