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Heme Oxygenase-1 Is a Critical Regulator of Nitric Oxide Production in Enterohemorrhagic *Escherichia coli*-Infected Human Enterocytes[1]

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Enterohemorrhagic *Escherichia coli* (EHEC) are the causative agent of hemolytic-uremic syndrome. In the first stage of the infection, EHEC interact with human enterocytes to modulate the innate immune response. Inducible NO synthase (iNOS)-derived NO is a critical mediator of the inflammatory response of the infected intestinal mucosa. We therefore aimed to analyze the role of EHEC on iNOS induction in human epithelial cell lines. In this regard, we show that EHEC down-regulate IFN-γ-induced iNOS mRNA expression and NO production in Hct-8, Caco-2, and T84 cells. This inhibitory effect occurs through the decrease of STAT-1 activation. In parallel, we demonstrate that EHEC stimulate the rapid inducible expression of the gene *hmox-1* that encodes for the enzyme heme oxygenase-1 (HO-1). Knock-down of *hmox-1* gene expression by small interfering RNA or the blockade of HO-1 activity by zinc protoporphyrin IX abrogated the EHEC-dependent inhibition of STAT-1 activation and iNOS mRNA expression in activated human enterocytes. These results highlight a new strategy elaborated by EHEC to control the host innate immune response. *The Journal of Immunology,* 2008, 180: 5720–5726.

Infection with enterohemorrhagic *Escherichia coli* (EHEC)[4] may lead to diseases ranging from watery diarrhea to life-threatening complications such as hemolytic-uremic syndrome, the main cause of acute renal failure in children worldwide. Human infection occurs by ingestion of contaminated food derived from rearing animals, including meat, milk, or soiled vegetables (1). EHEC reach and colonize the lower intestine, initiating the formation of attaching and effacing lesions to the epithelium and the development of a mucosal innate immune response. Clinical data have established that type 1 cytokines and chemokines are over-expressed in EHEC-infected patients (2, 3). More particularly, an elevated ratio between pro- and anti-inflammatory cytokines is a reliable marker of the severity of EHEC infection and development of hemolytic-uremic syndrome (4, 5). Therefore, the capacity of bacteria to modulate the enterocyte innate immune response may be a critical step in pathogenesis.

Enterocytes are the first cells in contact with EHEC. This interaction yields to the locus of enterocyte effacement (LEE)-dependent structural modifications of the epithelium, i.e., attaching/effacing lesions, and to the development of a nonspecific defense program. In vitro studies have highlighted that EHEC induce the synthesis of chemokines and cytokines in human epithelial cell lines (6, 7), but may also subvert the innate immune response by inhibiting various signal transduction pathways and suppressing the activation of transcription factors such as NF-κB or STAT-1 (8–10). Other proinflammatory mediators induced by enteropathogenic bacteria may play a critical role in the pathophysiology of enteritis and related diseases. Among them, NO is a potent effector of the innate immune system, which plays a major role in the development of inflammation (11) and in the fight against pathogens (12). Under pathophysiologic conditions, NO is synthesized in large amounts by the inducible NO synthase (iNOS). The transcription of the gene encoding iNOS can be induced by bacteria (12, 13) or by proinflammatory cytokines (13) and requires the activation of diverse transcription factors including NF-κB or STAT-1 (14). We recently established that chemical and cellular sources of NO suppress the EHEC production of Shiga-toxin (Stx), the main virulence factor responsible for hemorrhagic symptoms, without affecting the growth and the survival of the bacteria (15). However, in the context of EHEC/NO interaction study, the effect of these bacteria on iNOS induction has not yet been investigated.

The aim of this study was to determine whether EHEC modulate iNOS expression and activity in human epithelial cell lines, stimulated or not by proinflammatory cytokines. We found that EHEC suppress the STAT-1-dependent transcription of the gene encoding iNOS in activated cells. This inhibition requires the activation of the enzyme heme oxygenase (HO)-1 by EHEC.

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[4] Abbreviations used in this paper: EHEC, enterohemorrhagic *Escherichia coli*; LEE, locus of enterocyte effacement; iNOS, inducible NO synthase; Stx, Shiga-toxin; HO, heme oxygenase; Bay 11–7082, (E)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile; ZnPP, zinc protoporphyrin IX; CO, carbon monoxide; CORM-2, tricarbonyldichlororuthenium-(II)-dimer; siRNA, small interfering RNA; EPEC, enteropathogenic *E. coli*.

Table I. *List of bacteria*

Strain	Origin[a]	Serotype	LEE	*stx1*/*stx2*	Source
EDL933	HC/O	O157:H7	+	+/+	ATCC # 700927
Sakaï	HUS/O	O157:H7	+	+/+	STEC Center[b]
8624	HC/O	O157:H7	+	−/+	STEC Center[b]
CL3	HUS	O113:H21	−	−/+	STEC Center[b]
CL15	HUS	O113:H21	−	−/+	STEC Center[b]
87307	HUS	O113:H21	−	−/+	STEC Center[b]
EDL933 Δ*eae*	NA	O157:H7	+	+/+	Ref. 19
EDL933 Δ*espA*	NA	O157:H7	+	+/+	Ref. 16
EDL933 Δ*espD*	NA	O157:H7	+	+/+	Ref. 17
EDL933 Δ*sepL*	NA	O157:H7	+	+/+	Ref. 18
EDL933 Δ*stx1/stx2*	NA	O157:H7	+	−/−	Ref. 10
DH5α	ND	ND	−	−/−	Stratagene
EcG1	F	ND	−	−/−	This work
EcG2	F	ND	−	−/−	This work

[a] HC, hemorrhagic colitis; HUS, hemolytic-uremic syndrome; O, outbreak; NA, not applicable; ND, not determined; F, healthy human feces.

[b] National Food Safety and Toxicology Center, Michigan State University, East Lansing, MI; STEC center: http://shigatox.net.

Materials and Methods

Pharmacological reagents

The NF-κB inhibitors, namely *Z*-Leu-Leu-Leu-CHO (20 μM) and (*E*)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile (Bay 11–7082; 20 μM), and the JAK-2 inhibitor 1,2,3,4,5,6-hexabromocyclohexane (20 μM) were purchased from Calbiochem. The STAT-1 inhibitor (2-fluoroadenine-9-b-D-arabinofuranoside also named fludarabine; 20 μM), the HO-1 inhibitor zinc protoporphyrin IX (ZnPP; 10 μM), bilirubin, and the carbon monoxide (CO) donor tricarbonyldichlororuthenium-(II)-dimer (CORM-2) were obtained from Sigma-Aldrich.

Bacteria

Bacteria used throughout this study are listed in Table I. Six O157:H7 and O113:H21 EHEC strains were isolated from patients with hemorrhagic colitis or hemolytic and uremic syndrome; these strains were selected because they express the major virulence factors usually carried by the majority of the bacteria belonging to both serotypes. The *eae*, *espA*, *espD*, *sepL*, and *stx1*/*stx2* isogenic mutants in the strain EDL933 were used and have been previously described (10, 16–19). Commensal *E. coli* (isolates EcG1 and EcG2) were obtained as follows: Feces from healthy human were 10^4-fold-diluted and plated overnight at 37°C on sorbitol MacConkey agar plates; purity of isolated colonies was monitored by direct microscopic observation after Gram staining, and phylum was determined by API gallery and 16S rRNA gene sequencing as previously described (20). We determined by PCR that the strains EcG1 and EcG2 do not possess the genes *stx2*, *espA*, and *eae* (data not shown).

Bacteria were isolated on Luria-Bertani agar plates. One clone was grown overnight at 37°C in Luria-Bertani broth then diluted and grown in cell culture medium for 2 h to the exponential growth phase ($A_{600} \sim 0.2$–0.4). These bacteria were used to infect epithelial cells.

Cultures of epithelial cells and infections

The human colonic epithelial cell lines Hct-8 and Caco-2 were maintained in DMEM, 10% FBS, 1% sodium pyruvate, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin. T84 cells were cultured in supplemented DMEM/Ham's F12 (1/1). Cells (10^6/ml) were cultured for 7–10 days at 37°C under 5% CO_2. Confluent cells were stimulated with 50 ng/ml IFN-γ, 20 ng/ml TNF-α, and/or 5 ng/ml IL-1β (each obtained from Pierce), in the presence or absence of CORM-2, bilirubin, or bacteria at a multiplicity of infection of 1–100 in complete medium without antibiotics; cytokines and bacteria were added at the same time. Pharmacological inhibitors were added to cell 30 min before infection. Following infection, cocultures were washed and 1) RNA or proteins were extracted from fixed cells, or 2) a fresh complete medium containing 2 mM l-arginine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin was added to determine NO production after 24 h.

Transfection and HO-1 silencing

Cells cultured in 6-well plates were transfected overnight with 200 nM small interfering RNA (siRNA) for human HO-1 (5′-AAGCCACACAG CACUAUGUAAdTdT-3′ and 5′-UUACAUAGUGCUGUGUGGCUU dTdT-3′; Ref. 21) or lamin as control (Dharmacon), using siLentFect Lipid Reagent (Bio-Rad). After 18 h, cells were washed and stimulated with cytokines and/or infected with EHEC.

mRNA analysis by real-time PCR

Total RNA of epithelial cells was isolated using TRIzol (Molecular Research Center) and was reverse-transcribed (2 μg) using oligo(dT) primers and 5 U/μl Superscript II reverse transcriptase (Invitrogen). PCR was conducted using 1 μl of cDNA, 0.25 U Platinum *Taq*DNA polymerase (Invitrogen), 0.12 pmol/μl each of 5′ and 3′ human iNOS (5′-ACCTCAG CAAGCAGCAGAAT-3′ and 5′-ATCTGGAGGGGTAGGCTTGT-3′, 269 bp) or *hmox-1* (the gene encoding HO-1) primers (5′-ACATCTAT GTGGCCCTGGAG-3′ and 5′-GGCAATCTTTTTGAGCACCT-3′, 287 bp), and 0.03 pmol/μl each of β-actin primers (436 bp; 10). One PCR cycle consisted of the following: 94°C for 30 s, 58°C for 30 s, and 72°C for 1.5 min; the total cycle number was 30. Real-time PCR was performed using the same primers and the SYBR Premix Ex *Taq* kit (Takara); reactions were conducted in the Mastercycler ep Realplex Silver apparatus (Eppendorf). One PCR cycle consisted of the following: 94°C for 30 s, 58°C for 30 s, and 72°C for 45 s. Results were calculated using the comparative cycle threshold method and are expressed as relative mRNA expression compared with uninfected cells.

EMSA

Nuclear proteins of cultured epithelial cells were purified using the NE-PER Nuclear and Cytoplasmic Extract kit (Pierce) and protein concentration was measured with the Protein Assay kit (Bio-Rad). NF-κB and STAT-1 DNA-binding activities were analyzed as previously described (10) using 2 μg proteins. The double-stranded oligonucleotide probes (40 fmoles) containing the NF-κB (5′-TCCAA*GGGGACTTTCC*ATG-3′) and the STAT-1 (5′-GACAT*TTCCCGTAA*ATC-3′) binding consensus sequence (italicized letters) were used.

Immunolocalization of transcription factors

Immunocytochemistry and confocal microscopy were performed as previously described (10). The anti-NF-κB p65 rabbit polyclonal Ab (1/500; Calbiochem), the anti-STAT-1 rabbit polyclonal Ab (1/100; Epitomics), and the goat anti-rabbit IgG DyLight™ 647 conjugated (1/1000; Pierce) were used.

HO-1 Western blot analysis

Immunoblots were performed as previously described using a rabbit anti-human HO-1 Ab (StressGen; Ref. 21). Equal transfer of proteins was confirmed by staining the nitrocellulose membrane with Ponceau Red. Proteins from chondrocytes of Coprotoporphyrin-treated mice were used as positive control (21).

Determination of NO_x concentration

In culture supernatants, the concentrations of the stable oxidized products of NO, NO_3^- and NO_2^-, were determined using the Nitrite/Nitrate Assay kit (Sigma-Aldrich), followed by spectrophotometric measure of absorbency at 540 nm.

Statistical analysis

Quantitative data are presented as the mean ± SEM. Student's *t* test was used to determine significant differences between two groups. ANOVA with the Student-Newman-Keuls test was used to analyze significant differences among multiple groups; $p = 0.05$ was considered significant.

Results

EHEC inhibits cytokine-stimulated iNOS induction in human enterocytes

Hct-8 cells were infected with different strains of EHEC in the presence or absence of IFN-γ. The six EHEC strains tested, the commensal *E. coli* isolates, or *E. coli* DH5α were not able to induce iNOS mRNA expression in the epithelial cell lines after 6 h of coculture (Fig. 1*A*). We then sought to determine the effect of bacteria on activated cells. As shown in Fig. 1*B*, iNOS mRNA expression was increased in Hct-8 cells treated 6 h with IFN-γ. When IFN-γ and EHEC isolates belonging to O157:H7 or O113:H21 serotypes were added together with the cells, the levels of

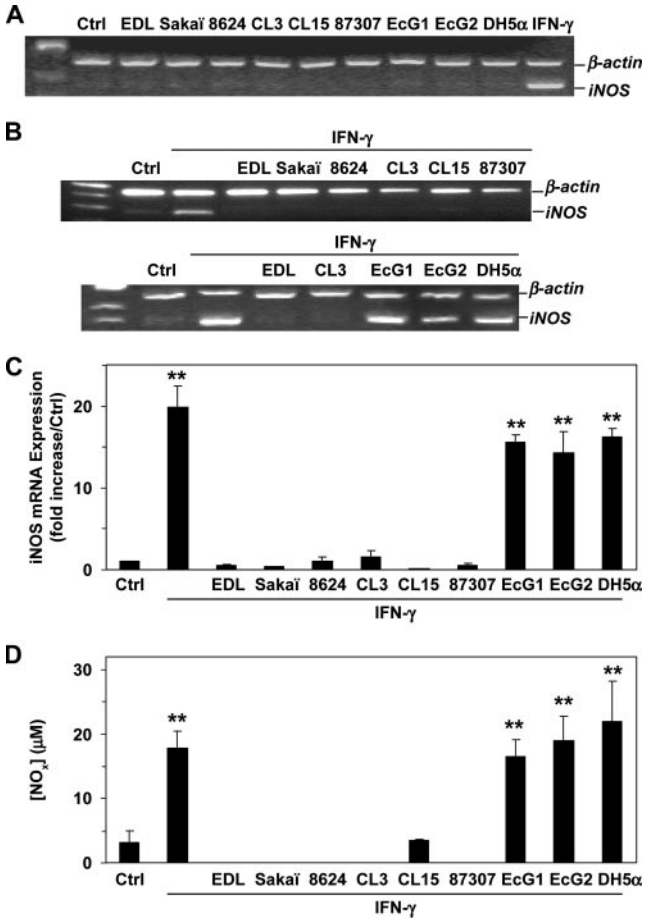

FIGURE 1. Effect of EHEC on iNOS induction in human enterocytes. Hct-8 cells were infected with bacteria for 6 h. RNA was purified, reverse transcribed, and the expression level of the gene encoding iNOS was analyzed by RT-PCR (*A*). Cells treated with IFN-γ were used as positive control for iNOS mRNA expression. Cells were treated with IFN-γ in the presence or absence of bacteria. Subsequently, iNOS gene transcription was analyzed by RT-PCR (*B*) and real-time PCR (*C*). The 6-h stimulated cells were washed and incubated for 18 h in complete medium containing antibiotics. $[NO_2^-]$ plus $[NO_3^-]$ were then measured in culture supernatants (*D*). For *C* and *D*, **, $p < 0.01$ vs control cells and cells treated with IFN-γ and infected with the EHEC strains ($n = 4$). Ctrl, Untreated cells; EDL, EDL933.

iNOS transcripts were dramatically decreased in comparison to cells treated with IFN-γ; the inhibitory effect was observed with a multiplicity of infection of 10 or 100, but not of 1 (data not shown). However, iNOS mRNA expression in activated epithelial cells was not inhibited by the commensal *E. coli* isolates or by the strain DH5α (Fig. 1*B*). These results were confirmed by real-time PCR (Fig. 1*C*). The expression of the iNOS gene was up-regulated by ~20-fold under IFN-γ stimulation when compared with control cells. This increase was significantly inhibited by 97–105% by the use of EHEC, according to the strain tested. Similarly, IFN-γ-induced NO synthesis in Hct-8 cells was completely inhibited using the six EHEC strains (Fig. 1*D*); NO generation was not inhibited by commensal *E. coli* isolates or by the strain DH5α (Fig. 1*D*).

Similar results were obtained with Caco-2 and T84 cells (Table II). Moreover, iNOS mRNA expression and NO production increased by ~20% when the cells were stimulated with a cytokine mix (IFN-γ, TNF-α, and IL-1β), in comparison to the cells that were only stimulated with IFN-γ; in this condition, the EHEC inhibitory effect was also observed.

Table II. *Induction of iNOS in human epithelial cell lines*[a]

	Ctrl	IFN-γ	IFN-γ + EDL933
Caco-2			
iNOS mRNA[b]	1	18.4 ± 1.9	0.5 ± 0.2
$[NO_x]$ (μM)	0.8 ± 0.3	20.2 ± 3.7	1.2 ± 0.7
T84			
iNOS mRNA[b]	1	17.3 ± 0.8	0.8 ± 0.2
$[NO_x]$ (μM)	0.6 ± 0.1	21.2 ± 0.6	1.1 ± 0.5

[a] Caco-2 or T84 cells were stimulated with IFN-γ, in the presence or absence of the strain EDL933, for 6 h. Subsequently, iNOS mRNA expression was analyzed by real-time PCR. Cells activated for 6 h were washed and incubated with fresh medium containing antibiotics for 18 h. NO concentrations were measured in the culture supernatants. Each data is the mean ± SEM of $n = 3$–4 independent experiments.
[b] Express as a fold increase/Ctrl.

Together these data demonstrate that O157:H7 and O113:H21 EHEC repress iNOS mRNA expression in human enterocytes activated by IFN-γ.

iNOS induction inhibition by EHEC is STAT-1-dependent

We next aimed to identify the molecular mechanism by which EHEC suppress IFN-γ-induced iNOS gene expression. We first demonstrated that iNOS mRNA expression (Fig. 2*A*) and NO production (Fig. 2*B*) in response to IFN-γ stimulation was completely

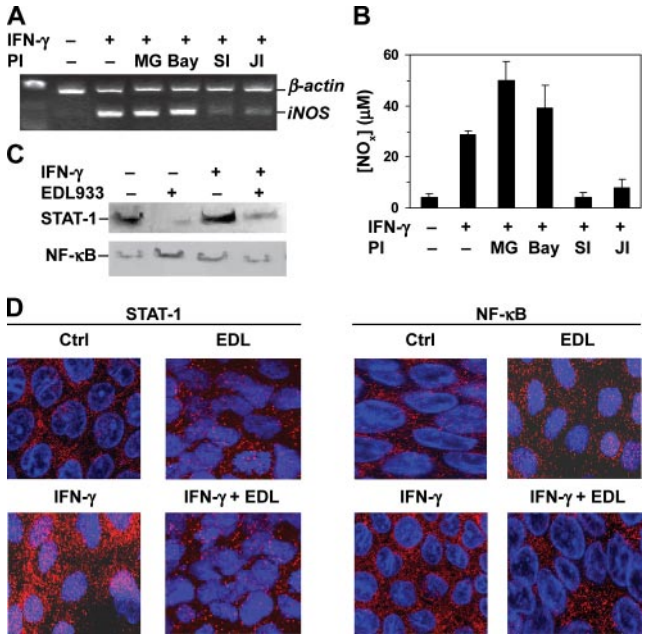

FIGURE 2. STAT-1 and NF-κB activation. *A* and *B*, Identification of the transcription factor implicated in iNOS induction. Hct-8 cells were treated for 30 min with pharmacological inhibitors (PI), namely the NF-κB inhibitors MG132 (MG) and Bay 11–7082 (Bay), the STAT-1 inhibitor (SI), or the JAK-2 inhibitor (JI); cells were then stimulated for 6 h with IFN-γ. The expression of the iNOS gene was analyzed by RT-PCR (*A*). Activated cells were incubated for 18 h in complete medium containing antibiotics and NO generation was measured in culture supernatants (*B*). *C*, Nuclear proteins from cells activated with IFN-γ ± EDL933 (EDL) for 3 h were extracted and analyzed by EMSA. Similar results were obtained in three independent experiments. *D*, Cellular localization of STAT-1 and NF-κB. Hct-8 cells were activated for 3 h and fixed on Lab-Tek slides. STAT-1 and NF-κB were immunodetected using an anti-p65 Ab (red) and with an anti-STAT-1 Ab (red), respectively. Nuclear outline was defined by DAPI staining (blue). Merged images are shown and overlay is presented in pink. The same results have been obtained in three independent experiments.

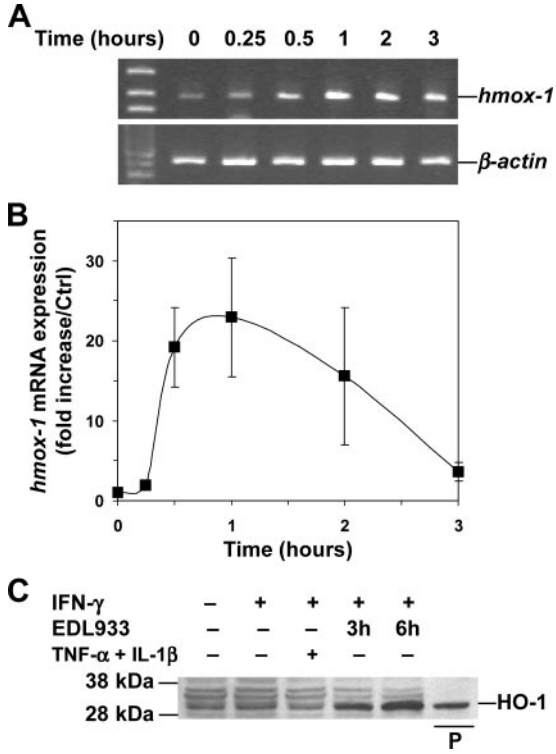

FIGURE 3. Induction of HO-1 by EHEC. *A* and *B*, Expression of *hmox-1* mRNA in response to EHEC. Hct-8 cells were infected with EDL933 in the presence or absence of IFN-γ and/or TNF-α and IL-1β. At indicated times, total RNA was extracted and the expression of *hmox-1* was analyzed by RT-PCR (*A*) and semiquantitative PCR (*B*; $n = 4$). *C*, The presence of HO-1 protein was monitored by Western blotting. Equal amounts of protein (25 μg) were loaded on the gel. P, Positive control.

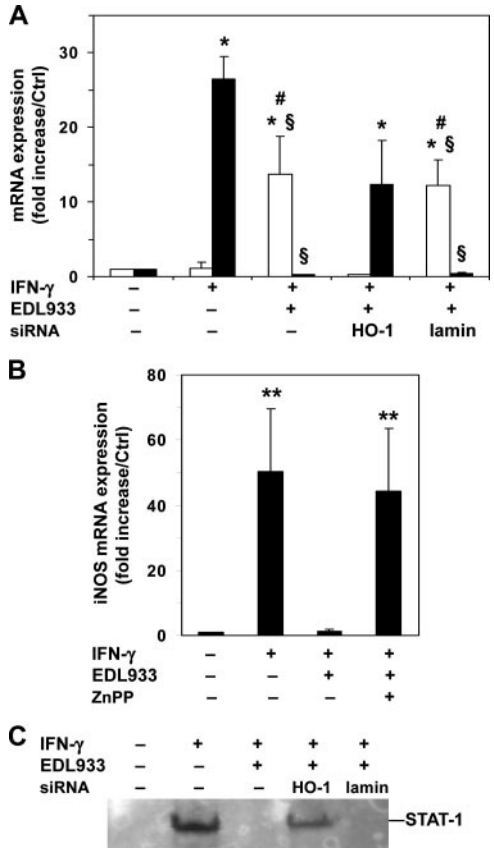

FIGURE 4. Inhibition of iNOS gene transcription by HO-1 in activated cells. *A*, *hmox-1* interference. Cells were transfected or not with HO-1 or lamin siRNA before a stimulation with IFN-γ, in the presence or absence of EDL933. Subsequently, the *hmox-1* (open bars) and *iNOS* (plain bars) mRNA expression levels were analyzed by real-time PCR after 2 and 6 h of infection, respectively. For both genes, $*$, $p < 0.05$ vs control cells; §, $p < 0.05$ vs cytokine-treated cells; #, $p < 0.05$ vs HO-1 siRNA-treated cells infected with EDL933 and stimulated with IFN-γ ($n = 4$). *B*, HO activity inhibition. Cells were treated with IFN-γ, EDL933, and/or ZnPP for 6 h. The level of iNOS mRNA was measured by real-time PCR. $**$, $p < 0.01$ vs control cells and EDL933-infected cells treated with IFN-γ ($n = 3$). *C*, Effect of HO-1 on STAT-1 activation. Nuclear proteins from cells transfected or not with HO-1 or lamin siRNA and activated for 3 h with IFN-γ and/or EDL933 were extracted and analyzed by EMSA. Similar results were obtained in two independent experiments.

inhibited using a STAT-1 or a JAK-2 inhibitor, but not by the NF-κB inhibitors Z-Leu-Leu-Leu-CHO and Bay 11–7082. Similar results were obtained when TNF-α and IL-1β were used to stimulate the cells together with IFN-γ (data not shown). Thus, these data suggest that iNOS transcription in activated epithelial cells involves the JAK-2/STAT-1 transduction pathway.

STAT-1 and NF-κB activation were then assessed by EMSA. As shown in Fig. 2*C*, we observed an increase in STAT-1 DNA-binding activity upon stimulation with IFN-γ in comparison to control cells. This enhancement was fully inhibited when EDL933 was added to the activated cells. Moreover, the basal STAT-1 activation observed in control cells was suppressed after infection with the bacteria. Oppositely, NF-κB DNA binding activity was not modulated by IFN-γ or by the strain EDL933.

These results were confirmed by immunofluorescence and confocal microscopy (Fig. 2*D*). The nuclear translocation of STAT-1 was evidenced in IFN-γ-treated Hct-8 cells, and was suppressed by the addition of the strain EDL933. NF-κB was detected only in the cytoplasm of control or IFN-γ-activated cells; its nuclear translocation was observed in $\sim$10% of EDL933-infected cells, as previously described (10).

We conclude that the inhibition of STAT-1 activation by EHEC blocks the induction of iNOS gene transcription.

The implication of HO-1 in iNOS mRNA expression inhibition

HO-1 activity has been previously described to inhibit iNOS induction (21–25). We therefore hypothesized that EHEC suppress iNOS mRNA expression through the activation of HO-1. We first analyzed *hmox-1* mRNA expression levels in Hct-8 cells in response to EHEC by RT-PCR (Fig. 3*A*) and semiquantitative PCR (Fig. 3*B*). The gene *hmox-1* was up-regulated 0.5 h after the beginning of the infection, reached a peak after 1 h, and decreased from this level at 2 and 3 h. Compared with uninfected cells, the level of HO-1 protein in human enterocytes was enhanced in response to a 3-h EHEC infection (Fig. 3*C*). IFN-γ or cytokines did not induce HO-1 in Hct-8 cells (Fig. 3*C*). Additionally, *hmox-1* was not up-regulated when the cells were infected with EcG1, EcG2, or DH5α (data not shown).

To investigate the role of HO-1 in EHEC-mediated iNOS induction inhibition in human enterocytes, we first used a siRNA-based strategy. Cells were first exposed to HO-1 or lamin siRNA, and were then were stimulated by IFN-γ and/or infected with EDL933; HO-1 and iNOS mRNA expression were subsequently assessed by real-time PCR (Fig. 4*A*). The HO-1 mRNA was expressed upon EDL933 infection when compared with uninfected cells, but was not induced by IFN-γ. Inversely, iNOS mRNA expression was up-regulated in the presence of IFN-γ but was not

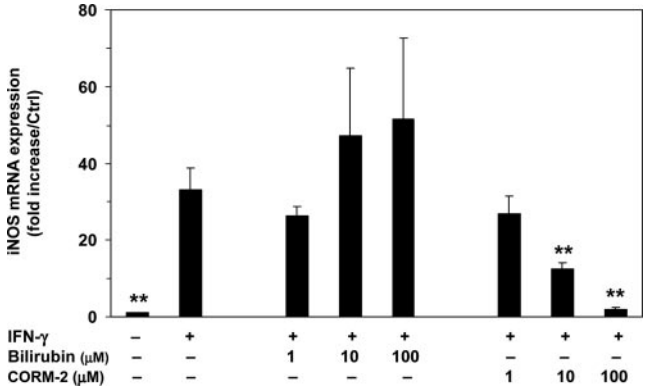

FIGURE 5. Hct-8 cells were stimulated for 6 h with IFN-γ in the presence or absence of various concentrations of bilirubin or CORM-2. Subsequently, iNOS gene transcription was analyzed by real-time PCR. **, $p < 0.01$ when compared with cells treated with IFN-γ ($n = 3$–4).

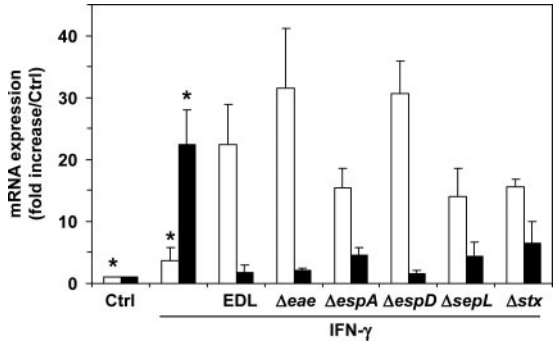

FIGURE 7. Effect of different mutant strains on *hmox-1* induction and iNOS transcription inhibition. Hct-8 cells were infected with EDL933 (EDL) or with isogenic mutants lacking *eae*, *espA*, *espD*, *sepL*, or *stx1/stx2* (*stx*) genes, in the presence of IFN-γ. The relative expression of *hmox-1* (open bars) and *iNOS* (plain bars) genes was measured by semiquantitative PCR after 2 and 6 h of infection, respectively. *, $p < 0.05$ vs the others conditions ($n = 3$–5).

stimulated by the bacteria. The expression of *hmox-1* in EDL933-infected cells was completely inhibited using HO-1 siRNA; this inhibition was concomitantly associated with the partial, but significant, restoration of iNOS mRNA expression. The lamin siRNA had no effect on *hmox-1* and iNOS mRNA expression in Hct-8 cells stimulated with IFN-γ and infected by EDL933. To further verify the association of HO-1 induction with the inhibition of iNOS mRNA expression, we used the HO inhibitor ZnPP in Hct-8 cells/EDL933 cocultures. In the presence of ZnPP, the EHEC-induced inhibition of iNOS mRNA expression was completely suppressed (Fig. 4*B*). Concomitantly, STAT-1 activation was partially restored using HO-1 siRNA, but not lamin siRNA, on cells activated by IFN-γ and infected with EDL933 (Fig. 4*C*).

To gain further insight into the mechanism by which HO-1 activity inhibits iNOS mRNA expression, we treated activated Hct-8 cells with the two main products of heme metabolism, namely bilirubin and CO. The IFN-γ-dependent iNOS gene expression was not modulated by various concentrations of bilirubin (Fig. 5). Inversely, a concentration-dependent inhibition of iNOS mRNA expression was observed using the CO donor CORM-2 (Fig. 5).

We next reasoned that EHEC-induced HO-1 activity may suppress iNOS mRNA expression in unstimulated epithelial cells. HO-1 expression was up-regulated by 11 ± 3.1-fold in EDL933-infected cells when compared with control cells; this increase was completely inhibited using HO-1 siRNA, but not lamin siRNA. However, iNOS mRNA expression was not induced in cells infected with EHEC in the presence or absence of HO-1 or lamin siRNA (Fig. 6).

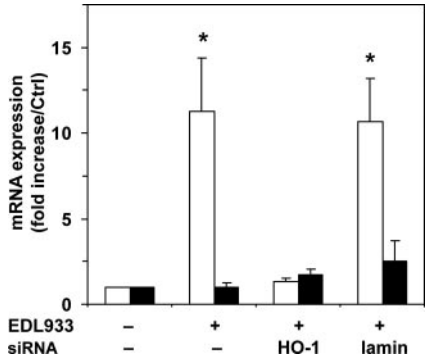

FIGURE 6. Cells were transfected or not with HO-1 or lamin siRNA before an infection with the strain EDL933. The expression of *hmox-1* (open bars) and *iNOS* (plain bars) genes were analyzed by real-time PCR after 2 and 6 h of infection, respectively. *, $p < 0.05$ vs control cells and infected cells treated with HO-1 siRNA ($n = 3$).

These results indicate that HO-1 activity is required for EHEC-mediated inhibition of STAT-1 activation and iNOS mRNA expression in IFN-γ-activated human enterocytes, but does not modulate iNOS transcription in untreated cells.

Toward the identification of the EHEC factor responsible for HO-1 induction

We analyzed the effect on *hmox-1* induction and on *iNOS* inhibition of the main bacterial factors implicated in the EHEC pathogenesis: intimin is encoded by the LEE gene *eae* and allows the intimate attachment of the bacteria to epithelial cells; EspA forms the needle of the type III secretion system; EspD protein is essential for the formation of surface appendages and is integrated in the cytoplasmic membranes of eukaryotic cells; SepL is involved in the translocation of bacterial factors; the main virulence factor is Stx encoded by the genes *stx1* and *stx2*. The gene *hmox-1* was up-regulated by 15–30-fold in Hct-8 cells cocultured with EDL933, *eae*-, *espA*-, *espD*-, *sepL*-, or *stx1-/stx2*- strains when compared with uninfected cells (Fig. 7). Concomitantly, there was no significant difference in the degree of down-regulation of iNOS mRNA in IFN-γ-treated Hct-8 cells between the wild-type strain and isogenic mutants (Fig. 7).

Discussion

Our results show that EHEC-induced HO-1 in human epithelial cells is a critical modulator of the innate immune response. Although HO-1 has been previously implicated in attenuating the inflammatory response (26, 27) and in the inhibition of iNOS gene expression (22–25), the present study is the first to demonstrate that a pathogenic bacterium suppresses NO production by inducing HO-1 in activated human colonic epithelial cells.

In our experimental conditions, activation of the transcription factor STAT-1 was required for induction of iNOS gene transcription in activated Hct-8 cells because a STAT-1 inhibitor or a JAK-2 inhibitor, but not NF-κB inhibitors, suppressed the IFN-γ-induced iNOS mRNA expression, as previously described with other cell types (14, 28). Similar to previous findings obtained with Hep-2 and T84 cells (9, 29), we found that EHEC interferes with STAT-1 activation in Hct-8 and Caco-2 cells activated with IFN-γ or with the three cytokines, without any effect on NF-κB. Nonetheless, we establish herein that EHEC inhibit the activation of STAT-1 by

inducing HO-1. The enzyme HO-1 catabolizes heme into CO, biliverdin that is rapidly converted to bilirubin, and iron (30). These products have antioxidant properties and may mediate the anti-inflammatory response of HO-1 (26, 31–33). It has been described (34) that the HO-1-dependent inhibition of the IFN-γ-STAT-1 signaling pathway in murine endothelial cells is dependent on the formation of bilirubin. Nonetheless, our experiments further indicate that bilirubin has no effect on iNOS gene expression in cytokine-treated Hct-8 cells; rather, iNOS mRNA expression was inhibited by the CO donor CORM-2. Therefore, we propose that CO is the key element of iNOS gene regulation, as recently established in Caco-2 cells (35). Aside from its role in heme degradation, HO-1 has a critical function in maintaining cellular homeostasis. HO-1-deficient mice do not normally survive and the animals that do survive die within a year while demonstrating signs of chronic inflammation in numerous organs (36). Thus, it is proposed that HO-1 serves as an adaptive mechanism to protect cells from oxidative stress (37). In this context, an increased expression of HO-1 has also been shown in pathophysiological states such as atherosclerosis (38), sepsis (39), *Helicobacter pylori*-positive and -negative gastritis, and ulcerative colitis (40); in these two last inflammatory diseases, HO-1 was immunodetected in superficial and cryptic epithelial cells. Additionally, the protective role of HO-1 has been demonstrated in experimental colitis in trinitrobenzene sulfonic acid and dextran sodium sulfate models (41, 42), and in IL-10-deficient mice (43). The preventive effect of HO-1 was correlated with decreased free radical production and the inhibition of iNOS expression in colonic tissues (41). Together with our findings, these reports suggest that HO-1 could be over-expressed in the human colonic epithelium during EHEC infection and may modulate the host inflammatory response and the development of the hemorrhagic symptoms.

When *hmox-1* expression was inhibited by siRNA, a partial restoration of STAT-1 activation and iNOS mRNA expression was observed. However, when HO-1 activity was inhibited by ZnPP, the transcription of the gene encoding iNOS was completely restored. According to this result, we hypothesize that the constitutive isoforms of HO, namely HO-2 and HO-3, could be implicated in part in iNOS gene down-regulation. Nonetheless, we might speculate EHEC suppress STAT-1 activation by others mechanisms than HO-1 induction or inhibit iNOS transcription by acting on different transcription factors. Intriguingly, O157:H7 EHEC express an HO encoded by the gene *chuS* (44). This gene is shared by other pathogenic bacteria such as *Corynebacterium diphtheriae* (45) or *Neisseria meningitides* (46), but is not present in nonpathogenic *E. coli* laboratory strains. It has been previously demonstrated that a bacterial enzyme, namely the arginase of *Helicobacter pylori*, can act as a potent regulator of iNOS activity in eukaryotic cells (12). Thus, we constructed a *chuS* mutant in the EDL933 background to determine the implication of bacterial HO on host iNOS mRNA expression. We did not find any differences in the levels of iNOS induction in cells infected with EDL933 or with the isogenic mutant *chuS*, in the presence or absence of cytokines (data not shown). These results demonstrate that EHEC HO does not modulate iNOS gene expression in human enterocytes.

High-output NO formation in pathological conditions may determine pathogen behavior and host susceptibility. Therefore, pathogenic bacteria have elaborated different strategies to modulate iNOS expression and NO production (12). The iNOS gene is up-regulated in human epithelial cells infected with enteroinvasive bacteria such as *E. coli* or *Salmonella dublin* (47, 48), or in IFN-γ-primed enterocytes infected with *S. dublin* (13). Conversely, we and others have observed that iNOS is not directly induced in colonic epithelial cells by EHEC (this work) and enteropathogenic *E. coli* (EPEC; Ref. 49) that develop extracellularly. Furthermore, these bacteria inhibit the IFN-γ-induced iNOS mRNA expression (this work and Ref. 49). However, NF-κB activation is the main target for EPEC (49), whereas our data highlight that EHEC disrupt the STAT-1 signaling. We suggest that this discrepancy can be explained by the different technical approaches to stimulate iNOS mRNA expression (cytokine concentration, time of stimulation). Alternatively, EPEC and EHEC may have elaborated different strategies to disrupt cellular signalization and limit NO production. In this respect, it has been described that EHEC but not EPEC disrupt the IFN-γ-STAT-1 signaling in human epithelial cells (29). Moreover, the EPEC inhibitory effect on iNOS induction is linked to the LEE-encoded type three secretion system because a *sep-2* mutant strain that lacks a functional secretion machinery fails to repress the cytokine-induced NO production (49), whereas we show that 1) LEE positive and LEE negative EHEC strains are potent inhibitors of iNOS mRNA expression, and 2) LEE gene isogenic mutants exhibit the same inhibitory effect as the parental LEE-positive strain EDL933. Thus, we suggest that adhesion and the type III secretion system are not involved in inhibition of NO production. Moreover, our experimental data indicate that Stx is not involved in HO-1 induction and iNOS inhibition. The identification of the bacterial factor(s) responsible for *hmox-1* expression and NO production inhibition deserves additional investigation. Such research is currently underway in our laboratory.

The immune function of NO refers to its ability to modulate the inflammatory process and to kill pathogens. Moreover, we recently demonstrated that chemical or cellular sources of NO inhibit the expression of the gene *stx2* and the synthesis of Stx2, without affecting the growth and the survival of the bacteria (15). It should be noted that, in our previous report, we first stimulated the cells with cytokines for 18 h to induce iNOS, before adding EHEC (15); in the present study, Hct-8 cells were stimulated with cytokines and EHEC at the same time. Based on these both findings, it is tempting to speculate that EHEC suppress iNOS mRNA expression to decrease the NO-dependent Stx production inhibition. Therefore, limiting host NO production in human enterocytes may represent a strategy elaborated by EHEC to favor their own virulence.

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Disclosures

The authors have no financial conflict of interest.

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