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Bacteroides fragilis Enterotoxin Upregulates Intercellular Adhesion Molecule-1 in Endothelial Cells via an Aldose Reductase-, MAPK-, and NF-κB–Dependent Pathway, Leading to Monocyte Adhesion to Endothelial Cells

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Enterotoxigenic Bacteroides fragilis (ETBF) produces a ~20-kDa heat-labile enterotoxin (BFT) that plays an essential role in mucosal inflammation. Although a variety of inflammatory cells is found at ETBF-infected sites, little is known about leukocyte adhesion in response to BFT stimulation. We investigated whether BFT affected the expression of ICAM-1 and monocyte adhesion to endothelial cells (ECs). Stimulation of HUVECs and rat aortic ECs with BFT resulted in the induction of ICAM-1 expression. Upregulation of ICAM-1 was dependent on the activation of IκB kinase (IKK) and NF-κB signaling. In contrast, suppression of AP-1 did not affect ICAM-1 expression in BFT-stimulated cells. Suppression of NF-κB activity in HUVECs significantly reduced monocyte adhesion, indicating that ICAM-1 expression is indispensable for BFT-induced adhesion of monocytes to the endothelium. Inhibition of JNK resulted in a significant attenuation of BFT-induced ICAM-1 expression in ECs. Moreover, inhibition of aldose reductase significantly reduced JNK-dependent IKK/NF-κB activation, ICAM-1 expression, and adhesion of monocytes to HUVECs. These results suggest that a signaling pathway involving aldose reductase, JNK, IKK, and NF-κB is required for ICAM-1 induction in ECs exposed to BFT, and may be involved in the leukocyte–adhesion cascade following infection with ETBF. The Journal of Immunology, 2011, 187: 1931–1941.

Infection by enterotoxigenic Bacteroides fragilis (ETBF) strains is associated with diarrheal illness, colitis, inflammatory bowel diseases (1–3), and colorectal cancer (4). B. fragilis enterotoxin (BFT), a ~20-kDa heat-labile metalloprotease, is regarded as a virulence factor for these diseases. Exposure to BFT results in the infiltration of a variety of inflammatory cells and destruction of the mucosal epithelial cell layer (3, 5, 6). The initial recruitment and activation of inflammatory cells are important for bacterial pathogenesis at the site of ETBF infection.

Inflammatory cell recruitment is a fundamental phenomenon of the tissue response to bacterial infection. Endothelial cells (ECs) play a critical role in this response via their ability to express cell surface adhesion molecules that mediate interactions with leukocytes in the bloodstream (7). Several surface adhesion molecules are well characterized in ECs, such as ICAM-1 (CD54) and VCAM-1 (CD106). In addition, ECs can express surface molecules involved in rolling (e.g., P- and E-selectin) and can secrete an array of chemokines involved in attracting inflammatory cells (e.g., IL-8 [CXCL8] and MCP-1 [CXCL2]) (8).

ICAM-1 interacts with LFA-1 or macrophage adhesion ligand-1 (Mac-1), members of the β2 integrin family, which are expressed on activated leukocytes. The interaction between LFA-1 and ICAM-1 or Mac-1 and ICAM-1 is responsible for the firm interaction between leukocytes and ECs, and is therefore important for subsequent processes (9). Expression of ICAM-1 on ECs is upregulated by several stimuli, including inflammatory cytokines (e.g., IL-1 and TNF-α) and bacterial infection. Interestingly, Clostridium difficile toxin A markedly enhanced the gene expression of ICAM-1 within 2 h of treatment of mice with this toxin (10). Porphyromonas gingivalis strains or their flagellin proteins significantly increased the expression of surface ICAM-1 molecules (11, 12), and flagellin from clinical isolates of Escherichia and Salmonella strains increased expression of ICAM-1 and enhanced leukocyte transendothelial cell migration (13). In light of these reports, it is possible that increased ICAM-1 expression may be one of the inflammatory responses to stimulation with ETBF-derived BFT.

Activation of NF-κB or AP-1 is known to be important for the induction of leukocyte adhesion molecules in ECs (14, 15). In addition, endothelial expression of ICAM-1 and VCAM-1 is facilitated by MAPK (16–18). Aldose reductase (AR) catalyzes the NADPH-dependent conversion of glucose to sorbitol, the first step in polyol pathway of glucose metabolism. AR is known to be involved in the expression of adhesion molecules in ECs (19). In addition, AR was required for TNF-α–induced activation of the MAPK pathway and neutrophil adhesion to ECs (20). These ob-
servations raise the possibility that the signaling molecules may be activated in BFT-exposed ECs to regulate the expression of adhesion molecules. However, there is no evidence that BFT-induced signaling leads to ICAM-1 expression in ECs, although some of those signaling molecules have been reported to be activated in intestinal epithelial cells stimulated with BFT (5, 21–26).

In the current study, we investigated ICAM-1 induction in response to BFT stimulation, and found that EC exposure to BFT resulted in the upregulation of ICAM-1 through activation of a signaling pathway involving AR, JNK MAPK, IκB kinase (IKK), and NF-κB, finally culminating in monocyte adhesion to ECs.

Materials and Methods

Reagents

Medium 199, LPS-free FBS, antibiotics, l-glutamine, TRIzol, and Ca²⁺ and Mg²⁺-free HBSS were obtained from Life Technologies BRL (Gaithersburg, MD). Calcein AM, curcumin, endothelial cell growth supplement, heparin, BSA, and RPMI 1640 medium were purchased from Sigma-Aldrich (St. Louis, MO). mAbs against IκBα, IKK-α, IKK-β, phospho-IKK-α/β, pan-ERK 1/2 (ERK1/2, p44/p42), phospho-ERK1/2, pan-JNK (p54/p46), phospho-JNK, pan-p38, phospho-p38, and actin were acquired from Cell Signaling Technology (Beverly, MA). Abs against ICAM-1, endothelin-1/2/3, p50, p52, p65, c-Rel, Rel B, c-Jun, c-Fos, Jun-B, Jun-D, and Fos-B were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit and anti-mouse secondary Abs conjugated to HRP were purchased from Transduction Laboratories (Lexington, KY). Anti-mouse FITC-conjugated secondary goat Ab was obtained from Chemicon (Temecula, CA). BMS345541, PD98059, SB203580, and SP600125 were acquired from Calbiochem (La Jolla, CA).

Purification of BFT and cell culture conditions

BFT was purified from the culture supernatants of a highly toxigenic strain of ETBF, as described previously (5, 21–25). The purity of the BFT preparations was confirmed by SDS-PAGE. Typical preparations of BFT contained 0.5–1.2 mg protein/ml as measured by the bicinchoninic acid protein assay. Buffers used in the purification were prepared using LPS-free HBSS.

FIGURE 1. Expression of ICAM-1 surface molecules in ECs stimulated with BFT. HUVECs (A) and RAOECs (B) were treated with BFT (100 ng/ml) for 24 h. Cells were stained with a mAb against ICAM-1, and were analyzed using flow cytometry. Results are representative of more than five independent experiments. C, HUVECs were incubated with or without BFT (100 ng/ml) for 24 h. Cells were stained with anti–ICAM-1 Ab (green) and DAPI (blue, nucleus), and were visualized under a fluorescent microscope (original magnification ×400). Results are representative of three independent experiments.
free distilled water. The activity of LPS in BFT solutions (1 mg/ml) was less than 1 endotoxin U/ml (Pyroset test kit, quantitative chromogenic Limulus amebocyte lysate; Associates of Cape Cod). Using the HEK-Blue LPS detection kit (InvivoGen, San Diego, CA), with a detection limit of 3 ng/ml, the amount of LPS in BFT solutions (1 mg/ml) was <3 ng/ml. BFT was frozen in aliquots at −80°C immediately after purification.

HUVECs (purchased from Cambrex Bio Science, Walkersville, MD) were cultured in medium 199 containing 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), heparin (50 µg/ml), and endothelial cell growth supplement (50 µg/ml). Primary rat aortic ECs (RAOECs) were obtained from Genlantis (San Diego, CA) and were cultured according to the manufacturer’s instructions. Third to seventh passages of ECs were used for experiments. The human monocytic THP-1 cell line (ATCC TIB-202) was cultured in RPMI 1640 medium, supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 1.5 mM L-glutamine, 1 mM sodium pyruvate, and 2-ME (0.05 mM). Low-passage–number THP-1 cells (<passage 20) were used for all experiments. HUVECs, RAOECs, and THP-1 cells were maintained in a 5% CO2 incubator at 37°C.

Quantitative RT-PCR

Cells were treated with BFT, after which total cellular RNA was extracted using TRIzol. Reverse transcription and PCR amplification were performed, as described previously (27). The primers and expected PCR product sizes were as follows: human ICAM-1, 5′-CTG GGT TGT CCT TGG ACA CT-3′ (antisense), 3′-CTG GCC ACC GCA GCA CC-3′ (sense), 260 bp (NM_001101.3 Homo sapiens ICAM1, mRNA) (28); human VCAM-1, 5′-ATG ACA TGC TTG AGC CAG G-3′ (antisense), 5′-TGA CAC GGT GAA GGT TTT-3′ (sense), 99 bp (NM_001199834.1 Homo sapiens VCAM1, transcript variant 3, mRNA) (11); human β-actin, 5′-TCG CCT CAT GCT ATG GCT G-3′ (antisense), 5′-CTC AGC CTC GCT ATG GCT G-3′ (sense), 284 bp (NM_031144.2 Rattus norvegicus Vcam1, mRNA) (30); rat β-actin, 5′-GTC GGC CGC TCT AGG CAC CAA-3′ (sense), 5′-CTT TTC GAT GTC ACG CAC GAT TTC-3′ (antisense), 648 bp (NM_012889.1 Rattus norvegicus Icam1, mRNA) (31). To quantify mRNA molecules, standard RNAs for human ICAM-1, human VCAM-1, rat ICAM-1, rat VCAM-1, and rat β-actin were generated by in vitro transcription using T7 RNA polymerase, as described previously (32). Standard RNA for human β-actin was provided by M. F. Kagnoff (University of California, San Diego, CA). The sizes of PCR products generated from standard RNAs for human ICAM-1, human VCAM-1, human β-actin, rat ICAM-1, rat VCAM-1, and rat β-actin are 480, 384, 520, 482, 396, and 746 bp, respectively.

Flow cytometric analysis

After the indicated periods of incubation with BFT, monolayers of HUVECs or RAOECs were washed with cold Ca2+- and Mg2+-free HBSS twice and trypsinized at 37°C for 3 min. Resuspended cells were centrifuged at 200 × g for 5 min at 4°C, and then washed with HBSS containing 0.5% BSA. The cells were transferred to flow cytometry tubes and centrifuged at 200 × g for 5 min at 4°C, and then the supernatants were discarded. Thereafter, the cells were incubated with anti-human ICAM-1 mouse mAb with 0.5% BSA for 3 h on ice. The cells were washed with cold HBSS containing 0.5% BSA twice, and then incubated with anti-mouse FITC-conjugated secondary goat Ab on ice and in the dark. After 1 h, the cells were washed with cold HBSS with 0.5% BSA twice. The cells were analyzed by flow cytometry (FACSCalibur cytometer; BD Biosciences, San Jose, CA). Ten thousand cells were analyzed per sample, and the expression level of ICAM-1 is expressed as mean fluorescence intensity (MFI) (33).

Immunofluorescence assay

To measure adhesion of monocytes to ECs, THP-1 cells were stained with calcein AM (4 µM) for 30 min at 37°C. Stained THP-1 cells (1 × 10⁶/ml) were added to BFT-exposed HUVECs. After a further incubation for 30 min at 37°C, cells were fixed with cooled methanol/acetone (1:1) at −20°C for 10 min. To stain ECs, primary Ab (anti–endothelin-1/2/3) was added for 1 h. Cells were washed with PBS before the secondary Ab (anti-rabbit

FIGURE 2. Time course of ICAM-1 mRNA expression in ECs after treatment with BFT. HUVECs (A) and RAOECs (B) were treated with BFT (100 ng/ml) for the indicated periods. Levels of ICAM-1, VCAM-1, and β-actin mRNA were analyzed by quantitative RT-PCR using standard RNAs. The values are expressed as mean ± SD (n = 3). Asterisks indicate statistical significance in comparison with unstimulated controls (p < 0.05).
Cy3) was added for an additional 1 h. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) was applied to the cells, and cells were then analyzed under a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany).

To detect surface ICAM-1 molecules, BFT-treated HUVECs were fixed with 1% paraformaldehyde for 1 h, after which cells were incubated with anti-human ICAM-1 mouse mAb for 3 h and then incubated with anti-mouse FITC-conjugated secondary goat Ab for 1 h. After treatment with 0.5% DAPI in HBSS for 5 min, cells were analyzed by a fluorescence microscopy.

**EMSAs**

Cells were harvested and nuclear extracts were prepared, as described previously (25). Concentrations of protein in the extracts were determined by the Bradford assay (Bio-Rad, Hercules, CA). EMSAs were performed, according to the manufacturer’s instructions (Promega, Madison, WI). In brief, 5 µg nuclear extract was incubated for 30 min at room temperature with a γ-32P-labeled oligonucleotide probe (5’-AGT TGA GGG GAC TTT CCC AGG C-3’ for the NF-κB binding site; 5’-CGC TTG ATG ACT CAG CCG GAA-3’ for the AP-1 binding site). After incubation, both bound DNA and free DNA were resolved on 5% polyacrylamide gels, as described previously (25, 34). Supershift assays were used to identify the specific members of the NF-κB or AP-1 families activated by BFT stimulation. EMSAs were performed, as described above, except that rabbit Abs (1 µg/reaction) against NF-κB proteins p50, p52, p65, c-Rel, or Rel B were added during the binding reaction period. For AP-1 supershift assays, rabbit Abs (1 µg/reaction) against c-Jun, c-Fos, Jun-B, Jun-D, or Fos-B were used, as described previously (25, 34).

**Plasmids, transfection, and luciferase assays**

Small interfering RNAs (siRNA) against the p65 subunit of NF-κB and c-jun were obtained from Qiagen (Valencia, CA) and Santa Cruz Biotechnology, respectively. Negative (nonsilencing) control RNA (NS-RNA) was added for an additional 1 h. Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA) was applied to the cells, and cells were then analyzed under a fluorescence microscope (Leica DM 5000B, Wetzlar, Germany).

**FIGURE 3.** Activation of NF-κB and IKK in ECs stimulated with BFT. A, HUVECs were treated with BFT (100 ng/ml) for the indicated periods. NF-κB DNA-binding activity was assessed by EMSA. B, Immunoblot results for concurrent IkBα, phospho-IKK-α/β, IKK-α, IKK-β, and actin expression levels in HUVECs under the same conditions are provided beneath the EMSA. The results are representative of three independent experiments. C, RAOCs were treated with BFT (100 ng/ml) for the indicated periods. NF-κB DNA-binding activity was assessed by EMSA. The results are representative of three independent experiments. D, Supershift assays using nuclear extracts from HUVECs treated with BFT (100 ng/ml) for 1 h were performed using Abs to p50, p52, p65, c-Rel, and Rel B. The results are representative of three independent experiments.

**FIGURE 4.** Effects of NF-κB suppression on ICAM-1 expression in HUVECs stimulated with BFT. A, HUVECs were transfected with either retrovirus containing IkBα-superrepressor (IkBα-AA) or control virus (GFP). At 48 h after transfection, the cells were stimulated with BFT (100 ng/ml) for 1 h. NF-κB–binding activity was assayed by EMSA. +, Represents the positive control in which HUVECs were treated with TNF-α (20 ng/ml). B, Transfected cells were treated with BFT (100 ng/ml) for 9 h. Levels of ICAM-1 mRNA were analyzed by quantitative RT-PCR using standard RNA. The values are expressed as mean ± SD (n = 5). The β-actin mRNA levels in each group remained relatively constant throughout the same period (∼106 transcripts/mg total RNA). C, HUVECs were transfected with an ICAM-1–luciferase transcriptional reporter together with retrovirus containing IkBα-superrepressor (IkBα-AA) or control virus (GFP), as indicated. After 48 h, the transfected cells were stimulated with BFT (100 ng/ml) for another 9 h, after which luciferase assays were performed. Data are expressed as mean fold induction in luciferase activity relative to unstimulated controls ± SEM (n = 5). The mean fold induction of the β-actin reporter gene relative to unstimulated controls remained relatively constant throughout each experiment. Asterisks indicate statistical significance in comparison with untransfected cells stimulated with BFT (p < 0.05).
was purchased from Qiagen. Cells in 6-well dishes were transfected with siRNA or nonsilencing siRNA using FuGene6 transfection reagent (Roche, Mannheim, Germany), as described previously (33). Briefly, 1 μg siRNA or nonsilencing siRNA was diluted in serum-free medium to produce a final volume of 100 μl, which was incubated with 3 μl Fugene 6 for 15 min at room temperature. The transfection mixture was added to the respective wells, each containing 300 μl medium (10% FBS content). Transfections were incubated for 48 h prior to the assay.

Reporter plasmids containing pICAM-1, p2x NF-κB-, pβ-actin-, and pRSV-β-galactosidase-luciferase were provided by M. F. Kagnoff (University of California, San Diego, CA) (35). Cells in 6-well dishes were transfected with 1.5 μg plasmid DNA using FuGene6 transfection reagent (Roche). The transfected cells were incubated for 24 h at 37˚C in a 5% CO2 incubator, and were then treated with BFT for the indicated times. Luciferase activity was determined in accordance with the manufacturer’s instructions (Tropix, Bedford, MA). Light release was quantitated for 10 s using a luminometer (MicroLumat Plus; Berthold, Bad Wildbad, Germany), as described previously (21).

A retroviral system containing a mammalian expression vector encoding a hemagglutinin epitope-tagged mutant IκBa (IκBa-AA) with substitutions of serine for alanine at positions 32 and 36 was used to block NF-κB activation, as described previously (36).

Immunoblots and ELISA

Cells were washed with ice-cold PBS and lysed in 0.5 ml/well lysis buffer (150 mM NaCl, 20 mM Tris [pH 7.5], 0.1% Triton X-100, 1 mM PMSF, and 10 μg/ml aprotonin). Fifteen to 50 μg protein per lane was size fractionated on a 6% polyacrylamide minigel (Mini-PROTEIN II; Bio-Rad) and electrophoretically transferred to a nitrocellulose membrane (0.1-μm pore size). The immunoreactive proteins to which the primary Abs had bound were visualized using goat anti-rabbit or anti-mouse secondary Abs conjugated to HRP, followed by ECL (ECL system; Amersham Life Science, Buckinghamshire, U.K.) and exposure to x-ray film.

The role of AR was evaluated by pretreatment with specific pharmacologic inhibitors, sorbinil and zopolrestat, for 24 h at the indicated concentration. The activity of JNK, p38, and ERK1/2 following BFT stimulation was evaluated by FACE ELISA kits (Active Motif, Carlsbad, CA), in accordance with the protocols provided with the kits. A HTScan IKK-b kinase assay kit was purchased from Cell Signaling Technology. The assay was performed, according to the manufacturer’s instruction (37).

Statistical analyses

Data of quantitative RT-PCR are presented as mean ± SD, and data of MFI, luciferase assays, and ELISA are presented as mean ± SEM. Wilcoxon’s rank sum test was used for statistical analysis. The p values <0.05 were considered statistically significant.

Results

BFT upregulates ICAM-1 expression in ECs

Stimulation of HUVECs with BFT increased the expression of ICAM-1 surface molecules, as assessed by flow cytometry (Fig. 1A). Similar increases in ICAM-1 expression were observed fol-
lowing BFT stimulation of RAOECs (Fig. 1B). To confirm the expression of ICAM-1 surface molecules, immunohistochemical analyses were performed using the anti–ICAM-1 Ab. As shown in Fig. 1C, the surface expression of activated ICAM-1 molecules in BFT-stimulated HUVECs was higher than that of unstimulated cells.

We next measured the expression of ICAM-1 mRNA using quantitative RT-PCR. Increased ICAM-1 mRNA expression in HUVECs was first noted at 1 h after stimulation. Levels of ICAM-1 mRNA peaked at 12 h poststimulation, and decreased to baseline thereafter (Fig. 2A). Similar to HUVECs, BFT-stimulated RAOECs also showed increased ICAM-1 mRNA expression compared with control cells (Fig. 2B). In contrast to ICAM-1, expression of VCAM-1 mRNA in HUVECs and RAOECs was constitutive and not affected by BFT stimulation. Levels of VCAM-1 transcripts in each cell ranged from \( \sim 10^6 \) to \( \sim 10^7 \) transcripts/\( \mu \)g cellular RNA. The \( \beta \)-actin mRNA levels in each experiment remained relatively constant throughout the same period.

The stimulation of HUVEC with increasing concentration of BFT was paralleled by increased promoter activity of ICAM-1 gene. At BFT concentrations of 10, 100, and 500 ng/ml, the promoter activity of ICAM-1 increased 102, 138, 188, and 179\% 24 h after the stimulation, respectively (mean percentage induction of luciferase activity relative to untreated controls, \( n = 3 \)). However, heat-inactivated BFT, which was heated for 30 min at 60 °C, did not activate the ICAM-1 reporter genes in HUVECs. To confirm the absence of an effect on ICAM-1 by contaminated LPS, HUVECs were treated with small amount of Escherichia coli LPS (3 ng/ml) for 24 h. The treatment of LPS did not significantly increase ICAM-1 expression, assessed by flow cytometry (control, 19.1 ± 2.5; LPS, 18.1 ± 2.4; mean of MFI ± SD, \( n = 3 \)).

**FIGURE 6.** Effects of AP-1 inhibition on ICAM-1 expression in ECs stimulated with BFT. HUVECs (A) and RAOECs (B) were stimulated with BFT (100 ng/ml) for the indicated periods of time. AP-1 activity was assessed by EMSA. The results are representative of three repeated experiments. C, Supershift assays were performed using Abs to c-Jun, c-Fos, Jun B, Jun D, and Fos B in HUVECs stimulated with BFT for 2 h. Results are representative of three independent experiments. D, HUVECs were transfected with siRNA against c-jun or NS-RNA for 48 h. The transfected cells were stimulated with BFT (100 ng/ml) for 2 h. AP-1 activity was assessed by EMSA. The results are representative of three repeated experiments. E, HUVECs were transfected with siRNA against c-jun or NS-RNA for 48 h. Upper panel, The transfected cells were stimulated with BFT (100 ng/ml) for 9 h. Levels of ICAM-1 mRNA were analyzed by quantitative RT-PCR using standard RNA. The values are expressed as mean ± SD (\( n = 5 \)). The \( \beta \)-actin mRNA levels in each group remained relatively constant throughout the same period (\( \sim 10^6 \) transcripts/\( \mu \)g total RNA). Bottom panel, Transfected or untransfected cells were stimulated with BFT (100 ng/ml) for 24 h. Cells were stained with mAb against ICAM-1 and were analyzed using flow cytometry. The data represent the MFI ± SEM (\( n = 5 \)). *\( p < 0.05 \).

**NF-κB activation is required to upregulate ICAM-1 expression in BFT-stimulated ECs**

Because the transcription factor NF-κB has been shown to be activated by stimulation of intestinal epithelial cells with BFT (21, 22, 24–26), we examined whether BFT stimulation could activate NF-κB in ECs. For this experiment, DNA-binding studies for NF-κB were performed after stimulation with BFT. As shown in Fig. 3A, stimulation of HUVECs with BFT increased NF-κB DNA binding, as assessed by EMSA. In contrast, degradation of IκB was observed in BFT-stimulated cells. Stimulation of HUVECs with BFT increased levels of phosphorylated IκK-α/β, which were first observed 10 min after stimulation and increased during the experimental period (Fig. 3B). Similar NF-κB DNA-binding results were observed in BFT-treated RAOECs (Fig. 3C).

NF-κB exists as either homo- or heterodimeric complexes (38). To identify the specific NF-κB subunits comprising the NF-κB signal detected by EMSAs in BFT-stimulated HUVECs, supershift assays were performed. Abs specific to p50, p52, p65, c-Rel, and Rel B were used for these experiments. The supershift experiments demonstrated that Abs to p65 and p50 shifted the NF-κB complex.
signal significantly. In contrast, anti-p52, anti–c-Rel, or anti-Rel B Abs did not shift the NF-κB signal (Fig. 3D). These results suggest that NF-κB activation by BFT stimulation is mediated predominantly by heterodimers of p65/p50 and homodimers of p65/p65.

We next evaluated whether NF-κB activation by BFT stimulation was associated with ICAM-1 expression in HUVECs. For this experiment, HUVECs were transfected with retrovirus-IκB-α AA. The transfected cells were then stimulated with BFT for 1 h, and the NF-κB DNA-binding activity was assessed by EMSA. Transfection with retrovirus-IκB-α AA suppressed NF-κB activity to control level in BFT-stimulated HUVECs; however, control retrovirus containing a GFP plasmid (retrovirus-GFP) did not reduce NF-κB activation (Fig. 4A). Cells transfected with retrovirus-IκB-α AA were stimulated with BFT for 9 h, and the level of ICAM-1 mRNA was determined by quantitative RT-PCR. Transfection with retrovirus-IκB-α AA inhibited ICAM-1 mRNA expression (Fig. 4B). Consistent with this, the ICAM-1–luciferase activity induced by BFT stimulation decreased significantly when the NF-κB signal was blocked (Fig. 4C). In this experimental system, transfection with retrovirus-IκB-α AA resulted in a significant decrease in the MFI values of ICAM-1 compared with untransfected or retrovirus-GFP–transfected cells treated with BFT (Fig. 5A, left panel). Because activation of p65/p50 heterodimeric and p65/p65 homodimeric NF-κB in response to BFT stimulation was observed (Fig. 3D), we performed another experiment using p65 siRNA to suppress the NF-κB signal. Blocking NF-κB with p65 siRNA significantly attenuated the BFT–induced increase in ICAM-1 expression, as assessed by MFI values (Fig. 5A, right panel). However, the NS-RNA had no significant effect.

To confirm the association between NF-κB activation and ICAM-1 expression in RAOECs, RAOECs were preincubated with the NF-κB inhibitor MG-132 for 30 min and then treated with BFT for another 9 h. Levels of ICAM-1 mRNA were analyzed by quantitative RT-PCR using standard RNA. As shown in Fig. 5B, combined treatment of RAOECs with the NF-κB inhibitor MG-132 and BFT led to a significant decrease in ICAM-1 mRNA expression compared with treatment with BFT alone. In addition, combined treatment with MG-132 and BFT resulted in lower ICAM-1 MFI values than BFT alone (Fig. 5C). These results demonstrate a direct connection between NF-κB–dependent signaling and ICAM-1 induction in ECs stimulated with BFT.

**AP-1 is not involved in the induction of ICAM-1 in BFT-stimulated ECs**

Because the promoter region of the ICAM-1 gene contains binding sites for NF-κB and AP-1, we determined whether BFT could activate AP-1 in ECs. DNA-binding studies were performed using nuclear extracts after stimulation of HUVECs or RAOECs with BFT. Stimulation of these cells with BFT increased AP-1-DNA–binding activity, as shown by EMSA (Fig. 6A). Similar results were obtained in RAOECs (Fig. 6B). To further explore the effect of BFT on AP-1 activation, the DNA-binding activities of individual members of the AP-1 family were examined using a supershift assay. As shown in Fig. 6C, the entire AP-1 signal disappeared after treatment with Abs to c-Jun and c-Fos. However, the addition of Abs to Jun B, Jun D, or FosB did not affect the AP-1 signal induced by BFT. These results indicate that stimulation of ECs with BFT can activate AP-1 with c-Jun/c-Fos heterodimers.

Transfection with siRNA against c-Jun was used to suppress AP-1 activity in HUVECs. The c-Jun siRNA almost completely suppressed AP-1 activity in HUVECs stimulated with BFT (Fig. 6D). In this experimental system, HUVECs transfected with c-Jun siRNA or NS-RNA were stimulated with BFT for 9 h, and the level of ICAM-1 mRNA was determined by quantitative RT-PCR. ICAM-1 mRNA expression was not significantly inhibited by transfection with c-Jun siRNA compared with untransfected cells under the BFT-treated condition. In addition, transfection with c-Jun siRNA did not reduce the increase in ICAM-1 protein expression induced by BFT stimulation (Fig. 6E). Consistent with these results, pretreatment of cells with the AP-1 inhibitor curcumin before BFT stimulation did not result in a significant change in mRNA expression or MFI values of ICAM-1, although stimulation with BFT increased the levels of ICAM-1 mRNA transcript and the MFI values in ROAECs (Fig. 6F). These results suggest that AP-1 signaling is not involved in the induction of ICAM-1 in ECs stimulated with BFT.

**Suppression of NF-κB activation inhibits monocyte adhesion to ECs in response to BFT stimulation**

To evaluate whether BFT exposure could affect monocyte adhesion to the endothelium, monocytic THP-1 cells were incubated with...
BFT-treated HUVECs. BFT-treated HUVECs promoted a significant increase in THP-1 cell adherence (Fig. 7A). In this experimental system, pretreatment of HUVECs with the NF-κB inhibitor MG-132 before BFT stimulation resulted in a significant decrease in monocyte adhesion to HUVECs. In addition, pretreatment of HUVECs with the IKK inhibitor BMS345541 significantly reduced monocyte adhesion to HUVECs. In contrast, pretreatment of HUVECs with the AP-1 inhibitor curcumin did not influence monocyte adhesion to HUVECs compared with BFT alone (Fig. 7B).

**MAPKs are associated with ICAM-1 induction in BFT-stimulated ECs**

BFT strongly activated the phosphorylation of ERK1/2, p38, and JNK in HUVECs. Activation of all three MAPK signaling molecules was first noted 5 min after stimulation. Maximum activation of all three MAPKs was seen after 30 or 60 min, and then subsequently decreased (Fig. 8A). We next evaluated whether inhibition of MAPK activity influenced ICAM-1 induction in BFT-stimulated HUVECs. The following kinase inhibitors were used: PD98059, an inhibitor of MEK1/2, a MAPK that phosphorylates ERK1/2; pyridinyl imidazole SB203580, which specifically inhibits p38; and SP600125, which inhibits JNK. Pretreatment of HUVECs with SB203580 (≥50 μM), SP600125 (≥10 μM), or PD98059 (≥20 μM) for 30 min significantly inhibited the BFT-induced activation of ICAM-1 (Fig. 8B). Notably, the JNK inhibitor SP600125 was superior to PD98059 at reducing ICAM-1 gene activation. To confirm these results, MFI values of ICAM-1 surface molecules in BFT-stimulated ECs were measured using flow cytometric analyses. As shown in Fig. 8C, SP600125 and PD98059 significantly reduced the MFI of ICAM-1 in BFT-stimulated HUVECs compared with BFT alone. In this experimental system, the JNK inhibitor SP600125 decreased the MFI of ICAM-1 to greater extent than PD98059. Similar results were observed in ROAECs (Fig. 8D). Together, these results suggest that BFT-induced JNK signaling may be more indispensable for the induction of ICAM-1 expression than ERK or p38 signaling.
Inhibition of AR suppresses JNK, IKK, and NF-κB signaling, leading to ICAM-1 induction in BFT-stimulated ECs

We further investigated whether AR activity may be required for the induction of ICAM-1 molecules in BFT-stimulated HUVECs. To address this hypothesis, the effect of AR inhibitors such as sorbinil and zoporelatstat was evaluated. HUVECs were pretreated with AR inhibitors before cells were stimulated with BFT. In a dose-dependent manner, sorbinil and zoporelatstat inhibited the activation of BFT-induced JNK and ERK (Fig. 9A, 9B). Notably, sorbinil and zoporelatstat definitely inhibited BFT-induced JNK activation at entire experimental period compared with BFT-induced ERK activation. Activation of p38 MAPK, however, was not significantly inhibited by pretreatment with sorbinil and zoporelatstat (Fig. 9C). In this experimental system, AR inhibitors significantly inhibited IKK activity in HUVECs compared with control cells under BFT-stimulated conditions (Fig. 10A). In addition, AR inhibitors significantly decreased the promoter activities of the NF-κB and ICAM-1 genes following BFT stimulation (Fig. 10B, 10C). Moreover, AR inhibitors resulted in a significant decrease in monocyte adhesion to BFT-exposed HUVECs (Fig. 10D). These results suggest that the exposure of ECs to BFT can activate a signaling cascade involving AR/JNK MAPK/IKK/NF-κB, leading to ICAM-1 induction and monocyte adhesion.

Discussion

Infiltration of inflammatory cells at the site of ETBF infection in the gut is well observed (3). Molecular interactions are required for inflammatory cells to cross the endothelium at infected sites. The first step is the capture of inflammatory cells and their rolling on ECs via firmer adhesion to the ECs, followed by diapedesis and transvasation across the endothelium. This process is regulated by the expression of adhesion molecules on the endothelium in response to specific environmental stimuli, which is termed endothelial activation (39, 40). In the current study, we investigated whether BFT activated the endothelium by inducing the expression of the adhesion molecule ICAM-1, which is necessary for monocyte adhesion. We found that one of the inflammatory responses to ETBF-derived BFT was the rapid induction of ICAM-1 expression in ECs.

ICAM-1 and VCAM-1 are surface molecules that mediate leucocyte adhesion to ECs. However, the induction of specific adhesion molecules seems to depend on the species of infecting microbe. For example, Aspergillus fumigatus infection increased the expression of VCAM-1, but suppressed the expression of ICAM-1 in ECs (41). The lethal toxin of Bacillus anthracis enhanced TNF-induced VCAM-1 expression, but not ICAM-1 expression in ECs (42). A previous report showed that human microvascular endothelial cell line (HMEC-1) stimulated with B. fragilis LPS had suppressive effect on ICAM-1 expression, but ICAM-1 expression was augmented when stimulated with enterotoxin preparations (Tox 1 and Tox 2) (43). In addition, the expression of VCAM-1 was augmented by immunosporas when endothelium was stimulated with LPS or enterotoxin preparations at concentration of 10 mg/ml (44). These reports suggest that bacterial products of ETBF can influence the adhesion molecules in ECs. In the current study, stimulation of HUVECs and RAECs with BFT did not result in a change in VCAM-1 expression, whereas ICAM-1 was upregulated. Therefore, we focused on the induction of ICAM-1 in ECs.

Transcriptional factors such as NF-κB and AP-1 regulate inflammatory responses (38, 45). The promoter region of the ICAM-1 gene contains binding sites for both NF-κB and AP-1. Thus, the induction of ICAM-1 by cytokines, Gram-negative bacteria, or bacterial products requires the activation of NF-κB and/or AP-1 and their binding to the promoter region (15, 35, 46–48). We demonstrated that treatment of ECs with BFT strongly activated NF-κB and AP-1. Suppression of NF-κB activity by transfection with retrovirus-IκBα-AA or p65 siRNA significantly inhibited BFT-induced ICAM-1 expression in HUVECs. The importance of
NF-κB for ICAM-1 expression was confirmed in RAOEcs stimulated with BFT. In contrast to NF-κB signaling, suppression of AP-1 activity did not result in a significant change in ICAM-1 expression in BFT-exposed ECs. Considering those results, NF-κB–dependent and AP-1–independent expression of ICAM-1 may be a unique signature of ECs exposure to ETBF-derived BFT.

Endothelial ICAM-1 is involved in the adhesion of leukocytes to the endothelium (49). In the current study, BFT exposure induced a significant increase in monocyte THP-1 cell adherence to ECs. In addition, the NF-κB inhibitor MG-132 and the IKK inhibitor BMS345541 significantly decreased monocyte adhesion to BFT-treated HUVECs. However, the AP-1 inhibitor curcumin did not influence monocyte adhesion to HUVECs. These results indicate that a signaling pathway including IKK, NF-κB, and ICAM-1 is most likely indispensable for adhesion of monocytes to the endothelium in response to BFT stimulation.

MAPK signaling is known to be an important event underlying ICAM-1 expression (7, 16, 17). Although BFT can induce MAPK-dependent NF-κB signaling in intestinal epithelial cells (26, 34), the cooperation between NF-κB and MAPK signaling is not clear in ECs stimulated with BFT. To gain insight into the signaling pathways involved in BFT-induced ICAM-1 upregulation, we attempted to determine whether NF-κB and MAPK signaling were associated with ICAM-1 expression in ECs. We found that pre-treatment of ECs with the JNK inhibitor SP600125 was superior to the ERK inhibitor PD98059 or the p38 inhibitor SB203580 in the inhibition of ICAM-1 expression. These results suggest that JNK is more essential for ICAM-1 induction in BFT-stimulated ECs than ERK or p38. Moreover, JNK regulated ICAM-1–induced monocyte–EC interactions under the condition of BFT stimulation.

The polyl pathway enzyme AR was required for TNF-α–induced activation of the JNK pathway in human pulmonary microvascular ECs and was required for neutrophil adhesion to TNF-α–stimulated ECs (20). In addition, inhibition of the AR activity prevented the expression of adhesion molecules in TNF-α–stimulated ECs (19). In contrast, TNF-α–induced ICAM-1 expression was not inhibited by treatment with AR inhibitor zopolrestat in ECs (20). In the current study, inhibition of AR activity resulted in the suppression of BFT-induced JNK, IKK, and NF-κB activation. In addition, treatment with AR inhibitors demonstrated the significant inhibition of ICAM-1 expression following BFT stimulation and the significant decrease in monocyte adhesion to BFT-exposed HUVECs. These data present the evidence that AR may play an important role in regulating the JNK/NF-κB–dependent ICAM-1 expression in BFT-stimulated ECs. Although the BFT receptor is predicted to be a membrane protein other than E-cadherin or known protease-activated receptor 1–4 in intestinal epithelial cells (50), the primary receptor on ECs has not been identified. Further study should be needed.

The initial response to BFT was in fact shown to be induction of an antiapoptotic protein such as cellular inhibitor of apoptosis protein-2 (23). BFT treatment of colonic epithelial monolayers resulted in delayed apoptosis. In addition, after BFT treatment of T84 cells or human colon biopsies in vitro, a delayed loss of cell viability and apoptosis of detached epithelial cells were noted (3). The delayed loss of cell viability and apoptosis after BFT exposure may be important to the host because it can provide sufficient time for cells to generate signals for eliminating the chances of bacterial colonization. Consistent with this, expression of antimicrobial peptide human β-defensin-2 was enhanced in intestinal epithelial cells during the early period of BFT stimulation (34). Based on these results, it is plausible that the ETBF-infected host may adapt to react to BFT as a countermeasure.

Leukocyte migration is seemingly potentially detrimental to the pathogen. BFT initially interacts with a single layer of epithelial cells that lines the intestinal mucosa. Intestinal epithelial cells can induce IL-8 and MCP-1 upon stimulation with BFT (21, 25). In addition, chemokine expression in intestinal epithelial cells exposed to BFT led to the development of enteritis murine model (5). Based on these results, leukocyte migration is most likely a pathogenic process in ETBF-infected condition.

In summary, we demonstrated that exposure of ECs to BFT resulted in the rapid activation of MAPK signaling. Activated MAPK then mediated the expression of ICAM-1 genes via IKK and NF-κB signaling. In addition, the signaling was regulated by AR activity. Based on these findings, we suggest that in ECs exposed to BFT, a signaling cascade involving AR, JNK, IKK, and NF-κB that induces ICAM-1 expression is activated, leading to the infiltration of inflammatory cells at sites infected with ETBF.

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Disclosures

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References


