***Bacteroides fragilis* Enterotoxin Induces Formation of Autophagosomes in Endothelial Cells but Interferes with Fusion with Lysosomes for Complete Autophagic Flux through a Mitogen-Activated Protein Kinase-, AP-1-, and C/EBP Homologous Protein-Dependent Pathway**

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ABSTRACT *Bacteroides fragilis* enterotoxin (BFT), a virulence factor of enterotoxigenic *B. fragilis* (ETBF), plays an essential role in mucosal inflammation. Although autophagy contributes to the pathogenesis of diverse infectious diseases, little is known about autophagy in ETBF infection. This study was conducted to investigate the role of BFT in the autophagic process in endothelial cells (ECs). Stimulation of human umbilical vein ECs (HUVECs) with BFT increased light chain 3 protein II (LC3-II) conversion from LC3-I and protein expression of p62, Atg5, and Atg12. In addition, BFT-exposed ECs showed increased indices of autophagosomal fusion with lysosomes such as LC3-lysosome-associated protein 2 (LAMP2) colocalization and the percentage of red vesicles monitored by the expression of dual-tagged LC3B. BFT also upregulated expression of C/EBP homologous protein (CHOP), and inhibition of CHOP significantly increased indices of autophagosomal fusion with lysosomes. BFT activated an AP-1 transcription factor, in which suppression of AP-1 activity significantly downregulated CHOP and augmented autophagosomal fusion with lysosomes. Furthermore, suppression of Jun N-terminal protein kinase (JNK) mitogen-activated protein kinase (MAPK) significantly inhibited the AP-1 and CHOP signals, leading to an increase in autophagosomal fusion with lysosomes in BFT-stimulated ECs. These results suggest that BFT induced accumulation of autophagosomes in ECs, but activation of a signaling pathway involving JNK, AP-1, and CHOP may interfere with complete autophagy.

KEYWORDS autophagy, *Bacteroides fragilis* enterotoxin, endothelial cells

Enterotoxigenic *Bacteroides fragilis* (ETBF) is known to be associated with diarrheal diseases, inflammatory bowel diseases, and colorectal cancers. *B. fragilis* enterotoxin (BFT), a virulence factor of ETBF, is responsible for these diseases (1). Exposure to BFT results in the infiltration of inflammatory cells through endothelial cells (ECs) (2–4). Although aspects of pathogenesis regarding ETBF infection have been investigated, a more detailed understanding of the interaction between BFT and the host, especially BFT-induced changes in host cells, could reveal indispensable characteristics of ETBF infection.

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Macroautophagy (here referred to as autophagy) is a degradation process that involves the nonspecific bulk degradation of cytoplasmic components such as damaged organelles and foreign pathogens (5). Autophagy consists of at least three steps (6). It is initiated by the formation of an isolation membrane, also known as a phagophore. The autophagy-related genes (*atg*) are known to regulate autophagosome formation. The first conjugation system contributes to the coupling of Atg12 with Atg5 to form a covalently linked heterodimer, which then recruits Atg16 to generate phagophores. The phagophore elongates and expands to engulf intracellular cargo, which is followed by final enclosure of the double-membrane autophagosome. The second conjugation system couples microtubule-associated protein 1 light chain 3 (LC3) to phosphatidylethanolamine, leading to the formation of LC3-II protein. LC3-II is present on both the inner and outer isolation membranes, serving as a recognition site for LC3-binding chaperones such as p62/SQSTM1 (p62) that deliver their cargo to autophagosomes (7, 8). Once formed, autophagosomes traffic along microtubules to reach lysosomes, where they fuse to form autolysosomes, allowing for inner autophagosome membrane and the autophagic cargo degradation by lysosomal acid hydrolases (7, 9, 10). Together, all of these steps form the so-called “autophagic flux” (9, 10).

Autophagy is, in part, considered to function in decisions of cell survival and death (11). Activation of autophagy has potential risks as it has the potential to rescue diseased cells from death (12, 13). In addition, it plays a prominent role in resistance to bacterial infections in eukaryotic cells by capturing and degrading invading microbes or toxins (14). Antibacterial autophagy provides potent cell-autonomous immunity against bacterial attempts to colonize the cytosol of mammalian cells (15–18); therefore, it is possible that pathogens may inhibit the autophagic process to spread their infection or survive at infected sites. For example, phosphoprotein of human parainfluenza virus type 3 and matrix protein 2 of influenza A virus are known to block autophagosome-lysosome fusion to increase virus production (19, 20). Knowledge of antimicrobial autophagy has allowed us to analyze autophagy regulation in response to BFT stimulation; however, the detailed mechanisms of how BFT regulates the autophagic process remain elusive.

Inflammatory cell recruitment is a fundamental phenomenon of the tissue response to bacterial infection. 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. We have already demonstrated that BFT upregulates adhesion molecules in ECs leading to monocyte adhesion (4); however, there is no evidence that BFT regulates the autophagic processes in ECs. In the present study, we investigated autophagic processes in the early response to stimulation of ECs with BFT and found that EC exposure to BFT resulted in the accumulation of autophagosomes; however, activation of a signaling pathway involving Jun N-terminal protein kinase (JNK) mitogen-activated protein kinase (MAPK), AP-1, and C/EBP homologous protein (CHOP) was associated with interruption of the complete autophagy.

RESULTS

BFT triggers the accumulation of autophagosomes in ECs. To characterize the autophagic process in BFT-stimulated HUVECs, we first determined whether BFT stimulation could trigger autophagosome formation. Since the LC3 precursor (LC3-I) is proteolytically cleaved for incorporation into the autophagosomal membrane as LC3-II during autophagosome formation (21), we assessed conversion of LC3-I to LC3-II via immunoblot assay. At 3 h after BFT stimulation, LC3-II levels were notably increased (Fig. 1A and B), indicating that there was a cumulative increase in autophagosome formation as stimulation progressed. The p62 protein is selectively incorporated into autophagosomes through direct binding to LC3 and is efficiently degraded by autophagy (22). Because total cellular p62 expression levels inversely correlate with autophagic activity (21), we measured the expression of p62 protein in BFT-exposed human umbilical vein endothelial cells (HUVECs). As shown in Fig. 1A and C, increased p62 band intensity in BFT-exposed HUVECs was first noted 1 h after stimulation, with

FIG 1 Expression of proteins associated with autophagosome formation in HUVECs stimulated with BFT. (A) Primary HUVECs were treated with BFT (100 ng/ml) for the indicated time periods. Conversion of LC3-I to LC3-II protein and expression of p62 and actin proteins were analyzed by immunoblot assays. Results are representative of three independent experiments. (B and C) Densitometric analysis of expressed LC3-II (B) and p62 (C) proteins on primary HUVECs. Values represent relative densities of each protein compared with actin (mean $\pm$ standard deviation [SD]; $n = 3$). *, $P < 0.05$ compared with the untreated control. (D) CRL-1730 cells were treated with BFT (100 ng/ml) for the indicated time periods. Conversion of LC3-I to LC3-II protein and expression of p62 and actin proteins were analyzed by immunoblot assays. Results are representative of three independent experiments. (E) Primary HUVECs were treated with BFT (100 ng/ml), bafilomycin A1 (BA [20 nM]), or rapamycin (Rapa [100 nM]) for the indicated time periods. Conversion of LC3-I to LC3-II protein and expression of actin proteins were analyzed by immunoblot assays (left panels). Results are representative of three independent experiments. The right panels are the results from densitometric analysis of expressed LC3-II proteins. Values represent relative densities of each protein compared with actin.

a peak observed 6 h poststimulation, which gradually diminished. Similar results were also observed in a human endothelial cell line (CRL-1730) stimulated with BFT (Fig. 1D).

We next examined the effects of bafilomycin A1, as an inhibitor of the late phase of autophagy, on BFT-induced LC3-II levels in ECs. As a positive control, we used rapamycin. As shown in Fig. 1E (top panel), LC3-II levels 12 h after treatment with bafilomycin A1 and BFT were higher than at time zero. In addition, combined treatment

FIG 2 (A) Primary HUVECs (top panel) and CRL-1730 cells (bottom panel) were treated with BFT (100 ng/ml) for the indicated time periods. Atg5 and Atg12 protein expression was analyzed by immunoblot assays. Results are representative of three independent experiments. (B) Primary HUVECs were treated with the indicated concentrations of BFT for 6 h. Protein expression of p62 was measured using ELISA kits. Data are expressed as mean fold induction $\pm$ SEM relative to that of the untreated controls ($n = 5$).

with bafilomycin A1 and BFT increased LC3-II levels compared with BFT alone during the entire experimental period. These results were similar to those obtained from treatment with bafilomycin A1 and rapamycin (Fig. 1E, bottom panel). To confirm autophagosome formation, protein expression of Atg5 and Atg12 was observed. The Atg5-Atg12 conjugate interacts noncovalently with Atg16 to form the Atg5-Atg12-Atg16 complex, which localizes to autophagosome precursors and plays an essential role in autophagosome formation (23). As shown in Fig. 2A (top panel), protein expression of Atg5 and Atg12 in HUVECs treated with rapamycin (positive control) was observed at 1 h after stimulation, with a peak observed 6 h poststimulation, which gradually diminished. The kinetics of Atg5 and Atg12 protein expression were similar to those in BFT-exposed HUVECs (Fig. 2A, bottom panel). These results suggest that stimulation of HUVECs with BFT can induce autophagosome formation. The magnitude of increased p62 activity was dependent on the concentration of BFT, as assessed by enzyme-linked immunosorbent assay (ELISA) (Fig. 2B). The 50% effective concentration (EC_{50}) of BFT was 91.2 ng/ml, as calculated by SigmaPlot 10.0 software (Systat Software, Inc., San Jose, CA, USA). Based on this result, 100 ng/ml of BFT was used in subsequent experiments.

BFT partially induces autophagosomal fusion with lysosomes in ECs. We next asked whether BFT-induced autophagosome accumulation could lead to fusion with lysosomes in HUVECs. We examined autophagy flux in BFT-stimulated cells through three independent assays (24). For all assays, HUVECs treated with rapamycin, an autophagy inducer, were used as a positive control. In the first assay, we performed experiments using immunofluorescence microscopy to observe autophagosomes and

FIG 3 BFT partially induces autophagosomal fusion with lysosomes in HUVECs. (A and B) HUVECs were stimulated with BFT (100 ng/ml) or rapamycin (Rapa [100 nM]) for 6 h. Cells were stained with anti-LC3 and anti-LAMP2 Abs, and images were captured using fluorescence microscopy. Colocalization of LC3 and LAMP2 staining was quantified (mean $\pm$ SEM; $n = 5$ [B]) as described in Materials and Methods. *, $P < 0.05$. (C and D) HUVECs were stimulated with BFT (100 ng/ml) or rapamycin (100 nM) for 6 h. Cells were stained with anti-LC3 and anti-p62 Abs, and images were captured using fluorescence microscopy. Colocalization of LC3 and p62 staining was quantified as described in Materials and Methods (mean $\pm$ SEM; $n = 5$ [D]). *, $P < 0.05$. (E) CRL-1730 cells transduced with RFP-GFP-LC3-expressing baculoviruses were stimulated with BFT (100 ng/ml) or rapamycin (100 nM) for 6 h and analyzed with fluorescence microscopy. Results for the unstimulated control cells are not shown because yellow or red vesicles in unstimulated control cells were rarely seen. (F) Yellow dots represent autophagosomes, while red dots indicate autolysosomes in which the GFP signal was faded out. Unstimulated control cells could not be shown because yellow or red vesicles were rarely seen in unstimulated control cells. The graph represents the percentage of red vesicles among the total vesicles per cell (mean $\pm$ SEM) as described in Materials and Methods. *, $P < 0.05$.

lysosomes. For these experiments, BFT-stimulated HUVECs were stained with anti-LC3 and anti-LAMP2 antibodies (Abs). As shown in Fig. 3A, there was a considerable level of association between autophagosomes and lysosomes in BFT-exposed HUVECs, suggesting that BFT induces autophagosomal fusion to lysosomes in HUVECs. However,

fewer overlapping cells stained with anti-LC3 and anti-LAMP2 Abs were observed in BFT-stimulated cells than in rapamycin-treated cells. To objectively measure the association between autophagosomes and lysosomes, fluorescence images were analyzed using ImageJ. The density of LC3-LAMP2 colocalization in BFT-stimulated cells was higher than in unstimulated cells; however, the colocalization in BFT-stimulated cells was lower than in rapamycin-treated cells (Fig. 3B).

We performed immunofluorescence microscopy to analyze formation of LC3 puncta (LC3-positive bodies) and the expression of p62 proteins. As shown in Fig. 3C, BFT-exposed HUVECs promoted the increase in both LC3 puncta (green) and p62 (red), in which LC3 puncta and p62 appeared to overlap in identical sites. In contrast to the LC3-LAMP2 colocalization, the colocalization of LC3 and p62 in BFT-stimulated cells was significantly higher than in rapamycin-treated cells (Fig. 3D). These results suggest that many autophagosomes induced by BFT are not associated with lysosomal fusion in HUVECs.

We also used the autophagy flux indicator RFP-GFP-LC3, which initially fluoresces both red (red fluorescent protein [RFP]) and green (green fluorescent protein [GFP]) as it labels autophagosomes. Upon fusion with lysosomes that expose the RFP-GFP fluorophore to an acidic environment, the GFP is denatured and loses its green fluorescence, while RFP maintains its red fluorescence. Therefore, the fusion of autophagosomes with lysosomes results in the loss of yellow fluorescence and the appearance of only red RFP fluorescence. Rapamycin-treated HUVECs showed red color; however, cells stimulated with BFT were observed as yellow in color (Fig. 3E and F). We did not show a group for unstimulated control cells on the figure because yellow or red vesicles in unstimulated control cells were rarely seen. These results collectively suggest that stimulation of HUVECs with BFT can induce autophagosome formation and lysosomal fusion; however, large numbers of accumulated autophagosomes are not associated with lysosomal fusion.

Limiting autophagosome-lysosome fusion induced by BFT is associated with CHOP expression in ECs. Since CHOP is known to play a dual role in both inducing apoptosis and limiting autophagy through the transcriptional control of specific target genes (25), we asked whether CHOP might be involved in the BFT-induced inhibition of autophagosome-lysosome fusion. As shown in Fig. 4A, stimulation of HUVECs with BFT increased CHOP expression. Similar results were also observed in CRL-1730 cells stimulated with BFT. To evaluate the role of CHOP on BFT-induced autophagy, *CHOP* shRNA was used. *CHOP* shRNA almost completely suppressed cellular CHOP protein expression in BFT-stimulated CRL-1730 cells; however, a control lentivirus did not reduce CHOP expression (Fig. 4B). In this experimental system, blocking CHOP expression with shRNA significantly reduced the BFT-induced increase in p62 expression as assessed by immunoblot assays. In immunofluorescence microscopy, BFT-exposed ECs increased the number of LC3 puncta; however, transfection with *CHOP* shRNA did not significantly change the number of LC3 puncta compared with untransfected cells under BFT-stimulated conditions (Fig. 4C). These results suggest that CHOP signaling may not influence autophagosomal formation in BFT-exposed ECs.

Colocalization assays were performed to determine autophagosomal fusion with lysosomes. As shown in Fig. 4D, transfection with *CHOP* short hairpin RNA (shRNA) increased LC3-LAMP2 colocalization compared with untransfected cells under BFT-stimulated conditions. Consistent with these results, cells transfected with *CHOP* shRNA changed from yellow to red fluorescence when BFT was exposed to the *CHOP* shRNA-transfected cells (Fig. 4E), while untransfected or control RNA-transfected cells maintained yellow fluorescence under BFT-stimulated conditions. These results indicate that CHOP-dependent signaling is involved in the inhibition of autophagosome-lysosome fusion in BFT-stimulated ECs.

CHOP-mediated autophagic process is not associated with NF- κ B activation in BFT-exposed ECs. Since the *CHOP* gene promoter region has binding sites for transcription factors such as NF- κ B and AP-1, we determined whether chemical NF- κ B or AP-1 inhibitors could change the protein expression of CHOP and p62 in BFT-stimulated

FIG 4 BFT-induced CHOP expression is associated with interference with autophagy in HUVECs. (A) Primary HUVECs and CRL-1730 cells were stimulated with BFT (100 ng/ml) for the indicated time periods. Expression of CHOP and actin proteins was analyzed by immunoblot assays. Results are representative of more than three independent experiments. (B to E) CRL-1730 cells were transfected with *CHOP*-specific shRNA or control RNA. Transfected cells were combined with BFT (100 ng/ml) for 3 h (CHOP) or 6 h (p62). (B) Expression of CHOP and p62 proteins was analyzed by immunoblot assays. Results are representative of three independent experiments. (C) Cells were stained with anti-LC3 Ab and analyzed by fluorescence microscopy. Results are representative of three independent experiments. In the right panel, the number of LC3 puncta was determined by a method in which images of approximately 50 cells were taken in each experiment and three experiments were analyzed. *, $P < 0.05$; NS, not statistically significant. (D) Cells were stained with anti-LC3 and anti-LAMP2 Abs and analyzed by fluorescence microscopy. Results are representative of three independent experiments. In the right panel, colocalization of LC3 and LAMP2 staining was quantified as described in Materials and Methods (mean $\pm$ SEM; $n = 3$). *, $P < 0.05$. (E) Cells were treated with a baculovirus system containing an autophagy flux indicator, RFP-GFP-LC3, after which cells were stimulated with BFT (100 ng/ml) for 6 h and analyzed by fluorescence microscopy, as described in Materials and Methods. Results for unstimulated control cells are not shown because yellow or red vesicles in unstimulated control cells were rarely seen. The graph represents the percentage of red vesicles among the total vesicles per cell (mean $\pm$ SEM). *, $P < 0.05$.

FIG 5 Effects of NF- κ B and AP-1 chemical inhibitors on CHOP and p62 protein expression in HUVECs stimulated with BFT. HUVECs were preincubated with the NF- κ B inhibitor Bay 11-7082 (50 μ M) or AP-1 inhibitor SR11302 (10 μ M) for 30 min, followed by stimulation with BFT (100 ng/ml) for an additional 3 h (CHOP) or 6 h (p62). Expression of CHOP and p62 proteins was analyzed by immunoblot assays. Results are representative of more than three independent experiments.

HUVECs. For this experiment, primary HUVECs were preincubated with NF- κ B inhibitor Bay 11-7082 or AP-1 inhibitor SR11302 for 30 min, followed by BFT treatment. As shown in Fig. 5, combined treatment of HUVECs with SR11302 plus BFT resulted in a decrease in CHOP and p62 protein expression compared to treatment with BFT alone. However, any changes in CHOP and p62 protein levels were not found in the combined treatment with Bay 11-7082 plus BFT compared with BFT treatment alone.

CHOP-mediated autophagic process is largely due to AP-1 activation in BFT-exposed ECs. We next determined whether AP-1 could be associated with the CHOP-mediated autophagic process. DNA-binding studies for AP-1 showed that stimulation of HUVECs with BFT increased AP-1 DNA binding (Fig. 6A). Concurrently, phospho-c-jun protein expression was increased in BFT-exposed HUVECs. Similar results were also observed in human endothelial CRL-1730 cells treated with BFT (Fig. 6B).

To evaluate whether BFT-induced AP-1 activation might be associated with the autophagic process, transfection with lentivirus containing a dominant-negative *c-jun* plasmid (lentivirus-dn-*c-jun*) was used. Transfected CRL-1730 cells were stimulated with BFT for 1 h, and the AP-1 DNA binding activity was assessed by electrophoretic mobility shift assay (EMSA). Transfection with lentivirus-dn-*c-jun* suppressed AP-1 activity to control levels in BFT-treated cells, while transfection with control lentivirus did not reduce AP-1 activation (Fig. 6C, top panel). In this experimental system, cells transfected with or without lentivirus-dn-*c-jun* were treated with BFT and the level of CHOP protein expression was determined by immunoblot assays. The results showed that transfection with lentivirus-dn-*c-jun* attenuated CHOP protein expression (Fig. 6C, middle panel). In addition, the p62 protein expression was decreased in dn-*c-jun*-transfected cells compared with untransfected cells under BFT-exposed conditions (Fig. 6C, bottom panel).

In another experiment, using immunofluorescence microscopy, there was a significant difference in LC3-LAMP2 colocalization between dn-*c-jun*-transfected and untransfected cells under BFT-stimulated conditions (Fig. 6D). In addition, dn-*c-jun*-transfected cells stimulated with BFT showed red fluorescence compared with BFT-stimulated untransfected cells, showing yellow fluorescence (Fig. 6E). These results suggest that the BFT-induced autophagic process is conducted through a pathway including CHOP and AP-1 in HUVECs.

Inhibition of JNK MAPK suppresses AP-1 and CHOP signaling, leading to promotion of autophagosomal fusion with lysosomes in BFT-exposed ECs. BFT was found to activate the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and JNK in HUVECs (Fig. 7A). Similar activation of MAPK signals in CRL-1730 cells was obtained following BFT stimulation (Fig. 7B). We next evaluated whether inhibition of MAPK activity influenced the activation of AP-1 and CHOP 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,

FIG 6 Effects of AP-1 suppression on autophagy in HUVECs stimulated with BFT. (A and B) HUVECs (A) or CRL-1730 cells (B) were treated with BFT (100 ng/ml) for the indicated times. AP-1 DNA binding activity was assessed by EMSA. (+) represents the positive control using nuclear extracts obtained from HUVECs treated with TNF- α (20 ng/ml) for 1 h, and (-) represents the negative control. Immunoblot results for concurrent phospho-c-jun and lamin B in nuclear extracts under the same conditions are provided beneath the EMSA panels. Results are representative of three independent experiments. (C) CRL-1730 cells were transfected with either lentivirus containing dominant-negative *c-jun* plasmid (dn-c-jun) or control virus. Transfected cells were stimulated with BFT (100 ng/ml) for 1 h (EMSA), 3 h (CHOP), or 6 h (p62). AP-1 binding activity was assayed by EMSA (top panel). Expression of CHOP and p62 proteins was analyzed by immunoblot assays (middle and bottom panels). Results are representative of more than three independent experiments. (D) Transfected CRL-1730 cells were treated with BFT (100 ng/ml) for 6 h, and immunofluorescence microscopy was then performed using anti-LC3 and anti-LAMP2 Abs. Results are representative of three independent experiments. In the right panel, colocalization between LC3 and LAMP2 staining was quantified as described in Materials and Methods (mean $\pm$ SEM; $n = 3$). *, $P < 0.05$; NS, statistically nonsignificant. (E) Transfected cells were treated with a baculovirus system containing RFP-GFP-LC3, after which cells were stimulated with BFT (100 ng/ml) for 6 h and analyzed by fluorescence microscopy, as described in Materials and

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FIG 7 MAPK signals are associated with CHOP expression in BFT-stimulated HUVECs. (A and B) Primary HUVECs (A) and CRL-1730 cells (B) were stimulated with BFT (100 ng/ml) for the indicated time periods. Expression of ERK1/2, p38, and JNK proteins was measured by immunoblot assays. Results are representative of more than three independent experiments. (C) Primary HUVECs were preincubated with SB203580 (open circles), PD98059 (open triangles), or SP600125 (open squares) for 30 min and then stimulated with BFT (100 ng/ml) for another 6 h. Levels of CHOP expression were determined by ELISA. Data are expressed as the mean percentage of increase relative to the level in unstimulated controls $\pm$ SEM ($n = 5$). *, $P < 0.05$ compared to results with BFT alone. (D) Primary HUVECs were preincubated with PD98059 (20 μ M), SB203580 (20 μ M), or SP600125 (20 μ M) for 30 min and then stimulated with BFT (100 ng/ml) for 10 min. Levels of expression of each protein were determined by immunoblot assays. Results are representative of more than three independent experiments.

which specifically inhibits p38; and SP600125, which inhibits JNK. Pretreatment of HUVECs with PD98059 ($\geq 50 \mu$ M), or SP600125 ($\geq 10 \mu$ M) significantly inhibited the BFT-induced upregulation of CHOP (Fig. 7C). In these experiments, each chemical inhibitor suppressed each activity of MAPK signals (Fig. 7D). SB203580 is known to inhibit p38 catalytic activity by binding to the ATP binding pocket, but does not inhibit phosphorylation of p38 by upstream kinases (26). SB203580 potently inhibits the activity of p38 MAPK by the suppression of the activation of MAPK-activated protein K2 (MAPKAPK2), a specific physiological substrate of p38 MAPK (26). Based on these

FIG 6 Legend (Continued)

Methods. Results are representative of three independent experiments. Results for unstimulated control cells are not shown because yellow or red vesicles in unstimulated control cells were rarely seen. The graph represents the percentage of red vesicles among the total vesicles per cell (mean $\pm$ SEM). *, $P < 0.05$.

results, we measured phospho-MAPKAPK2 for SB203580-induced inhibition of p38 activity.

We further investigated whether JNK activity is required for limiting induction of the autophagic process in BFT-stimulated HUVECs. For this study, we used a transfection model with a lentivirus containing a dominant-negative *JNK* plasmid (lentivirus-dn-*JNK*) transfected into CRL-1730 cells. Phosphorylation of JNK was clearly suppressed in CRL-1730 cells transfected with lentivirus-dn-*JNK* (Fig. 8A). In addition, transfection with lentivirus-dn-*JNK* significantly decreased protein expression, including phospho-c-jun, CHOP, and p62 following BFT stimulation. In this experimental system, ELISAs were performed to measure protein expression levels. As shown in Fig. 8B, suppression of JNK activity significantly reduced CHOP and p62 protein expression in dn-*JNK*-transfected cells compared with untransfected cells under BFT-stimulated conditions.

In another experiment, using immunofluorescence microscopy, there was a significant difference in LC3-LAMP2 colocalization between dn-*JNK*-transfected and untransfected cells under BFT-stimulated conditions (Fig. 8C). In addition, dn-*JNK*-transfected cells stimulated with BFT showed red fluorescence compared to BFT-stimulated untransfected cells, which showed yellow fluorescence (Fig. 8D). These results suggest that EC exposure to BFT activates a signaling cascade involving JNK MAPK, AP-1, and CHOP, leading to inhibition of autophagosomal fusion with lysosomes.

DISCUSSION

Autophagy plays a prominent role in resistance to bacterial infections in eukaryotic cells by capturing and degrading the invading microbes or toxins (14). As such, pathogens may inhibit the autophagic process to spread their infection or survive at infected sites. In the present study, we found that one of the early responses to BFT stimulation was the induction of autophagosomal formation but impairing their fusion with lysosomes in ECs.

During autophagosome formation, the LC3 precursor LC3-I is proteolytically cleaved for incorporation into the autophagosomal membrane as LC3-II. In addition, p62 is incorporated into autophagosomes through direct binding to LC3 and is gradually degraded by autophagic flux (22). Proteins such as Atg5 and Atg12 are essential for autophagosome formation. Considering that LC3-II, p62, Atg5, and Atg12 protein levels were increased in ECs stimulated with BFT, it is possible that BFT can induce autophagosome formation.

Complete autophagy indicates that autophagosomes fuse to form autolysosomes, allowing the degradation of both the inner autophagosome membrane and autophagic cargo by lysosomal acid hydrolases. To characterize BFT-induced autophagosomal fusion with lysosomes, we used immunofluorescence microscopy using anti-LC3B and anti-LAMP2 Abs or the autophagy flux indicator RFP-GFP-LC3B. Fusion of autophagosomes with lysosomes was observed in BFT-treated ECs; however, the amounts of LC3-LAMP2 colocalization and autolysosomes that were a percentage of the red vesicles, as monitored by the expression of dual-tagged LC3B (RFP-GFP-LC3B), were lower in BFT-treated ECs than in rapamycin-treated cells. In contrast, BFT-treated ECs showed higher colocalization of LC3 and p62 compared with rapamycin-treated cells. These results suggest that BFT-exposed HUVECs maintain the suppressed autophagic state even though BFT can in part induce autophagy. Based on these results, we investigated autophagy-suppressing factors in BFT-stimulated ECs.

CHOP encodes a ubiquitous transcription factor that is involved in autophagy. For example, the HP0175 secreted antigen of *Helicobacter pylori* activated transcription of CHOP, leading to autophagy (27). In addition, hypoxia promotes autophagy through an HIF-1-independent pathway involving transcriptional induction of Atg5 and LC3 through the PERK-responsive transcription factors ATF4 and CHOP (28). Another study demonstrated that CHOP upregulated a number of autophagy genes during the first 6 h of starvation; however, CHOP inhibited autophagy when amino acid starvation was prolonged (25). These results suggest that CHOP plays an important role in induction or suppression of autophagy according to the stimuli. In the present study, BFT

FIG 8 Effects of JNK MAPK suppression on autophagy in HUVECs stimulated with BFT. CRL-1730 cells were transfected with lentiviruses containing a dominant-negative *JNK* plasmid (dn-*JNK*) or control virus. (A) The transfected cells were stimulated with BFT (100 ng/ml) for 30 min, after which immunoblots for phospho-JNK protein expression were performed (top panel). Transfected cells were stimulated with BFT (100 ng/ml) for 1 h (AP-1), 3 h (CHOP), or 6 h (p62). AP-1 binding activity was assayed by EMSA (middle panel). Expression of CHOP and p62 proteins was analyzed by immunoblot assays (bottom panel). Results are representative of more than five independent experiments. (B) Transfected cells were stimulated with BFT (100 ng/ml) for 1 h (AP-1), 3 h (CHOP), or 6 h (p62). Protein expression of CHOP and p62 and AP-1 activity were measured using the respective ELISA kits. Data are expressed as the mean fold induction $\pm$ SEM relative to that of the untreated controls ($n = 5$). *, $P < 0.05$. (C) Transfected cells were treated with BFT (100 ng/ml) for 6 h, and immunofluorescence microscopy was performed using anti-LC3 and anti-LAMP2 Abs. Results are representative of three independent experiments. In the right panel, colocalization of LC3 and LAMP2 staining was quantified as described in Materials and Methods (mean $\pm$ SEM; $n = 3$). *, $P < 0.05$. (D) Cells were treated with a baculovirus system containing an autophagy flux indicator, RFP-GFP-LC3, after which cells were stimulated with BFT (100 ng/ml) for 6 h and analyzed

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upregulated CHOP signals in HUVECs. Suppression of CHOP increased the index of autophagy flux, including LC3-LAMP2 colocalization, p62 protein degradation, and the percentage of red vesicles in BFT-stimulated ECs.

The decrease in p62 protein expression (or the increase of p62 protein degradation) in BFT-exposed ECs transfected with *CHOP* shRNA may be interpreted in two ways, including (i) a decrease in p62 protein synthesis and autophagosomal formation or (ii) the degradation of synthetic p62 proteins by progressing autophagosomal fusion with lysosomes. CHOP upregulates a number of autophagy genes but is not involved in the first steps of the autophagic process during the first 6 h of starvation (25). In addition, the present study shows that LC3-positive bodies (LC3 puncta) were not different between *CHOP* shRNA-transfected and untransfected cells under BFT-stimulated conditions, indicating that BFT-induced activation of CHOP may not be involved in the formation of autophagosomes. Therefore, the increase in p62 protein degradation seems to be associated with augmentation of autophagosomal fusion with lysosomes.

LC3 is associated with the outer face of the outer membrane and inner face of the inner membrane of the double-membrane autophagosome. When the autophagosome matures into an autolysosome, outer membrane LC3 is liberated into the cytosol, whereas inner membrane LC3 is trapped within the autolysosome and is finally degraded by lysosomal proteases. Therefore, the fate of individual LC3-II molecules depends on their localization (29). LC3-II that is located on the outer face of the autophagosome is delipidated through a second cleavage by the Atg4 protease and is converted back into the LC3-I form in the cytosol (30). In contrast, p62 is only located on inner face of the inner membrane of the double-membrane autophagosome in a form that combines with LC3-II. Therefore, p62 protein degradation may not match the number of LC3 puncta because LC3 puncta assessed by immunofluorescence microscopy showed both LC3-I and LC3-II forms.

Transcription factors such as NF- κ B and AP-1 regulate a variety of inflammatory responses (31). Although promoter region of the *CHOP* gene has binding sites for NF- κ B and AP-1, it remains controversial as to whether CHOP expression is associated with NF- κ B or AP-1 in HUVECs. For example, *CHOP* deficiency in mice suppressed NF- κ B signaling (32) and HepG2 cells transfected with *CHOP* shRNA decreased NF- κ B activation under treatment with palmitate (33). The autophagy inhibitor chloroquine significantly inhibited bortezomib-induced I κ B α degradation, increased complex formation with NF- κ B, and reduced NF- κ B nuclear translocation and DNA binding activity in lymphoma cells (34). In addition, inhibition of NF- κ B promoted autophagy in porcine granulosa cells (35). AP-1 proteins such as junB and c-jun, but not junD or c-fos, have been shown to inhibit autophagy (36, 37). In the present study, exposure of HUVECs to BFT activated transcription factors such as NF- κ B and AP-1. Inhibition of NF- κ B activity did not influence CHOP expression and the CHOP-mediated autophagic process in BFT-exposed ECs. In contrast, inhibition of AP-1 activity significantly reduced CHOP expression in BFT-stimulated ECs. In addition, suppression of AP-1 increased the index of autophagy flux, including LC3-LAMP2 colocalization, p62 protein degradation, and the percentage of red vesicles. Based on these results, BFT-induced AP-1 activation seems to be associated with CHOP induction and inhibition of the autophagic process in ECs.

Although MAPK signaling is a regulating event underlying autophagic process (13, 38, 39), there is no evidence of BFT-induced activation of MAPK, AP-1, and CHOP. In the present study, pretreatment of HUVECs with the JNK inhibitor SP600125 was superior to treatment with the ERK inhibitor PD98059 or the p38 inhibitor SB203580 in inhibiting CHOP expression. In addition, suppression of JNK MAPK signals in BFT-treated ECs using

FIG 8 Legend (Continued)

by fluorescence microscopy, as described in Materials and Methods. Results are representative of three independent experiments. Results for unstimulated control cells are not shown because yellow or red vesicles in unstimulated control cells were rarely seen. The graph represents the percentage of red vesicles among the total vesicles per cell (mean $\pm$ SEM). *, $P < 0.05$.

a lentiviral transfection system resulted in a significant decrease in AP-1-CHOP activation and a significant increase in autophagy. These results suggest that BFT-exposed ECs activate a signaling cascade involving JNK MAPKs, leading to AP-1-CHOP activation and partially inhibition of complete autophagic flux.

Cell death is a major determinant of inflammatory disease severity. Whether cells live or die during inflammation largely depends on the relative success of the pro-survival process of autophagy versus the prodeath process of apoptosis (40). In addition, promotion of autophagy enhances susceptibility toward apoptotic stimuli (41). Considering the present results, it is plausible that the autophagic process regulated by BFT may modulate the rate of apoptosis in ECs. Because this study focused exclusively on autophagy, further studies are required to clarify the effects of the autophagic process on apoptosis in BFT-stimulated cells.

In summary, we have identified a BFT-induced signaling pathway, including JNK MAPK, AP-1, and CHOP, that may play a role in impairing autophagosomal fusion with lysosomes in BFT-exposed ECs.

MATERIALS AND METHODS

Reagents. Vascular cell basal medium (ATCC PCS100030), an endothelial cell growth kit (ATCC PCS100041), F12K medium (ATCC 30-2004), and fetal bovine serum (FBS; ATCC 30-2020) were obtained from ATCC (Manassas, VA, USA). Recombinant human basic fibroblast growth factor (bFGF) was obtained from Invitrogen (Carlsbad, CA, USA). Heparin, bovine serum albumin (BSA), and rapamycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Monoclonal Abs (MAbs) against pan-extracellular signal-regulated kinase 1/2 (pan-ERK1/2, p44/p42), phospho-ERK1/2, pan-JNK (p54/p46), phospho-JNK, pan-p38, phospho-p38, phospho-MAPKAPK2, CHOP, Atg5, Atg12, lamin B, and actin were acquired from Cell Signaling Technology, Inc. (Beverly, MA, USA). Abs against p62/SQSTM1 and LC3B were acquired from Abnova (Taipei City, Taiwan) and Novus Biologicals (Saint Charles, MO, USA), respectively. Abs against lysosome-associated membrane protein 2 (LAMP2), consisting of goat anti-rabbit or anti-mouse secondary Abs conjugated to horseradish peroxidase, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 488- and DyLight 549-conjugated secondary Abs were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Abcam (Cambridge, MA, USA), respectively. PD98059, SB203580, SP600125, and Bay 11-7085 were acquired from Calbiochem (La Jolla, CA, USA). SR11302 and bafilomycin A1 were obtained from Tocris Bioscience (Bristol, United Kingdom).

Purification of BFT and cell culture conditions. BFT was purified from culture supernatants of a toxigenic ETBF strain (ATCC 43858) as described previously (42–44). The purity of BFT preparations was confirmed by SDS-PAGE. The activity of lipopolysaccharide (LPS) in BFT solution (1 mg/ml) was less than 1 endotoxin unit/ml (Pyrostat test kit, quantitative chromogenic *Limulus* amoebocyte lysate; Associates of Cape Cod, Inc., East Falmouth, MA, USA). Using a HEK-Blue LPS detection kit (InvivoGen, San Diego, CA, USA) with a detection limit of 3 ng/ml, the amount of LPS in BFT solutions (1 mg/ml) was found to be less than 3 ng/ml. BFT was frozen in aliquots at -80°C immediately after purification.

Primary human umbilical vein ECs (HUVECs; PCS-100-010) were obtained from the ATCC. Primary HUVECs were cultured in vascular cell basal medium supplemented with an endothelial cell growth kit containing vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), bFGF, ascorbic acid, L-glutamine, heparin sulfate, hydrocortisone hemisuccinate, and FBS. The HUVEC cell line (ATCC CRL-1730) was cultured in F12K medium containing heparin (0.1 mg/ml), bFGF (20 ng/ml), and 10% FBS, according to ATCC instructions. Cells were maintained in a 5% CO_2 incubator at 37°C , and the third to seventh passages of HUVECs were used for experiments.

Immunoblots and ELISA. Cells were washed with ice-cold phosphate-buffered saline (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 phenylmethylsulfonyl fluoride [PMSF], and 10 $\mu\text{g/ml}$ aprotinin). Protein (15 to 50 μg 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). Immunoreactive proteins were visualized using goat anti-rabbit or anti-mouse secondary Abs conjugated to horseradish peroxidase, followed by enhanced chemiluminescence (ECL system; Amersham Life Science, Buckinghamshire, England) and exposure to X-ray film.

The activity of AP-1 following BFT stimulation was evaluated using the FACE (Fast activated cell-based ELISA) kit (Active Motif, Carlsbad, CA, USA), in accordance with the protocols provided with the kit (45, 46). ELISA kits for measuring total SQSTM1/p62 and CHOP proteins were acquired from Cell Signaling Technology and ImmunoWay Biotechnology (Plano, TX, USA), respectively. The assays were performed according to the manufacturer's instructions.

Immunofluorescence assay and image analysis. For immunofluorescence staining, cells were grown on a chamber slide (Thermo Fisher Scientific), fixed in methanol-acetone (1:1) at -20°C for 10 min, and permeabilized in PBS containing 1% BSA and 0.3% (vol/vol) Triton X-100 for 30 min at room temperature. Cells were stained with primary Abs (rabbit anti-LC3 and mouse anti-p62/SQSTM1 Abs or rabbit anti-LC3 and mouse anti-LAMP2 Abs) for 30 min at room temperature. Cells were then treated with Alexa Fluor 488-conjugated secondary Ab (green) against rabbit IgG and DyLight 549-conjugated secondary Ab (red) against mouse IgG for 30 min. Vectashield mounting medium with DAPI (4',6-diamidino-2-phenylindole [Vector Laboratories, Inc., Burlingame, CA, USA]) was applied to the cells, and

images were captured using a DMI4000B (Leica Microsystems GmbH, Wetzlar, Germany) fluorescence microscope. Quantitative colocalization analysis was performed using the JACoP (Just Another Colocalization Plugin) plugin in the ImageJ program (NIH Image). For analysis, images of approximately 50 to 60 cells were taken in each experiment and three to five experiments were analyzed, bringing the total number of cells to 150 to 300 per determination, as previously described (47). The value shown represents Pearson's coefficient. A linear equation describing the relationship between the intensities in two images is calculated by linear regression. The Pearson's coefficient provides an estimate of the goodness of this approximation. Its value can range from 1 to -1 , with 1 representing complete positive correlation and -1 a negative correlation; zero represents no correlation (47).

To measure maturation of the autophagosome to the autolysosome, the Premo Autophagy Tandem Sensor red fluorescent protein (RFP)-green fluorescent protein (GFP)-LC3B kit (catalog no. P36239; Life Technologies Co., Carlsbad, CA, USA) was used according to the manufacturer's instructions. By combining an acid-sensitive GFP with an acid-insensitive RFP, the change from autophagosome (neutral pH) to autolysosome (acidic pH) is visualized by imaging the specific loss of the GFP fluorescence (green) upon acidification of the autophagosome following lysosomal fusion, leaving only red fluorescence. Images were observed using a DMI4000B fluorescence microscope. In experiments where the RFP-GFP-LC3B construct was used, vesicles were counted to calculate the percentage of red vesicles among the total number of vesicles (yellow $\pm$ red); the average percentage (% divided by total no. of cells = n) was used to plot the graph. For this, the following equation was used, as previously described (48): % of red vesicles = [avg no. of red vesicles in n cells/avg no. of (yellow $\pm$ red) vesicles] $\times$ 100. In this study, the number of fluorescent bodies per cell among 50 cells (n) was calculated from three independent experiments (over 30 fields).

EMSAs. Cells were harvested and nuclear extracts were prepared as described previously (42, 44). Concentrations of protein in the extracts were determined using the Bradford assay (Bio-Rad, Hercules, CA, USA). Electrophoretic mobility shift assays (EMSAs) were performed according to the manufacturer's instructions (Promega, Madison, WI, USA). In brief, 5 μ g of nuclear extract was incubated for 30 min at room temperature with a γ - 32 P-labeled oligonucleotide probe (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 (42, 44).

Transfection assay. Lentiviral systems containing mammalian expression vectors were used to block CHOP, AP-1, and MAPK activation, as described previously (44, 45). Specifically, lentiviral systems containing mammalian expression vectors carrying a hemagglutinin epitope-tagged mutant *c-jun* gene (TAM67) with deletions of amino acids at positions 3 to 122 were used to block AP-1. Lentiviral systems containing mammalian expression vectors carrying a FLAG epitope-tagged mutant *JNK1* gene with threonine substituted for alanine at position 183 and tyrosine for phenylalanine at position 185 were used to block JNK activation. Viral systems were supported by BioCore at the Institute of Biomedical Science (Seoul, South Korea). Lentiviruses containing *CHOP* shRNA plasmid and control lentiviruses were purchased from Santa Cruz Biotechnology. Transfection experiments were performed according to the manufacturer's instructions.

Statistical analyses. Student's *t* test was used for statistical analysis. *P* values of <0.05 were considered statistically significant.

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