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Bacterium-Generated Nitric Oxide Hijacks Host Tumor Necrosis Factor Alpha Signaling and Modulates the Host Cell Cycle *In Vitro*

Brian Mocca and Wei Wang

Food and Drug Administration, Center for Biologics Evaluation and Research, Office of Vaccine Research and Review, Division of Bacterial, Parasitic and Allergenic Products, Bethesda, Maryland, USA

In mammalian cells, nitric oxide (NO·) is an important signal molecule with concentration-dependent and often controversial functions of promoting cell survival and inducing cell death. An inducible nitric oxide synthase (iNOS) in various mammalian cells produces higher levels of NO· from L-arginine upon infections to eliminate pathogens. In this study, we reveal novel pathogenic roles of NO· generated by bacteria in bacterium-host cell cocultures using *Moraxella catarrhalis*, a respiratory tract disease-causing bacterium, as a biological producer of NO·. We recently demonstrated that *M. catarrhalis* cells that express the nitrite reductase (AniA protein) can produce NO· by reducing nitrite. Our study suggests that, in the presence of pathophysiological levels of nitrite, this opportunistic pathogen hijacks host cell signaling and modulates host gene expression through its ability to produce NO· from nitrite. Bacterium-generated NO· significantly increases the secretion of tumor necrosis factor alpha (TNF-α) and modulates the expression of apoptotic proteins, therefore triggering host cell programmed death partially through TNF-α signaling. Furthermore, our study reveals that bacterium-generated NO· stalls host cell division and directly results in the death of dividing cells by reducing the levels of an essential regulator of cell division. This study provides unique insight into why NO· may exert more severe cytotoxic effects on fast growing cells, providing an important molecular basis for NO·-mediated pathogenesis in infections and possible therapeutic applications of NO·-releasing molecules in tumorigenesis. This study strongly suggests that bacterium-generated NO· can play important pathogenic roles during infections.

In mammalian cells, nitric oxide (NO·) is a highly reactive and diffusible signaling molecule that plays key roles in modulating both physiological and pathological processes, such as immune response, cell survival, and cell death (reviewed in references 8 and 36). The diverse functions of NO· are often determined by its concentration or its source of production. Low levels of endogenous NO· are produced from L-arginine by constitutively expressed nitric oxide synthase in neuronal cells (nNOS, also known as NOS1) and endothelial cells (eNOS, also known as NOS3) to mediate normal physiological processes. Higher levels of NO· can be produced by an inducible nitric oxide synthase (iNOS, also known as NOS2) in different cell types, but mainly in macrophages, upon infection to kill pathogens (reviewed in reference 9). The higher levels of NO· produced by iNOS have been implicated in various human inflammatory diseases and neurodegenerative diseases (reviewed in references 9 and 12) and in chronic inflammation-related tumorigenesis (18, 33). Recent studies have also shown that chemical-generated higher levels of exogenous NO· can induce tumor cell apoptosis *in vitro* and in animal studies (37; reviewed in reference 1), raising interests in therapeutic exploration of NO·-releasing reagents as antitumor prodrugs.

On the other hand, a complete bacterial denitrification pathway involves a set of denitrification enzymes to sequentially reduce nitrate (NO_3^-) to gaseous nitrogen (N_2), with NO· as an intermediate product (reviewed in reference 69). This complete denitrification pathway has been studied mostly as an alternative means of generating energy used by anaerobes under oxygen-limited or strict anaerobic growth conditions. Recently, various truncated denitrification pathways have been identified in several human pathogens, such as *Mycobacterium tuberculosis* (56), *Neisseria meningitidis* (3), and *Neisseria gonorrhoeae* (48). However, the possible involvement of NO· generated by bacteria in bacterial pathogenesis was not previously elucidated. Recently, we discovered and characterized a truncated denitrification pathway (see Fig. 1A) that was highly upregulated in *Moraxella catarrhalis* cells grown in a biofilm (64–66).

Moraxella catarrhalis is a Gram-negative obligate aerobe that has been recognized as one of the top three bacterial causes of the most common childhood infectious disease, acute otitis media (AOM) (reviewed in reference 45). *M. catarrhalis* forms biofilms on the middle ear mucosa in children with otitis media (29). In addition, *M. catarrhalis* is the second most common bacterial cause of exacerbations of chronic obstructive pulmonary disease (COPD) (46). *M. catarrhalis* infection increases airway inflammation and the levels of proinflammatory cytokines interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) in sputum samples from COPD patients (49, 54). It was unknown which bacterial gene function may affect host secretion of TNF-α in exacerbations of COPD caused by *M. catarrhalis*. Little is known about the pathogenic mechanism used by this opportunistic pathogen due to the lack of an animal model for studying *M. catarrhalis* pathogenesis.

Recent studies have shown that the *M. catarrhalis* nitrite reductase AniA (also known as a major anaerobically induced outer membrane protein) can produce NO· from nitrite (64, 66) and is most likely expressed *in vivo* (52). The NO· generated by the AniA protein is further detoxified by the *M. catarrhalis* nitric oxide reductase NorB (64). Importantly, a combined nitrate/nitrite con-

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Address correspondence to Wei Wang, wei02.wang@fda.hhs.gov.

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TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Description or genotype	Source or reference
Bacterial strains		
ATCC 43617	Wild-type strain	ATCC
O35E	Wild-type strain	31
O35E *narGH*	A *narGH* deletion mutant of strain O35E	65
O35E *aniA*	The DNA sequence between primer pair WW217-WW218 (Fig. 1C) was replaced with a *kan* cartridge	66
O35E *aniA*(pWW115)	*aniA*::*kan* mutant containing a cloning vector pWW115	This study
O35E *aniA*(pWW161)	*aniA*::*kan* mutant containing a wild-type *aniA* gene in *trans*	This study
O35E *norB*	The DNA sequence between primer pair WW248-WW349 (Fig. 1C) was replaced with a *kan* cartridge	64
ETSU-9	Wild-type strain	Steven Berk
7169	Wild-type strain	Anthony Campagnari
Plasmids		
pWW115	Spec^r; cloning vector for *M. catarrhalis*	63
pWW161	pWW115 containing the wild-type *aniA* gene of *M. catarrhalis* ATCC 43617	This study

centration in middle ear effusion (MEE) from patients with otitis media was as high as 0.5 to ~1 mM (35), providing *in vivo* substrates for bacterial denitrification enzymes to produce NO·.

In this study, we investigated the possible pathogenic effects of bacterium-generated NO· using the human bronchial epithelial cell line 16HBE14o (hereafter abbreviated as HBE) (17) as host cells in a bacterium-host cell interaction model. We utilized wild-type *M. catarrhalis* O35E and its well-defined denitrification mutant strains as biological producers of NO· or as controls. Our data show that in the presence of pathophysiological levels of nitrite, the *M. catarrhalis* cells exert adverse effects on the host cell monolayer and reduce host cell viability by the bacterium's ability to generate NO· from nitrite. Using state-of-the-art confocal microscopy live-cell imaging technology, we reveal that bacterium-generated NO· stalls host cell division, resulting in a cascade of apoptosis of dividing cells followed by death of the adjacent cell with apoptotic-cell-like morphological changes. These observations are further mechanistically supported by the results of investigating NO·-modulated host gene expression using human protein arrays. Our data show that bacterium-generated NO· significantly induces host cell secretion of TNF-α, elevates the levels of a crucial apoptosis effector (activated caspase-3) and decreases the levels of inhibitors of apoptosis proteins (IAPs, including cellular IAP 1 [cIAP1], cIAP2, X-linked inhibitor of apoptosis [XIAP], and survivin), therefore triggering host cell programmed death partially through TNF-α signaling. Importantly, the reduced levels of survivin, an essential physiological regulator of cell division (reviewed in reference 2), may be mechanistically linked to the observation of NO· stalling host cell division and inducing dividing cell apoptosis.

To the best of our knowledge, this is the first study to provide novel insight into important pathogenic roles of bacterium-generated NO·, including modulating host gene expression and host cell cycle, stimulating the secretion of the most potent cell death signaling molecule and the proinflammatory cytokine TNF-α, and inducing host cell death.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. catarrhalis* strains used in this study are listed in Table 1. The base medium and bacterial culture conditions were described previously (64).

Cocultures of *M. catarrhalis* and human airway epithelial cells. HBE cells were routinely cultured in tissue culture medium MEM* [minimal essential medium (MEM) (Cellgro) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS) (HyClone) and 1× Glutamax-I (Gibco)] with supplementation of 1× penicillin-streptomycin (MP Biomedicals, LLC) in a 5% CO_2 tissue culture incubator at 37·C. *M. catarrhalis*-HBE cell cocultures (multiplicity of infection [MOI] of 10 to 50) were incubated for up to 24 h in MEM* with or without nitrite (up to 5 mM $NaNO_2$). Human hemoglobin (1 mg/ml) was added as an NO· scavenger, caspase inhibitors (caspase-3 fmk inhibitor Z-DEVD-fmk [benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethyl ketone] [R&D Systems], pan-caspase fmk inhibitor Z-VAD-fmk [benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethyl ketone] [R&D Systems], or caspase fmk inhibitor control Z-FA-fmk [z-Phe-Ala-fluoromethyl ketone] [R&D Systems]) were added (to a final level of 100 μM) as appropriate.

Cloning of *M. catarrhalis aniA* gene for complementation study. A DNA fragment containing the wild-type *aniA* gene was amplified using the oligonucleotide primer pairs CTGGATCCATGTTCATCTTGACAC GCCAT and TGGAGCTCAATGAAAACCAGCGGAT together with the *M. catarrhalis* ATCC 43617 genomic DNA as the template. (The extra nucleotides encoding restriction digestion sites BamHI and SacI are underlined.) The PCR amplicon was digested with both BamHI and SacI and then ligated into cloning vector pWW115 (63) that had been digested with the same restriction enzymes. The ligation reaction mixture was used to transform *M. catarrhalis* O35E *aniA* mutant cells (66). A plasmid (designated pWW161) was isolated from one kanamycin- and spectinomycin-resistant transformant and was confirmed to contain an ATCC 43617 wild-type *aniA* gene by DNA sequencing and Western blotting (data not shown). The cloning vector pWW115 was also used to transform *M. catarrhalis* O35E *aniA* mutant cells to generate O35E *aniA*(pWW115) as a control.

Examination of the host HBE cell morphology. In tissue cultures, a low population of dead cells (1 to 5%) is normally observed. Therefore, for unbiased observations, we routinely monitor the morphology of HBE cell monolayer using a low-magnification (20×) objective. The cells were fixed by incubation in methanol for 5 min and then stained with a modified Giemsa stain reagent (Sigma-Aldrich). Cell images were captured using a Olympus IX51 microscope (20× objective), an Olympus DP72 camera, and the manufacturer's imaging software (DP2-BSW [Olympus]).

Confocal microscopy study together with immunofluorescent labeling was included in the investigation of possible host cell death mechanisms. For confocal microscopy live-cell image studies, *M. catarrhalis*-HBE cells (MOI of ~10) were cocultured in chambered coverglass slides in Dulbecco modified Eagle medium (DMEM) (GIBCO) containing 10% FBS (HyClone) and 1× Glutamax-I (GIBCO) with or without 1 mM nitrite. The cocultures were incubated at 37·C in a CO_2 incubator (XL-multi S DARK LS [PECON]) and were imaged using Axio Observer Z.1

and a Yokogawa CSU-X1 Spinning Disk confocal microscope (Zeiss) (63× objective) in 10-min or 15-min intervals for up to 15 h. The following immunofluorescent dyes were also added to the media to distinguish apoptotic cells from necrotic cells: (i) a cell-permeant DNA dye, Hoechst 33258 (Invitrogen), to stain the nuclei (pseudocolored dark blue) of all cells; (ii) a cell-impermeant DNA dye, YOYO-1 iodide (Invitrogen), to detect extracellular DNA (pseudocolored green) that was released by necrotic cells or to costain cellular DNA (pseudocolored turquoise) of late apoptotic and necrotic cells that lost membrane integrity; and (iii) a cell-impermeant annexin V Alexa Fluor 594 conjugate (Invitrogen) to detect apoptotic cells (38, 61). Because early apoptotic cells lose membrane asymmetry resulting in the phosphatidylserine translocation to the outside of cells, annexin V specifically stains phosphatidylserine red (pseudocolor) to visualize apoptotic cells.

For fixed-slide confocal microscopy analysis, *M. catarrhalis*-HBE cell cocultures in chambered glass slides were fixed with 4% formaldehyde at 24 h postinfection. The host cell actin cytoskeleton was stained (pseudocolor) red with phalloidin-Alexa Fluor 594 conjugate (Invitrogen). Bacterial cells were labeled with the mouse monoclonal antibody 24B5 (15) against the *M. catarrhalis* outer membrane protein UspA1 and then stained (pseudocolored) green with Alexa Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen). The ProLong Gold (Invitrogen) antifade reagent was used to mount coverslips. The fixed slides were imaged using the confocal microscope (100× objective).

Measurement of host HBE cell viability. At 0 h and 24 h postinfection, HBE cells from each coculture were collected, and the live HBE cell counts were determined using an automated cell counter (Countess [Invitrogen]) following the manufacturer's protocol. This study was performed in three repeated infection experiments, and in each experiment, cocultures were performed in triplicate for statistical analysis.

Detection of the expression of inflammatory cytokines by HBE cells. HBE cells, with or without infection (MOI of ~10) with wild-type *M. catarrhalis* O35E or O35E *aniA* mutant cells, were cultured in MEM* with or without the addition of 1 mM nitrite. We examined the secretion of HBE cell inflammatory cytokines using a Proteome Profiler Human Cytokine Antibody Array (R&D Systems) following the manufacturer's protocol. The human cytokine antibody array (spotted in duplicate) blots from two repeated experiments were scanned and quantified using a FluorChemQ MultiImage III system and software (Alpha Innotech). The intensities of cytokine protein arrays were quantified using the FluorChemQ software, and the resulting arbitrary densitometry units were used as measurements of cytokine levels. The positive control of HBE cells cultured alone was used to normalize cytokine protein arrays. The relative levels of cytokines secreted by HBE cells that were cocultured with wild-type O35E cells or O35E *aniA* mutant cells versus HBE cells alone were calculated.

In addition, the levels of secreted TNF-α and IL-1α were measured using the LEGEND MAX Human TNF-α and IL-1α enzyme-linked immunosorbent assay (ELISA) kits (BioLegend, Inc.) following the manufacturer's protocols.

Determination of host apoptotic protein levels. To determine host apoptotic protein levels, HBE cells were cocultured with *M. catarrhalis* O35E (wild type) or O35E *aniA* mutant cells (MOI of ~10) in MEM* supplemented with 1 mM nitrite. At 24 h postinfection, the medium was removed, and cell lysates were prepared for detecting host apoptotic protein levels using the Proteome Profiler human apoptosis array kit (R&D Systems) following the manufacturer's protocol. The human apoptosis array blots from at least two repeated experiments were scanned using a FluorChemQ MultiImage III system (Alpha Innotech). The intensities of apoptotic protein arrays were quantified using the FluorChemQ software, and the resulting arbitrary densitometry units were used as measurements of apoptotic protein levels. The positive control of HBE cells cocultured with *M. catarrhalis* O35E *aniA* cells was used to normalize apoptotic protein arrays. The relative levels of apoptotic proteins in HBE cells cocultured with wild-type O35E cells versus with O35E *aniA* mutant cells were calculated.

RESULTS

Establishing an *in vitro M. catarrhalis*-human airway epithelial cell coculture model for studying *M. catarrhalis* pathogenesis. *M. catarrhalis* is a strict human pathogen, and a workable animal model for studying *M. catarrhalis* pathogenesis remains to be developed. Chinchilla nasopharyngeal colonization studies showed that *M. catarrhalis* colonized the nasopharynxes of chinchillas without apparent disease symptoms (4, 32, 43). To investigate whether bacterium-generated NO· could be involved in pathogenesis, we optimized an *in vitro* system using HBE cells as the host in bacterium-host cell cocultures. After 24 h of coculture in MEM*, the number of CFU of *M. catarrhalis* O35E cells that grew attached to HBE cells was shown to be approximately 2 orders of magnitude higher than that of unattached cells [$(6.8 \pm 2.4) \times 10^5$ CFU/well and $(5.8 \pm 2.1) \times 10^3$ CFU/well, respectively], indicating that the majority (~99.9%) of *M. catarrhalis* cells grow attached to host cells in biofilms (see the proposed pathogenic model of *M. catarrhalis*-generated NO· in Discussion).

When *M. catarrhalis* O35E-HBE cell cocultures were incubated in MEM* containing low levels of sodium nitrite, O35E cells had little or no apparent effect on the host cell monolayer in the presence of low levels (0 to 100 μM) of nitrite (Fig. 1B). At increased nitrite levels (500 μM or higher), *M. catarrhalis* O35E cells disrupted the newly developed monolayer of host cells in a nitrite dose-dependent manner, progressing from the edges to the inner area of the host cell monolayer (Fig. 1B). This study showed that *M. catarrhalis* O35E cells are able to disrupt a monolayer of host cells in MEM* containing nitrite at approximately 500 μM, a level equivalent to pathophysiological conditions (35).

Importantly, the nitrite-dependent disruption of the newly formed host cell monolayer was observed during coculturing with all wild-type *M. catarrhalis* strains tested, including *M. catarrhalis* 7169 and ETSU-9 (data not shown).

***M. catarrhalis* O35E AniA is responsible for nitrite-dependent disruption of host cell monolayer.** To identify the denitrifying enzyme of *M. catarrhalis* responsible for the observed adverse effect on host cells, the wild-type *M. catarrhalis* O35E strain and its denitrification-deficient mutant derivatives, O35E *narGH* (65), O35E *aniA* (66), and O35E *norB* (64) strains, were used to infect HBE cells. The wild-type *M. catarrhalis* O35E strain and *narGH* and *norB* mutant strains all expressed detectable levels of AniA protein in cocultures by Western blot analysis (data not shown), whereas the O35E *aniA* mutant did not express AniA protein (66). Infection with the above-mentioned *M. catarrhalis* O35E strains does not have an apparent effect on host cell monolayer in MEM* (data not shown). However, in MEM* containing a low level of nitrite, only the *aniA* mutant cells had no apparent effect on the morphology of a host cell monolayer at 24 h postinfection (Fig. 1C), suggesting that AniA is required for the nitrite-dependent disruption of a host cell monolayer. As expected, the monolayer of HBE cells was not affected by the addition of nitrite alone (Fig. 1C, HBEC-only panel).

To confirm that the *M. catarrhalis* AniA protein is required for the nitrite-dependent disruption of a host cell monolayer, we performed a complementation study. As expected, the *M. catarrhalis* O35E *aniA* strain carrying the plasmid vector did not have apparent harmful effects on a monolayer of host cells [Fig. 1D, *aniA*

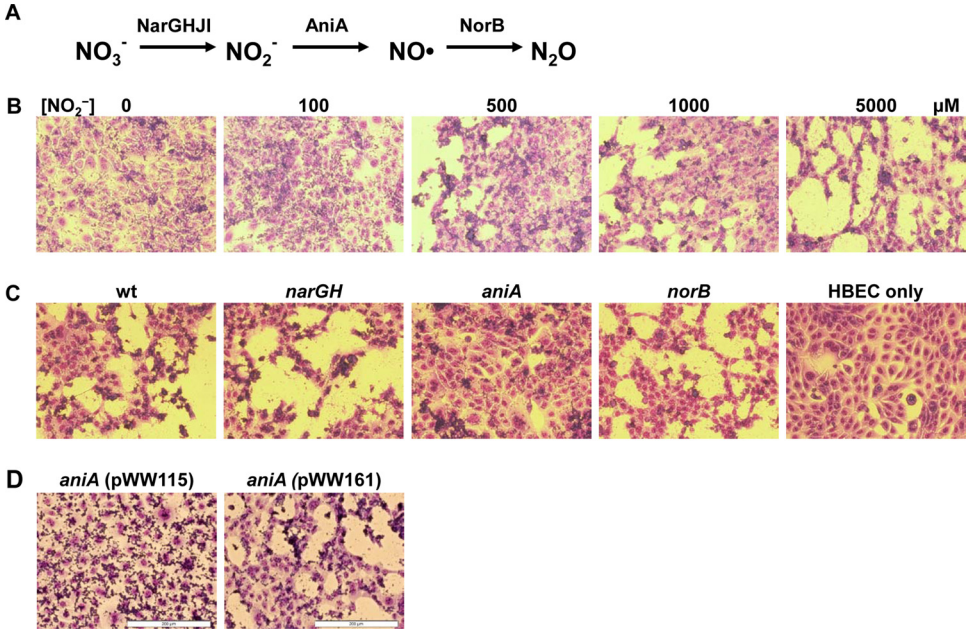

FIG 1 *M. catarrhalis* truncated denitrification genes exert nitrite-dependent adverse effects on host cells. (A) The truncated denitrification pathway in *M. catarrhalis* involves reduction of nitrate (NO_3^-) to nitrite (NO_2^-) by the nitrate reductase complex NarGHJI, reduction of NO_2^- to nitric oxide (NO) by nitrite reductase AniA, and reduction of NO· to nitrous oxide (N_2O) by nitric oxide reductase NorB. (B) Nitrite dose-dependent disruption of the host HBE cell monolayer by *M. catarrhalis* O35E. Microscopic images of Giemsa-stained HBE cells that were cocultured with wild-type *M. catarrhalis* O35E cells in medium containing different levels of nitrite (0, 100, 500, 1,000 and 5,000 μM) for 24 h. (C) Identification of the required *M. catarrhalis* denitrification enzyme for the nitrite-dependent disruption of a host cell monolayer. Microscopic images of HBE cells that were cocultured with wild-type *M. catarrhalis* O35E (wt) and O35E *narGH* mutant, O35E *aniA* mutant, and O35E *norB* mutant in MEM* containing 5 mM nitrite at 37·C in a CO_2 incubator for 24 h. (D) Confirmation of the *M. catarrhalis* AniA protein responsible for NO·-mediated pathogenesis *in vitro*. Microscopic images of HBE cells that were cocultured with the mutant *M. catarrhalis* O35E *aniA*(pWW115) or the complemented mutant O35E *aniA*(pWW161) in MEM* containing 5 mM nitrite for 24 h. Each image in panels B to D shows a representative image from at least three repeated experiments. All microscopic images were captured using a 20× objective. Bars, 200 μm.

(pWW115) panel]. The O35E *aniA* mutant containing a wild-type *aniA* gene in *trans* regained the ability to exert deleterious nitrite-dependent effects on the host cell monolayer [Fig. 1D, *aniA* (pWW161) panel]. To the best of our knowledge, this is the first study identifying and directly confirming that a functional *M. catarrhalis* AniA protein is responsible for an opportunistic pathogenic effect of *M. catarrhalis* on the host cell monolayer exacerbated by the presence of nitrite.

Bacterium-generated NO· is responsible for reduced host cell viability. The above study (Fig. 1) indicated that *M. catarrhalis*-expressed AniA might be responsible for the nitrite-dependent adverse effects on the host cell monolayer by reducing nitrite to NO· (64, 66). We monitored the viability of HBE cells to assess cytotoxicity. As expected, at 24 h postinfection, HBE viability was not significantly affected by nitrite when cultured alone (Fig. 2A, HBEC bars with and without nitrite [$P < 0.184$]) or cocultured with an *M. catarrhalis* O35E *aniA* mutant (Fig. 2A, *aniA* bars with and without nitrite [$P < 0.438$]). In contrast, when HBE cells were cocultured with AniA-expressing wild-type O35E or O35E *norB* mutant cells, live HBE cell counts were significantly lower ($P < 0.034$ or $P < 0.003$) in MEM* containing 5 mM nitrite (Fig. 2A, wt bars with and without nitrite) than in MEM* alone (without nitrite).

To confirm that NO· is responsible for the AniA-mediated nitrite-dependent cytotoxicity, we added human hemoglobin as a major NO· scavenger (at a final concentration of 1 mg/ml) into the growth medium. As shown (Fig. 2B), supplementing with human hemoglobin was able to reverse most of the nitrite-dependent cytotoxicity to HBE cells (Fig. 2B), except that the HBE cell viability was still significantly lower ($P < 0.006$) when cocultured with O35E *norB* mutant cells (Fig. 2B, *norB* bar with nitrite) compared with HBE cells only in MEM* containing 5 mM nitrite. This study confirms that bacterium-generated NO· is responsible for observed nitrite-dependent pathological effects.

Furthermore, by utilizing cell culture inserts (Millipore) to physically separate *M. catarrhalis* cells from HBE cells but allowing NO· to diffuse into HBE cell culture, we observed reduced nitrite-dependent disruption of the host cell monolayer (data not shown), strongly suggesting that the colonization of *M. catarrhalis* on host cells is important for NO·-mediated pathological effects.

Bacterium-generated NO· stalls host cell division and induces the host cell to undergo programmed death. One interesting observation that was overlooked initially may be important for understanding why NO·-mediated pathogenesis causes more severe damage on fast growing cells such as those in a newly developed monolayer. A well-developed HBE cell monolayer seemed to be more resistant to the disruption by bacterium-generated NO· (Fig. 3A, indicated by a solid black arrow), whereas the less-confluent and newly developed HBE cell monolayer (Fig. 3A, indicated by an open arrow) was severely damaged by bacterium-generated NO·.

To explore whether dividing host cells are more susceptible to NO· and to investigate host cell death mechanisms, we performed a confocal microscopy live-cell image study to monitor the effects of bacterium-generated NO· on host cell morphological changes using immunofluorescent dyes to distinguish apoptotic cells from

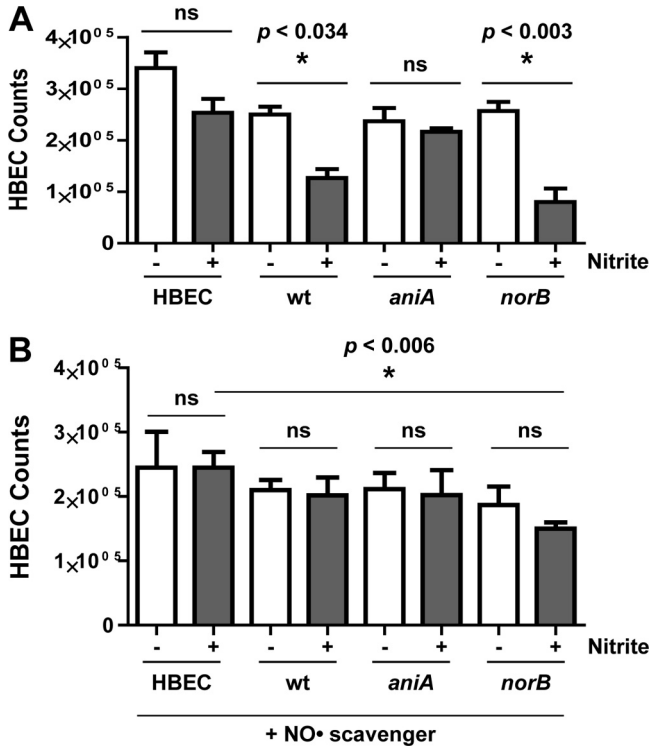

FIG 2 *M. catarrhalis* AniA-generated NO· reduces host cell viability. Viable HBE cell counts were determined in HBE cell culture (HBEC) or in cocultures with the wild-type *M. catarrhalis* O35E (wt) or O35E *aniA* mutant or *norB* mutant for 24 h in the following media: MEM* with (+) or without (−) 5 mM nitrite (A) or MEM* containing human hemoglobin (1 mg/ml) with (+) or without (−) 5 mM nitrite (B). ns, not significant.

necrotic cells. The live-cell image study during infection is important, because it is known that apoptotic cells can eventually undergo secondary necrosis in tissue cultures due to the absence of a mechanism to remove apoptotic cells (10).

There were several interesting observations from the confocal microscopy live-cell image studies. When cocultured with wild-type *M. catarrhalis* O35E cells in MEM*, host cell division was apparently not affected, and dividing HBE cells were able to form two daughter cells (Fig. 3B). Interestingly, in medium containing 1 mM nitrite, dividing host cells moving around could not form daughter cells and became apoptotic (annexin V stains apoptotic cells red) at approximately 3 to 5 h postinfection (Fig. 3C, panel 1). At ~8 h postinfection, the apoptotic host cells underwent secondary necrosis and released cellular contents, including nuclear DNA (YOYO-1 iodide stains nuclear DNA green [Fig. 3C, panels 2 and 3]). The released cellular contents were observed to be internalized by adjacent host cells and seemed to trigger death of adjacent cells (Fig. 3C, panels 2 and 3). During the different stages of infection, the confocal microscopy live-cell images captured the progression of host cell anoikis, a term for adherent cell apoptosis (27). Dying host cells underwent sequential morphological changes of apoptosis, such as detaching from adjacent cells and rounding, membrane blebbing, losing membrane asymmetry, and eventually losing membrane integrity (Fig. 3B, panels 2 and 3).

Bacterium-generated NO· induces host cell secretion of proinflammatory cytokines TNF-α and IL-1α. To elucidate the possible molecular basis of observed pathogenic roles of bacterium-generated NO·, we investigated whether bacterium-generated

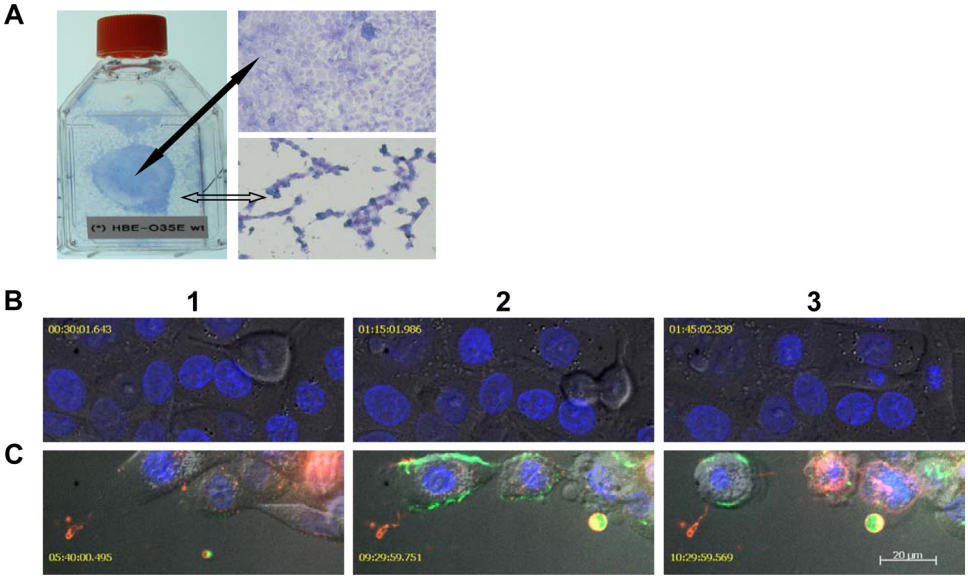

FIG 3 Dividing cells are more susceptible to the cytotoxicity of bacterium-generated NO·. (A) A photo of HBE cells cocultured with *M. catarrhalis* O35E cells in a T25 tissue culture flask and microscopic (20× objective) images of Giemsa-stained HBE cells in MEM* containing 1 mM nitrite. A fully confluent HBE cell monolayer (indicated by a solid black arrow) was not disrupted, whereas the newly developed HBE cell monolayer (indicated by an open arrow) was severely damaged by *M. catarrhalis* O35E. (B) Confocal microscopy live-cell images showed a commensal-like relationship of *M. catarrhalis* and HBE cells cocultured in MEM*. The infection of *M. catarrhalis* did not have apparent effects on HBE cell division. (C) Confocal microscopy live-cell images show *M. catarrhalis* exerting pathogenic effects on HBE cells in MEM* containing 1 mM nitrite. Dividing HBE cells failed to divide into two cells and became apoptotic (pseudocolored red) at 3 to 5 h postinfection (panel 1). HBE cells underwent secondary necrosis and released cellular contents, including DNA (YOYO-1 stained DNA green in panels 2 and 3). NO·-induced HBE cell death was associated with apoptotic-cell-like morphological changes (panels 2 and 3). The images in panels B and C were representative images from two replicate experiments. The confocal microscopic images in panels B and C were captured using a 63× objective at different time points after infection. Bar, 20 μm.

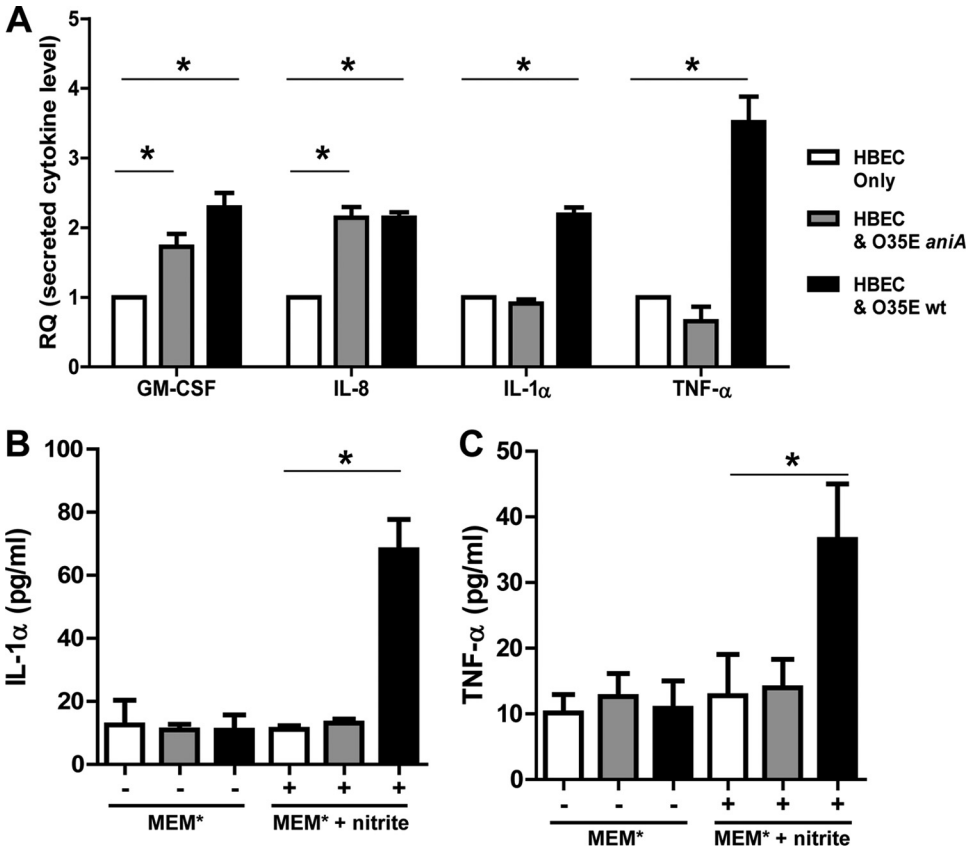

FIG 4 Bacterium-generated NO· induces host cell secretion of proinflammatory cytokines TNF-α and IL-1α. (A) Media of 24-h cultures of HBE cells alone (HBEC) (white bars) or of HBE cells cocultured with wild-type *M. catarrhalis* O35E (black bars) or *aniA* mutant cells (gray bars) were used for detecting the levels of secreted cytokines by host cells using a human cytokine array in MEM* containing 1 mM nitrite. Values are means plus standard deviations (error bars) from two repeated experiments (each in duplicate). (B) Detection of the secreted IL-1α by host cells using LEGEND MAX human IL-1α ELISA kit in MEM* with (+) or without (−) 1 mM nitrite. (C) Detection of the secreted TNF-α by host cells using LEGEND MAX human TNF-α ELISA kit in MEM* with (+) or without (−) 1 mM nitrite. Values in panels B and C are means plus standard deviations from two repeated experiments (each in triplicate).

NO· could affect host gene expression. We first utilized a Proteome Profiler human cytokine antibody array (R&D Systems) to examine the secretion of inflammatory cytokines by host HBE cells in MEM* with or without 1 mM nitrite.

In MEM* containing nitrite, the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-8 was significantly elevated by host cells that were cocultured with wild-type *M. catarrhalis* O35E or *aniA* mutant cells at 24 h postinfection (Fig. 4A, black and gray GM-CSF and IL-8 bars [*P* values of 0.0011 and 0.0001]) compared with HBE cell culture alone (Fig. 4A, white GM-CSF and IL-8 bars). The secretion of GM-CSF and IL-8 was also increased at least 2-fold in MEM* without the supplementation of nitrite (data not shown), confirming that the secretion of GM-CSF and IL-8 by HBE cells can be stimulated by *M. catarrhalis* infection independent of bacterium-generated NO· (as reported previously [55]).

More importantly, our study revealed that the secretion of TNF-α and IL-1α was significantly elevated only in host cells cocultured with *M. catarrhalis* O35E in response to bacterium-generated NO· (Fig. 4A, black IL-1α and TNF-α bars, [*P* values of 0.0002 and 0.0008]). The infection with *M. catarrhalis aniA* mutant cells did not increase host cell secretion of IL-1α and TNF-α under the same coculturing condition (Fig. 4A, gray IL-1α and TNF-α bars).

To confirm that the bacterium-generated NO· stimulates the secretion of TNF-α and IL-1α, we further measured the levels of TNF-α and IL-1α using the LEGEND MAX Human TNF-α and IL-1α ELISA kits (BioLegend, Inc.). Again, the levels of secreted TNF-α and IL-1α were shown to be significantly elevated only under coculturing conditions when bacterial cells produced NO· from nitrite (Fig. 4B and C, black bars with nitrite). The secretion of IL-1α and TNF-α by host cells was not affected by *M. catarrhalis* infection in MEM* without the supplementation of nitrite (Fig. 4B and C, gray and black bars without nitrite). It should be noted that both IL-1α and TNF-α are the most potent NF-κB activators and major endogenous pyrogens (21). This study suggests that bacterium-generated NO· may be involved in increasing inflammation and producing fever by stimulating the production of proinflammatory cytokines IL-1α and TNF-α.

***M. catarrhalis* AniA-generated NO· affects the levels of host proteins that play key roles in programmed death.** To investigate *M. catarrhalis* O35E-induced nitrite-dependent host cell death mechanisms, we utilized a Proteome Profiler human apoptosis array kit (R&D Systems) to detect the levels of apoptotic proteins in HBE cells cocultured with wild-type O35E (Fig. 5, black bars) or O35E *aniA* mutant cells (Fig. 5, gray bars) in MEM* containing 1 mM nitrite. In HBE cells cocultured with wild-type O35E, the balance of proapoptotic and anti-apoptotic proteins in the extrin-

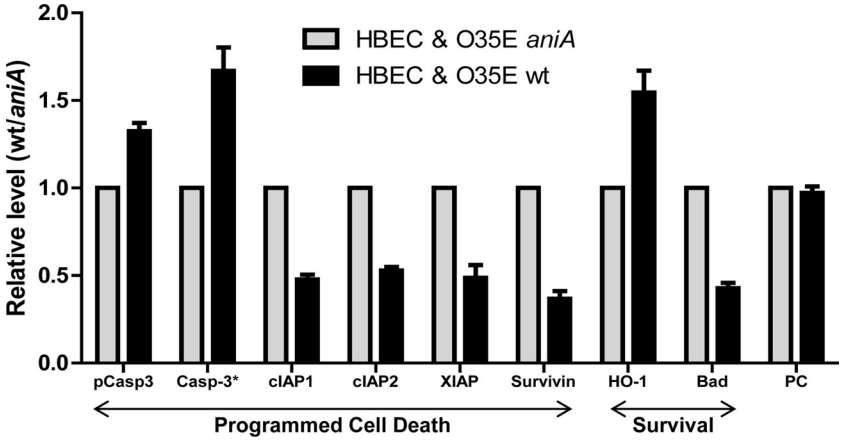

FIG 5 *M. catarrhalis* AniA-generated NO· affects the levels of host proteins that play key roles in programmed death. HBE cells were cocultured with wild-type *M. catarrhalis* O35E (black bars) or *aniA* mutant cells (gray bars) in MEM* containing 1 mM nitrite for 24 h. Cell lysates were used to determine the relative levels of apoptotic proteins using a human apoptosis protein array kit. Values are means plus standard deviations from at least two repeated experiments (each in duplicate). pCasp3, precursor of caspase-3; PC, positive control.

sic death pathway was shifted in favor of apoptosis. The relative level of activated caspase-3, one of the critical executioners of apoptosis (39, 41), was significantly increased (Fig. 5, black Casp-3* bar) ($P < 0.0041$), while the levels of anti-apoptotic proteins (reviewed in reference 2) cIAP1/2, XIAP, and survivin were significantly reduced (Fig. 5, black cIAP1, cIAP2, XIAP, and survivin bars [P values of 0.0001, 0.0002, 0.0009, and 0.0007]). The inactivated caspase-3 precursor was also increased significantly (Fig. 5, black pCasp3 bar) ($P < 0.0064$) in HBE cells cocultured with wild-type O35E. It should be noted that survivin is an essential regulator of cell division (reviewed in reference 2) and that cIAPs and XIAP can inhibit either apoptosis or necroptosis (25, 34).

On the other hand, the expression of a cytoprotector heme oxygenase-1 (HO-1) (reviewed in references 22 and 28) was significantly increased (Fig. 5, black HO-1 bar) ($P < 0.0074$), and the level of Bad (Bcl-associated death promoter) was significantly reduced (Fig. 5, black Bad bar) ($P < 0.0001$) by *M. catarrhalis* AniA-generated NO·. These data demonstrate that NO· is able to mediate controversial functions simultaneously, promoting cell survival and inducing cell death.

The cytotoxicity of bacterium-generated NO· can be partially inhibited by a pancaspase inhibitor. We further evaluated the effects of caspase inhibitors on NO·-mediated cytotoxicity. The addition of caspase-3 fmk inhibitor Z-DEVD-fmk at a final concentration of 100 μM provided little or no protection against bacterium-generated NO· cytotoxicity compared with the caspase fmk inhibitor control Z-FA-fmk (Fig. 6A, Z-FA and Z-DEVD bars). This result suggested that caspase-3 may not be the only apoptosis executioner in this study. The levels of other apoptosis executioners such as caspase-6 (16, 23) and caspase-7 (41, 62) were not examined in this study. Importantly, the addition of pancaspase fmk inhibitor Z-VAD-fmk at a final concentration of 100 μM resulted in a modest but statistically significant ($P < 0.0128$) increase in host cell viability (Fig. 6A, Z-VAD bar). This study confirms that bacterium-generated NO· reduces host cell viability by stalling cell division and inducing (at least a small population of) host cells to undergo caspase-dependent apoptosis.

DISCUSSION

Nitric oxide (NO·) is a highly reactive and diffusible radical that modulates various physiological and pathological processes in mammalian cells. In order to mediate normal physiological processes, the endogenous level of NO· must be precisely maintained through the production and scavenging of NO·. Recently, bacterial denitrification has been investigated for biofilm dispersal (5) and possible involvement in virulence (reviewed in reference 50). However, the possible pathogenic involvement of bacterium-generated NO· remained to be elucidated.

Our study suggests that bacterium-generated NO· can change the bacterium-host cell interaction from commensal-like to pathogenic, mostly exacerbated by the presence of pathophysiological levels of nitrite. Our study also suggested that *M. catarrhalis* colonization is important for NO·-mediated pathogenesis. A current working model for *M. catarrhalis* AniA-mediated nitrite-dependent pathogenesis is depicted in Fig. 6B. In the absence of pathogenic levels of nitrite, bacterial cells (Fig. 6B, *M. catarrhalis* cells were stained green) colonize on the surfaces of host HBE cells (Fig. 6B, HBE cells were stained red) in biofilms without apparent disease symptoms (Fig. 6B, HBEC & wt panel). It is possible that when bacterial cells form biofilms directly on the surfaces of human airway epithelial cells, bacterium-generated NO· could reach a deleterious level in localized microenvironments. Previous studies showed that the higher levels of NO· generated by chemicals or by iNOS can induce apoptosis or necrosis (reviewed in references 6 and 36). Higher levels of NO· result in XIAP *S*-nitrosylation which consequently abolishes XIAP's anticaspase-3 activity (58).

Our data show that bacterium-generated NO· stimulates host cell secretion of TNF-α (Fig. 4A and C). TNF-α is one of the most potent activators of NF-κB and endogenous pyrogens (21). The increased levels of TNF-α or reduced IAPs can induce either apoptosis or programmed necrosis, which is newly characterized as a caspase-independent necroptotic death (selected publications and reviews in references 13, 25, 34, 51, and 60). Our studies showed that the addition of pancaspase inhibitor Z-VAD-fmk had only a modest inhibitory effect on NO·-induced host cell death, even though the confocal microscopy live-cell image study

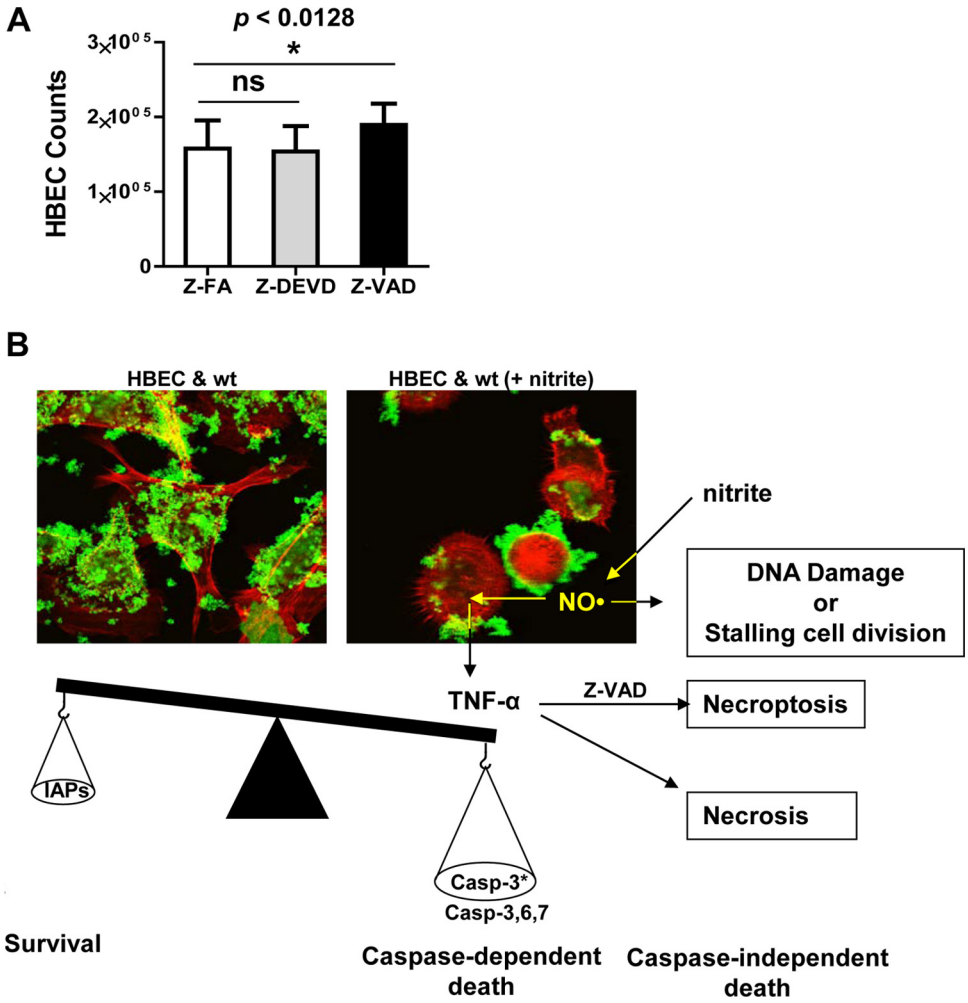

FIG 6 The cytotoxicity of bacterium-generated NO· can be partially inhibited by a pancaspase inhibitor. (A) HBE cells were cocultured with wild-type O35E in MEM* containing 1 mM nitrite and 100 μM caspase inhibitor control Z-FA-fmk, caspase-3 inhibitor Z-DEVD-fmk, or pancaspase inhibitor Z-VAD-fmk. The results shown are representative of data from three repeated experiments (each in triplicate). (B) A current working model of NO·-mediated bacterial pathogenesis. Bacterium-generated NO· may induce caspase-dependent and caspase-independent host cell death and modulate host cell gene expression through TNF-α signaling. The confocal microscopy images were captured using a 100× objective.

seemed to indicate that the levels of apoptotic death might be higher. It is possible that NO· also induces caspase-independent apoptosis (Fig. 6B) by damaging host DNA (7). Importantly, it should be noted that, while bacterium-generated NO· continually stimulates the secretion of elevated levels of TNF-α, the inhibition of caspase-dependent apoptosis with Z-VAD-fmk may induce a switch toward caspase-independent necroptosis (60).

Importantly, our data show that bacterium-generated NO· reduces the level of survivin (Fig. 5). It was also shown that chemical-generated NO· was able to reduce the level of survivin in lung cancer cells and induce apoptosis *in vitro* (13). Survivin has been shown to be a physiological regulator of cell division, and an increased level of this protein at mitosis is essential for cell division (reviewed in reference 2).

Clinical studies showed that higher levels of NO· were observed in the intestines of patients with infectious gastroenteritis (30) and in premature infants with necrotizing enterocolitis (NEC) (26). While infectious gastroenteritis is mostly acute and self-eliminating in adult patients, NEC is responsible for 1.8 million deaths in young children each year (reviewed in reference 24). NEC is a leading fatal intestinal disease in premature newborns, and NO· was suspected to play a key role in disruption of the gut epithelial barrier in NEC (14, 59). It is tempting to compare a fast developing monolayer of HBE cells with a fast developing epithelial barrier (for example, the length of the Eustachian tube doubles during the first few years of infancy) in young children and to assume that dividing epithelial cells in these populations are more vulnerable to NO·-mediated cytotoxicity. It might be understandable that higher levels of NO· (either iNOS or generated by bacteria) can exert more-noxious damage in infants and young children. In addition, this study may provide a novel insight into the molecular basis of NO·-releasing chemicals (to a certain degree) being able to differentially target the fast growing tumor cells from normal human cells *in vitro* (44).

Clinical studies reported that the *M. catarrhalis* infection in COPD patients is associated with increased levels of IL-8 and TNF-α (49, 54). The knowledge about how *M. catarrhalis* affects host secretion of TNF-α was previously absent. Studies showed

that the secretion of IL-8 and GM-CSF by human bronchial epithelial cells can be stimulated by either wild-type *M. catarrhalis* O35E cells or by the addition of both purified TNF-α and IL-1β (55). The *M. catarrhalis* lipooligosaccharides (LOS) (68) and the outer membrane protein UspA1 might be involved in stimulating the secretion of IL-8 *in vitro* (57), and MID/Hag might be involved in B-cell activation (47, 67). Importantly, serum samples from COPD patients who had recently cleared *M. catarrhalis* infection are reactive to *M. catarrhalis* LOS (53) and MID/Hag (40), indicating that *M. catarrhalis* extracellular components may contribute to the production of inflammatory disease. To the best of our knowledge, this is the first study to reveal that the *M. catarrhalis* AniA-generated NO· stimulates host cell secretion of TNF-α and IL-1α. Both TNF-α and IL-1α are potent NF-κB activators and major endogenous pyrogens (19). It has been shown that subnanomolar concentrations of IL-1 could produce fever in animals and humans (20). Our study suggests that *M. catarrhalis* AniA-generated NO· may be involved in increasing inflammation and producing fever in *M. catarrhalis* diseases.

Collectively, our data indicate that, in addition to stalling host cell division, bacterium-generated NO· induces host cell programmed death through TNF-α signaling with increased levels of proapoptotic proteins (including TNF-α and activated caspase-3) as well as decreased levels of antiapoptotic proteins (including XIAP, Survivin, and cIAP1/2). This study suggests that, in the presence of pathophysiological levels of nitrite, *M. catarrhalis* may employ a simple yet effective NO· production mechanism to fulfill contact-efficient virulence factor delivery and interkingdom signaling.

Our study also revealed an opposite function of NO·, promoting host cell survival by inducing the expression of the cytoprotector HO-1 and reducing the expression of Bad (Fig. 2B). Studies showed that chemical-generated NO· stimulates HO-1 expression in smooth muscle cells to promote cell survival (42), and HO-1 protects endothelial cells from TNF-α-induced apoptosis (11). It is important to further explore potential beneficial effects of bacterium-generated NO· on bacterium-host cell interaction. We are actively investigating how *M. catarrhalis* denitrification gene expression is regulated and whether the *M. catarrhalis* AniA protein is a potential vaccine candidate.

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