Oxidative stress enhances IL-8 and inhibits CCL20 production from intestinal epithelial cells in response to bacterial flagellin

Sabine M. Ivison,1 Ce Wang,1 Megan E. Himmel,1,2 Jared Sheridan,1 Jonathan Delano,1 Matt L. Mayer,3 Yu Yao,1 Arnawaz Kifayet,1 and Theodore S. Steiner1

1Division of Infectious Diseases, Department of Medicine; 2Department of Surgery; and 3Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

Submitted 2 March 2010; accepted in final form 23 June 2010

Oxidative stress enhances IL-8 and inhibits CCL20 production from intestinal epithelial cells in response to bacterial flagellin. Am J Physiol Gastrointest Liver Physiol 299: G733–G741, 2010. First published July 1, 2010; doi:10.1152/ajpgi.00089.2010.—Intestinal epithelial cells act as innate immune sentinels, as the first cells that encounter diarrheal pathogens. They use pattern recognition molecules such as the Toll-like receptors (TLRs) to identify molecular signals found on microbes but not host cells or food components. TLRs cannot generally distinguish the molecular signals on pathogenic bacteria from those found in commensals, yet under healthy conditions epithelial immune responses are kept in check. We hypothesized that, in the setting of tissue damage or stress, intestinal epithelial cells would upregulate their responses to TLR ligands to reflect the greater need for immediate protection against pathogens. We treated Caco-2 cells with the TLR5 agonist flagellin in the presence or absence of H2O2 and measured chemokine production and intracellular signaling pathways. H2O2 increased flagellin-induced IL-8 (CXCL8) production in a dose-dependent manner. This was associated with synergistic phosphorylation of p38 MAP kinase and with prolonged I-kB degradation and NF-kB activation. The H2O2-mediated potentiation of IL-8 production required the activity of p38, tyrosine kinases, phospholipase Cγ, and intracellular calcium, but not protein kinase C or protein kinase D. H2O2 prolonged and augmented NF-kB activation by flagellin. In contrast to IL-8, CCL20 (MIP3α) production by flagellin was reduced by H2O2, and this effect was not calcium dependent. Oxidative stress biases intestinal epithelial responses to flagellin, leading to increased production of IL-8 and decreased production of CCL20. This suggests that epithelial cells are capable of sensing the extracellular environment and adjusting their antimicrobial responses accordingly.

innate immunity; Toll-like receptor 5; NF-kB; MAP kinases

THE MAMMALIAN INNATE IMMUNE system relies on the recognition of microbial molecular signatures by pattern recognition receptors widely expressed on host cells. One problem with this system is the frequent inability to distinguish microbial signatures (also known as microbe-associated molecular patterns, or MAMPs) that derive from commensal organisms from those expressed on pathogens. Areas of heavy mucosal commensal colonization, such as the oral cavity, gut, and vagina, need ways to dampen inappropriate innate immune responses to these commensals. This can be achieved by various mechanisms, including downregulation or sequestration of pattern-recognition receptors such as the Toll-like receptors (TLRs), expression of signaling inhibitors, or mucous barriers between colonizing bacteria and host cells. Even with these safeguards in place, however, inappropriate innate immune responses to commensal bacteria still occur during pathological conditions [such as inflammatory bowel disease (IBD) and autoimmune arthritides].

Adaptive immunity handles the problem of inappropriate responses to commensal flora through the recognition of signals of tissue injury, which serve to upregulate antigen presentation in a proinflammatory manner. This “danger hypothesis” first proposed in the 1990s helps to explain why T cells and B cells reactive to commensal flora (which are not subjected to clonal deletion during maturation) do not normally proliferate and generate inflammatory responses (10, 21, 22). Similarly, the innate immune system can recognize tissue injury and upregulate responses appropriately. Endogenous danger signals or alarmins that are known to modulate TLR signaling include heat shock proteins (3), elements of the complement system (7, 28), and constitutive intracellular molecules such as IL-33 (25), HMGB1 (14, 44), and ATP (4) that are released into the extracellular environment by stressed, injured, or necrotic cells.

In this work, we examined the ability of an additional danger signal, oxidative stress, to modulate innate immune responses in intestinal epithelial cells (IECs). We induced oxidative stress by stimulating cells with H2O2, which is known to cause IL-8 production in other cell types, including airway epithelial cells and endothelial cells (6, 20, 24, 31). IECs treated with H2O2 undergo loss of junctional integrity and increased apoptosis (11, 23), but the result of this on microbial responses has never been reported. We tested the effect of H2O2 on Caco-2 colon carcinoma cells, which express TLR5 and respond to its ligand flagellin by secreting chemokines like IL-8/CXCL8 and MIP3α/CCL20 (8) as well as β-defensin (27). We found that Caco-2 cells, unlike airway epithelial cells, did not produce IL-8 or CCL20 in response to H2O2 alone, but that H2O2 significantly modulated production of these chemokines following flagellin treatment. This effect involved enhanced and sustained activation of NF-kB and p38 MAP kinase, suggesting that IECs possess a coordinated signaling response to oxidative stress as a danger signal to alter innate immune responses to flagellin.

MATERIALS AND METHODS

Cell culture. Caco-2 cells obtained from the American Type Culture Collection (ATCC, Rockville, MD) were grown in HyQ DMEM/High Glucose (HyClone; Logan UT) with 10% FBS (Invitrogen, Carlsbad, CA), nonessential amino acids (HyClone), penicillin (100 U/ml), and streptomycin (100 μg/ml) (Sigma, St. Louis, MO). Cells were seeded at 8 × 10^4/ml in polystyrene plates and used for...
experiments 6 days after becoming confluent; medium was changed three times per week. HeLa cells from ATCC were grown in Eagle’s minimal essential medium (StemCell, Vancouver, BC, Canada) supplemented with 10% heat-inactivated FBS (HyClone), 2 mM glutamine (StemCell), 1 mM sodium pyruvate (StemCell), and antibiotics as above. For transient transfection experiments, cells were seeded in 24-well plates and transfected the following day by using Lipofectamine LTX (Invitrogen) according to the manufacturer’s recommendations. Cells received the following constructs: pEGFP-N1 (Clontech) 25 ng, pEF6-hTLR5 (originally a gift from Alan Aderem, Institute for Systems Biology, Seattle, WA) 100 ng, and pSuper (oligoengine) constructs total p44/42, phospho-I-κB-α analysis. Antibodies (phospho-p38 T180/Y182, total p38, phospho-κB-α) according to the manufacturer’s recommendations. Cells received the following constructs: pEGFP-N1 (Clontech) 25 ng, pEF6-hTLR5 (originally a gift from Alan Aderem, Institute for Systems Biology, Seattle, WA) 100 ng, and pSuper (oligoengine) constructs 100 ng, and pSuper (oligoengine) constructs 500 ng. pSuper-PKD1 was constructed as previously described (8).

Medium was changed the following day, and cells were used for experiments the day after that.

Stimulation and inhibition of cells. Endotoxin-free recombinant H18 flagellin from enterohaemagglutinating Escherichia coli strain 042 (FlhC) was prepared as previously described (8) and was used at 100 ng/ml. Pharmacological inhibitors (U73122, PP2, Bay11–7082, BAPTA-AM, and U0126) were obtained from Calbiochem (San Diego, CA). H2O2 3% solution was purchased through a local pharmacy and used within 1 mo of purchase. Inhibitors were used at 10 μM except Bay11 (20 μM), G66976 (3 μM), G66983 (12 μM), LY294002 (30 μM), and BAPTA-AM (doses as indicated). Cells were pretreated with inhibitors or DMSO for 30 min before adding other stimuli. Human IL-8 and CCL20 contents of supernatants were determined by ELISA (OptEIA, BD Biosciences, San Jose, CA for IL-8 and Duo-set, R&D, Minneapolis, MN for CCL20) according to the manufacturers’ instructions. Results are expressed as picograms per milliliter, nanograms per milliliter, or fold of control, with error bars representing SD, except where noted; P values were calculated by Student’s t-test (Excel).

For detection of phosphorylated proteins, cells were stimulated for the indicated times, washed in cold PBS, and lysed in 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Nonidet P-40, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 2 mM Na2VO4, and protease inhibitors cocktail (Sigma). Equal amounts of proteins were separated by SDS-PAGE and blotted for Western blot analysis. Antibodies (phospho-p38 T180/Y182, total p38, phospho-p44/42 T202/Y204, total p44/42, phospho-I-κB-α) were from Cell Signaling. The anti-total I-κB-α antibody (C-15) was from Santa Cruz. Blots were scanned and densitometric analysis was done by using Alpha Innoteck software on at least three different experiments; density was adjusted relative to loading control and expressed as ratios to a fixed sample.

EMSAs. Caco-2 cells were lysed at the indicated times after stimulation as described above. Nuclear protein extracts were obtained and NF-κB binding to 32P-labeled oligonucleotide was measured by electrophoretic mobility shift assay (EMSA) as described (8). Specificity was verified by including a 100-fold excess of unlabelled NF-κB-binding oligonucleotide in one lane with flagellin-treated nuclear extract.

LDH assay. Cytotoxicity was measured as release of lactate dehydrogenase (LDH) into culture supernatant. Cells were treated with the indicated conditions for 3 h, and supernatants assayed for LDH by using the Cytotoxicity Detection Kit with minor modifications from the manufacturer’s instructions (Roche Applied Science). Cells were treated in parallel with culture media alone (untreated) or with Triton X-100 0.1% (which produced 100% cell death after 3 h as determined by Trypan blue staining). The % cytotoxicity of each sample was calculated as the LDH optical density (OD) of that sample minus OD of untreated, divided by the OD of the Triton-treated sample minus untreated.

Quantitative RT-PCR. Caco-2 cells were stimulated and total RNA was isolated using Trizol (Invitrogen), and 1 μg of RNA from each sample was reverse transcribed by using RevertAid H minus first-stand cDNA synthesis kit (Fermentas). Real-time PCR was performed with SYBRGreen master mix (Applied Biosystems, Foster City, CA or Fermentas, Burlington, ON, Canada) on an Opticon real-time thermal cycler. The following primer pairs were used: GAPDH (forward) 5'-gagagtaggtgtggcagtt (reverse) 5'-ttgaggatcctgcttggaga; CCL20 (forward) 5'-tgcaagattgctttcgcctagc (reverse) 5'-ttgcgtctggctactgg; and IL-8 (forward) 5'-atgacttcaagttgctgtggtggt (reverse) 5'-tctcgcccccctaaaccttctc. For determination of mRNA stability, 5 μg/ml actinomycin D (Sigma) was added to cells 1 h after stimulation and cells were harvested after a further 1, 2, and 3 h. Fold increases in chemokine mRNA were calculated based on the difference between cycle threshold values for the chemokine and GAPDH within each individual sample and were averaged from triplicate samples.

RESULTS

To determine whether oxidative stress can influence TLR5 signaling responses, we first measured IL-8 production from Caco-2 human colon carcinoma cells stimulated for 3 h with flagellin in the absence or presence of H2O2. As shown in Fig. 1A, cells secreted no IL-8 in the absence of flagellin, with or without H2O2. As expected 100 ng/ml flagellin caused significant IL-8 secretion (191 ± 28.7 pg/ml). Pretreatment of cells with H2O2 at doses from 0.5 to 2 mM for 20 min prior to the addition of 100 ng/ml flagellin caused a large and significant increase in IL-8 production over the ensuing 3 h, peaking at a mean 5.4-fold the concentration elicited by FlhC alone (P < 0.02) after pretreatment with 1 mM H2O2. This effect was dependent on the oxidative stress itself rather than contaminants in the H2O2 solution, since pretreatment with the oxidative scavenger N-acetylcysteine (N-AC) completely blocked the rise in IL-8 (from 5.4-fold to 1.3-fold increase with H2O2; P < 0.02, Fig. 1B). To confirm that other sources of oxidative stress can produce the same result, we tested the redox cyclers 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), which induces the formation of superoxide anions (41). At 5 μM (a relatively low dose), DMNQ increased flagellin-induced IL-8 production to the same extent as 1 mM H2O2 (Fig. 1C; P < 0.001 for DMNQ and H2O2 vs. FlhC alone).

We next sought to determine the basic signaling mechanisms underlying this effect. H2O2 has been shown to elevate intracellular calcium concentrations through both influx and release of intracellular stores (35). To measure the requirement for intracellular calcium in the augmentation of flagellin-induced IL-8, we pretreated Caco-2 cells with the cell-permeable calcium chelator BAPTA-AM prior to the addition of H2O2. IL-8 release by flagellin alone was not significantly inhibited at concentrations up to 25 μM but was reduced by 50 μM BAPTA-AM. In contrast, the combination of BAPTA-AM at 10 μM or higher with H2O2 substantially inhibited IL-8 release (Fig. 2A). To test whether this was due to an increase in H2O2 cytotoxicity in the absence of calcium (rather than impaired signaling), we performed LDH release assays on Caco-2 cells exposed to flagellin, H2O2, and BAPTA-AM alone or in combination (Fig. 2B). Although addition of 10 μM BAPTA-AM alone caused a small degree of cytotoxicity, addition of up to 50 μM BAPTA-AM did not significantly increase the cell death due to flagellin or flagellin plus H2O2. This degree of cytotoxicity alone would be unlikely to explain the loss of IL-8 production with addition of BAPTA-AM. Together, these results suggest that intracellular calcium is required for the production of IL-8 under conditions of oxidative stress, in contrast to under nonstress conditions. As expected, H2O2 itself increased cyto-
toxicity in a dose-dependent fashion, although at doses as high as 3 mM H$_2$O$_2$, only 20% of the cytotoxicity observed after 0.1% Triton X-100 treatment was observed (Fig. 2C).

We next examined the signaling pathways downstream of TLR5 that could be affected by H$_2$O$_2$. First we measured the effects of candidate kinase inhibitors on the ability of H$_2$O$_2$ to increase IL-8 production. Because some of these inhibitors reduce flagellin responses in the absence of any additional stimulus, it was necessary to normalize results to the amount of IL-8 produced by flagellin alone in the presence of each inhibitor. As shown in Fig. 3A, the NF-κB inhibitor Bay 111 completely eliminated the inflammatory effect of H$_2$O$_2$ ($P < 0.001$). Three other inhibitors also significantly reduced the effect of H$_2$O$_2$: the p38 MAPK inhibitor SB203580 (from 4-fold to 2.6-fold increase with H$_2$O$_2$; $P < 0.05$), PP2 (Src family tyrosine kinase inhibitor; 2.1-fold; $P < 0.05$), and the PLC-γ inhibitor U73122 (2.6-fold; $P < 0.05$). In contrast, the JNK inhibitor SP 600125, MEK/ERK inhibitor U0126, and PI3K inhibitor LY 294002 all significantly augmented the proinflammatory effect of H$_2$O$_2$.

One signaling pathway implicated in NF-κB-mediated, H$_2$O$_2$-induced signaling in other cell systems involves protein kinase D1 (PKD1). We previously reported that PKD1 is involved in TLR5 signaling in Caco-2 cells, and others have reported a broader involvement in MyD88-dependent TLR signaling (9, 30). Oxidative stress is a known trigger of PKD1 activation. Therefore, we tested Gö6976, which inhibits typical PKC toxicity in a dose-dependent fashion, although at doses as high as 3 mM H$_2$O$_2$, only 20% of the cytotoxicity observed after 0.1% Triton X-100 treatment was observed (Fig. 2C).

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isoforms along with PKD1, and Gö6983, which only inhibits PKC. However, neither compound significantly affected the ability of H2O2 to enhance flagellin-induced IL-8 production (Fig. 3A). To confirm this result, we reduced PKD1 expression using a short hairpin RNA (shRNA) construct in HeLa cells. As predicted, PKD1 knockdown on its own reduced flagellin-induced IL-8 production, but it did not affect the ability of H2O2 to enhance this response. Together, these results strongly suggest that the proinflammatory effect of H2O2 is independent of PKD1.

The pronounced effect of Bay11 led us to examine the involvement of the NF-κB pathway in the H2O2 response. First we measured I-κB phosphorylation and degradation by flagellin in the presence or absence of 1 mM H2O2. Flagellin alone caused transient I-κB degradation that peaked at 30–60 min, with resynthesis by 60–90 min. Although H2O2 alone did not induce I-κB degradation, it caused a significant and sustained degradation when added prior to flagellin (Fig. 4A and B). The pattern of I-κB phosphorylation was similar; H2O2 alone did not cause phosphorylation but enhanced and prolonged the flagellin-induced phosphorylation (Fig. 4C). We next measured NF-κB activation by EMSA. Consistent with the I-κB Western blots, H2O2 alone did not significantly activate NF-κB, but it caused a significant and sustained increase in flagellin-induced activation (Fig. 4D). Finally, to confirm specificity of the NF-κB activation, we performed immunofluorescent microscopy for NF-κB p65. As shown in Supplemental Fig. S1, H2O2 at 0.5 mM and 1 mM did not significantly increase nuclear migration of p65 at 30 min but prolonged and enhanced its activation at later time points. Together, these results strongly suggest that H2O2 can potentiate and prolong NF-κB activation following TLR5 stimulation.

Because the p38 inhibitor SB203580 reduced the effect of H2O2 on flagellin-induced IL-8 production, we measured activation of this kinase. As previously reported, cell stimulation with flagellin resulted in activation of p38, consistent with previous reports, and a similar (albeit faster) effect was observed with H2O2 (Fig. 5). Interestingly, the combination of the two stimuli, although additive at early time points, demonstrated synergistic sustained activation of p38 at later time.
points (Fig. 5, A and C). H₂O₂ also strongly activated ERK, although the addition of flagellin to H₂O₂ did not produce significantly higher phosphorylation than H₂O₂ alone (small additive effect). These results support the observation that pharmacological inhibition of p38 but not ERK reduced the effect of H₂O₂ on flagellin-induced IL-8 release, suggesting that synergistic activation of p38 contributes to the inflammatory effects of H₂O₂.

The results presented above would suggest that H₂O₂ induces a broadly active proinflammatory boosting of TLR responses in IECs. To test this, we measured the effect of H₂O₂ on CCL20 release from Caco-2 cells following flagellin stimulation. Surprisingly, CCL20 production was decreased by H₂O₂ in a dose-dependent fashion, with reduction to basal amounts at 2 mM (Fig. 6A). In the absence of flagellin, H₂O₂ alone did not reduce basal CCL20 production. To confirm that this effect was due to the oxidative stress itself, we repeated these experiments in the presence of N-AC. As shown in Fig. 6B, although N-AC alone inhibited both basal and flagellin-stimulated CCL20 production, addition of 1 mM H₂O₂ did not further reduce chemokine expression as it did in the absence of N-AC.

To examine the role of calcium-dependent pathways in this effect, we repeated these experiments in the presence of BAPTA-AM. If the inhibitory effect of H₂O₂ on CCL20 production were purely calcium dependent, we would expect to see a rise in CCL20 production with flagellin plus H₂O₂ in the presence of low concentrations of BAPTA-AM, but instead we saw a further decrease (Fig. 6C), suggesting that the inhibition of flagellin-induced CCL20 expression by H₂O₂ is a calcium-independent process. We were unable to test the effects of pharmacological inhibitors on CCL20 because those that were active reduced CCL20 production to basal levels, so that a further decrease with H₂O₂ was no longer observable. Nevertheless, these results clearly indicate divergent effects of oxidative stress on flagellin-induced IL-8 and CCL20 expression.

The effects of H₂O₂ on flagellin-induced MAPK and NF-κB suggest that the effects on chemokine production are likely to be at the transcriptional level. To test this, we measured IL-8 and CCL20 mRNA by quantitative real-time RT-PCR in Caco-2 cells stimulated with flagellin, H₂O₂, or both. As shown in Fig. 7, H₂O₂ alone had no effect on either transcript. Interestingly, H₂O₂ caused a delay in the rise in flagellin-induced IL-8 mRNA but greatly prolonged its increase, similar to what was observed with NF-κB activation. In contrast, H₂O₂ decreased CCL20 mRNA at all time points tested. To determine whether the effects of H₂O₂ were due to stabilization of IL-8 mRNA, we repeated these experiments but added actino-

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**Fig. 4.** H₂O₂ alters flagellin responses in IECs. A: Caco-2 cells were pretreated with media or H₂O₂ 1 mM for 20 min, followed by flagellin 100 ng/ml. At the indicated times, cells were lysed and protein extracts were analyzed for I-κBα by Western blot. B: densitometry from 4 experiments described in A. Band density of I-κBα was divided by p38 for each condition and normalized to the unstimulated control within each blot. Shown are means and SE from 3 experiments; *P < 0.05, FlIc/H₂O₂ vs. FlIc alone, t-test. C: Caco-2 cells treated with flagellin 100 ng/ml (F), H₂O₂ 1 mM (H), or both were lysed at the indicated time points and phospho-I-κB measured by Western blot. Blots were stripped and reprobed with GAPDH as a loading control. Results representative of 2 independent experiments. D: EMSA to detect NF-κB activation. Caco-2 cells were treated as above and nuclear extracts obtained at the time points shown. The arrow indicates the specific NF-κB-shifted band. Gel is representative of 5 independent experiments. E: densitometry of NF-κB activation by EMSA in these experiments is shown. Band density was normalized to the maximum value within each gel. Means and SE are shown for N = 3–5 for each time point. *P < 0.05, flagellin vs. flagellin + H₂O₂, t-test.
mycin D after 1 h, to inhibit further transcription. Interestingly, H2O2 partially negated the effect of actinomycin D, allowing IL-8 mRNA to continue to rise for at least 3 h, suggesting that H2O2 does significantly prolong IL-8 mRNA in addition to increasing its synthesis. The actinomycin was still active, since it significantly attenuated the rise in IL-8 mRNA (2,498 ± 508-fold increase over unstimulated cells, vs. 171.5 ± 22.6-fold, P < 0.005).

DISCUSSION

Oxidative stress is a common condition in both homeostatic and disease states. Cells are equipped with a number of ways to detect and regulate the concentrations of potentially damaging reactive oxygen species (ROS) such as superoxide anion and H2O2. In the gut, these ROS can be generated by the oxidative burst from infiltrating neutrophils or macrophages.

Fig. 5. Effects of H2O2 on flagellin-stimulated MAP kinase activation. A: Caco-2 cells treated as shown with H2O2 1 mM, flagellin 100 ng/ml, or both were lysed and proteins separated by SDS-PAGE. Phospho-p38 and phospho-ERK were measured by Western blot, and blots were stripped and reprobed with antibodies to the corresponding total protein. Results representative of at least 3 experiments. C, control (unstimulated cells); Ani, anisomycin 10 μM (positive control). B: densitometry of Western blots pooled from experiments shown in A. The band density of phospho-p38 was divided by density of total p38 and normalized as a ratio to the highest density value within each individual blot. Shown are mean values ± SE. *P < 0.05 **P < 0.001 vs. FliC alone, t-test. C: pooled densitometry as above, for phospho-ERK. *P < 0.05, both H2O2 groups vs. flagellin alone.

Fig. 6. H2O2 inhibits flagellin-induced CCL20 expression in Caco-2 cells. A: Caco-2 cells were pretreated with H2O2 at the doses shown for 20 min followed by flagellin 100 ng/ml for an additional 3 h. CCL20 concentrations were measured by ELISA. *P < 0.05 **P < 0.01, FliC/H2O2 vs. FliC alone, t-test. B: Caco-2 cells were treated as above in the presence or absence of N-AC. *P < 0.05 **P < 0.001, t-test. C: calcium independence of the effect of H2O2 on flagellin-induced CCL20 expression. Removal of intracellular calcium with BAPTA-AM prior to addition of H2O2 augments rather than inhibits the reduction of CCL20 secretion. Shown are means and SD.
or from commensal bacteria, particularly those that produce butyrate as a fermentation product (17, 18). A role for uncontrolled oxidative injury in IBD is suggested by several studies that have demonstrated increased expression of ROS-generating enzymes and/or decreased expression of oxidant scavengers in epithelial biopsies from patients with IBD (1, 15, 16, 43). Antioxidant activity is hypothesized to be one of the mechanisms of action of 5-ASA, one of the most commonly used IBD treatments (12).

There are several proposed mechanisms by which oxidative stressors like H2O2 can affect the intestinal epithelium. H2O2 has been shown to impair chloride transport in Caco-2 and other human IEC lines through a mechanism involving Fyn kinase activation, leading to PLC-γ-dependent release of intracellular calcium and activation of PKC-α (34). H2O2 can also disrupt IEC cell-cell barrier function, through activation of Src and EGFR tyrosine kinases (2, 32). H2O2 also increases expression of the atypical MHC molecules MICA and MICB on Caco-2 cells (47). Nemeth et al. (26) reported IL-8 release induced by H2O2 in Caco-2 cells, although this was only detected after 24 h of stimulation and was associated with cell necrosis. Similarly, Yamamoto et al. (48) found that H2O2 increased IL-8 production after IL-1 stimulation of Caco-2 cells. However, there are no previous reports of an effect of H2O2 on TLR5 signaling in any model system.

We found that H2O2 by itself activated both p38 and ERK MAP kinases but did not cause any measurable release of IL-8 from Caco-2 cells. However, the addition of H2O2 to flagellin substantially potentiated IL-8 release. This was associated with a synergistic activation of p38 and pronounced and sustained I-κB degradation, NF-κB activation, and IL-8 mRNA production. A similar proinflammatory activity of H2O2 has been reported in other cell systems. In airway epithelial cells, for example, H2O2 itself causes minimal IL-8 release, but augments IL-8 production in response to TNF-α, IL-1β, or the TLR3 agonist poly(I:C) (5, 24, 50). Enesa et al. (5) demonstrated that 100 μM H2O2 caused prolonged I-κB degradation and NF-κB activation in A549 cells and HeLa cells through the inhibition of a negative regulator, Cezanne, leading to polyubiquitination and degradation of newly synthesized I-κB molecules. This finding has not yet been reproduced in other studies or other cell lines. Other proposed mechanisms by which H2O2 potentiates inflammatory responses include tyrosine phosphorylation of I-κBα (42) and serine phosphorylation of I-κB kinases (13). However, in neutrophils, H2O2 can have the opposite effect, leading to decreased I-κB degradation and reduced inflammatory responses to the TLR4 agonist LPS (52). In contrast, H2O2 potentiates neutrophil responses to the TLR8 agonist resiquimod (50).

Several signaling pathways leading from H2O2 exposure to cellular phenotypes other than inflammation have been explored. Zhou et al. (51) showed that H2O2 activates p38, ERK, JNK, and PI3K in rat IECs, and that p38-mediated activation of PKCs is required for H2O2-induced apoptosis. Song et al. (37)
showed that PKD1 protects rat IECs from H2O2-mediated apoptosis through activation of NF-κB and downregulation of p38. This finding is consistent with previous reports that PKD is an important sensor of oxidative stress and may help provide survival signals through NF-κB activation (38–40).

On the basis of these results, we hypothesized that PKD1 might also be involved in potentiating IL-8 responses to flagellin, but we found no effect of either PKD1 shRNA or G66976. These findings suggest that the effect of H2O2 on TLR5 signaling is independent of PKD1. Our studies using other pharmacological inhibitors ruled out several additional candidate kinases that have been implicated in ROS signaling in other systems. For example, we found that blocking PI3K, MEKI/ERK, or JNK actually increased the potentiating effect of H2O2. In addition to p38 and NF-κB, PLC-γ was identified as a potential candidate for involvement in this pathway based on the inhibitory effect of U73122. Our data suggest that the effect of PLC-γ in this case is likely not due to activation of PKCs by diacylglycerol, since G66076 and G66983 failed to inhibit the H2O2 effect on IL-8. Instead, it may be due to IP3-induced release of calcium from intracellular stores.

Perhaps the most intriguing finding reported here is that under the same conditions in which it potentiates IL-8 release, H2O2 inhibits flagellin-induced CCL20 production. Like IL-8, CCL20 also possesses an NF-κB response element in its promoter (19). In the absence of H2O2, inhibition of NF-κB with Bay11 completely blocks flagellin-induced CCL20 expression by flagellin (data not shown). On the basis of those facts and on the potentiation of NF-κB signaling we report here, it is surprising that CCL20 expression was inhibited by H2O2. Of note, the CCL20 promoter contains response elements not found in the IL-8 promoter, including an ESE-1 site, which exerts important control over its expression (19).

We propose the following model for how H2O2 affects flagellin-induced chemokine production. The initial stages of the H2O2 response likely involve tyrosine kinase-mediated activation of PLC-γ. This in turn leads to activation of p38 and an as-yet-unknown inhibitor of IκB resynthesis. It is intriguing to speculate that Cezanne or the related zinc finger deubiquitinating enzyme A20 may be involved in this response, particularly in light of the report that A20 is upregulated in response to flagellin both in vitro and in the inflamed mouse gut in vivo (29). The end result of these effects would be increased and prolonged production (via NF-κB) and stabilization (via p38) of IL-8 mRNA, leading to increased protein expression, which is consistent with our findings. At the same time, there is an anti-inflammatory program in parallel that targets CCL20 expression. It is possible that proapoptotic or cytotoxic effects of H2O2 could preferentially target CCL20, as expression of CCL20 mRNA following flagellin stimulation is significantly slower than IL-8 expression (8).

In summary, we have shown for the first time that oxidative stress can modulate inflammatory responses to the TLR5 agonist flagellin. In the presence of H2O2, IECs upregulate IL-8 and downregulate CCL20. Interestingly, the same response pattern was observed when flagellin-stimulated Caco-2 cells were treated with the alternative stress signal extracellular ATP (9a), suggesting that this reaction may part of a global IEC response to stress. This chemokine profile would be predicted to lead to increased influx of neutrophils and decreased attraction of dendritic cells and lymphocytes expressing the CCL20 receptor, CCR6 (36, 45, 49). The result of this would be increased acute inflammation, with decreased antigen presentation and chronic inflammatory responses as a result. It is intriguing to speculate that IECs read the danger signal H2O2 as a sign of acute infection and recruit more neutrophils in response to flagellin detection to kill invading pathogens, while at the same time dampening the recruitment of CCR6+ cells to prevent unwanted and inappropriate T cell responses to flagellin and other components of the commensal flora. Perturbation of this regulatory pathway could contribute to the etiology of diseases involving inappropriate responses to commensal bacteria, such as IBD.

GRANTS

This work was supported by Canadian Institutes for Health Research (CIHR) operating grant III 160675 to T. S. Steiner and by the CHHR Team Grant in Immunoregulation and IBD (INN 84037 to T. S. Steiner). T. S. Steiner also held a New Investigator Award from CIHR. M. E. Himmel and M. L. Mayer hold CIHR Doctoral Research Awards. C. Wang was funded by the CHHR training award “Translational Research in Infectious Diseases.”

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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