Cyclooxygenase-2 Generates the Endogenous Mutagen \( \text{trans}-4\)-Hydroxy-2-nonenal in \textit{Enterococcus faecalis}–Infected Macrophages

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Abstract

Infection of macrophages by the human intestinal commensal \textit{Enterococcus faecalis} generates DNA damage and chromosomal instability in mammalian cells through bystander effects. These effects are characterized by clastogenesis and damage to mitotic spindles in target cells and are mediated, in part, by \textit{trans}-4-hydroxy-2-nonenal (4-HNE). In this study, we investigated the role of COX and lipoxygenase (LOX) in producing this reactive aldehyde using \textit{E. faecalis}–infected macrophages and interleukin (IL)-10–infected knockout mice colonized with this commensal. 4-HNE production by \textit{E. faecalis}–infected macrophages was significantly reduced by COX and LOX inhibitors. The infection of macrophages led to decreased Cox1 and Alox5 expression whereas COX-2 and 4-HNE increased. Silencing Alox5 and Cox1 with gene-specific siRNAs had no effect on 4-HNE production. In contrast, silencing Cox2 significantly decreased 4-HNE production by \textit{E. faecalis}–infected macrophages. Depleting intracellular glutathione increased 4-HNE production by these cells. Next, to confirm COX-2 as a source for 4-HNE, we assayed the products generated by recombinant human COX-2 and found 4-HNE in a concentration-dependent manner using arachidonic acid as a substrate. Finally, tissue macrophages in colon biopsies from IL-10–knockout mice colonized with \textit{E. faecalis} were positive for COX-2 by immunohistochemical staining. This was associated with increased staining for 4-HNE protein adducts in surrounding stroma. These data show that \textit{E. faecalis}, a human intestinal commensal, can trigger macrophages to produce 4-HNE through COX-2. Importantly, it reinforces the concept of COX-2 as a procarcinogenic enzyme capable of damaging DNA in target cells through bystander effects that contribute to colorectal carcinogenesis. Cancer Prev Res; 1–11. ©2013 AACR.

Introduction

COX and lipoxygenases (LOX) produce a variety of biologically important prostanoids, leukotrienes, hydroxyeicosatetraenoic acids, and lipoxins through the oxidation of polyunsaturated fatty acids such as arachidonic acid (1). These important signaling molecules help regulate numerous physiologic responses including inflammation, cellular proliferation, vascular and bronchial airway tone, and tissue hemostasis. COX occurs as a constitutive form (COX-1) in most tissues and in an inducible form (COX-2) that is typically expressed in response to environmental triggers such as infection, physical irritants, growth factors, and cytokines. There are several LOX isoforms with arachidonate 5-lipoxygenase (ALOX5 or 5-lipoxygenase) expressed primarily in inflammatory cells and dependent upon the arachidonate 5-lipoxygenase activating protein. COX and LOX pathways have been implicated in carcinogenesis. For example, prolonged exposure to aspirin, an irreversible COX inhibitor, significantly reduces the overall risk for colorectal cancer (CRC) and mortality from CRC in human trials (2). Observational and randomized clinical trials of nonsteroidal anti-inflammatory drugs and COX-2–specific inhibitors show reductions in colorectal adenomas (3). Similar decreases in adenoma formation are noted in \( \text{Apc}^\text{Min/+} \) and \( \text{Apc}^\text{Min/+} \) mice when Cox is inactivated (4, 5). Finally, ALOX5 is overexpressed in colon polyps and CRC (6), much like COX-2 (7), with inhibition impairing tumor growth and attenuating polyps in \( \text{Apc}^\text{Min/+} \) mice (8). The mechanisms by which COX-2 or ALOX5 initiate mutations to drive the adenoma-to-carcinoma sequence, however, remain unclear.

PGE\(_2\) and PGD\(_2\) are major COX-2–derived products that promote cancer cell growth by modulating signaling cascades for cellular proliferation, apoptosis, angiogenesis, and immune surveillance (9). ALOX5 produces 5(S)-hydroperoxyeicosatetraenoic acid, a precursor for leukotrienes that are signaling molecules in asthmatic, allergic, and inflammatory reactions (1). Although these lipid mediators...
promote diverse biologic functions, they are not mutagens and seem unlikely to initiate tumors. Each enzyme, however, produces products that can be reduced to hydroxyeicosatetraenoic or hydroxyoctadecadienoic acids (1). Arachidonic acid, the primary ω-6 polyunsaturated fatty acid substrate for COX-2, can also be oxidized by this enzyme to reactive carbonyl compounds such as 4-oxo-2-nonenal, 4-hydroperoxy-2-nonenal, 4-hydroxy-2E,6Z-dodecaadienal, and trans-4-hydroxy-2-nonenal (4-HNE; ref. 10). These bifunctional electrophiles can diffuse across cellular membranes to form adducts with proteins, phospholipids, and DNA. Among these, 4-HNE has been most intensively studied. This α,β-unsaturated aldehyde is a signaling molecule for cellular stress (11), induces COX-2 (12), inhibits DNA repair (13), reacts with DNA (1), damages microtubules and mitotic spindles (14, 15), and potentially contributes to chromosomal instability (CIN; refs. 15, 16).

The carcinogenic effects of non-prostanoid byproducts derived from COX-2 were investigated using rat intestinal epithelial cells overexpressing this enzyme (17). A 3-fold increase in heptanone-etheno-DNA adducts was observed in the presence of ascorbate that served to promote the decomposition of bifunctional electrophiles that were generated (17). Stereoisomeric analysis identified these adducts as lipid peroxidation byproducts of arachidonic acid that were generated by COX-2. Others investigators, however, using colon cancer cell lines failed to correlate DNA adduct levels with COX-2 activity (18). Finally, as mentioned above, ALOX5 also generates products that can be converted to lipid hydroperoxides and potentially decompose into DNA-damaging electrophiles (1).

COX-2 and ALOX5 are not ordinarily expressed in healthy colons. In colonic adenomas, however, COX-2 expression is found in mucosal macrophages but not in epithelial cells where cellular transformation leads to cancer (7). In contrast, ALOX5 is abundantly expressed in epithelial cells in adenomas (6). These temporospatial relationships, when considered in the context of clinical and animal data (2–6, 8), implicate these enzymes in colorectal carcinogenesis.

Using interleukin (IL)-10–knockout mice, we have recently linked the common intestinal commensal Enterococcus faecalis to COX-2 induction in macrophages and to transforming events in epithelial cells (19, 20). E. faecalis is a minority constituent of the mammalian intestinal microbiota that generates extracellular superoxide and oxidative stress under conditions of heme deprivation (21). The unusual redox physiology activates bystander effects (BSE) in macrophages and causes CRC in IL-10–knockout mice (15, 19). BSE is recognized by genomic damage in target cells that are exposed to diffusible clastogens (or chromosome-breaking factors) from activated myeloid and/or fibroblast cells (22, 23). Historically, these effects occur following irradiation. However, we discovered that macrophages infected by E. faecalis can also produce BSE that include double-strand DNA breaks, aneuploidy, tetraploidy, and CIN (15, 19, 20). This genomic damage, whether due to BSE from irradiated or infected cells, depends, in part, on COX-2 (19, 24), although the diffusible mediators remain ill-defined. We and others have suggested 4-HNE as one such mediator because it can cause DNA damage and act as a spindle poison to produce tetraploidy in target cells (15, 16). As such, this reactive aldehyde represents a potential link between COX-2 catalysis and CIN.

In this study we show that E. faecalis–infected macrophages produce increased amounts of 4-HNE in a COX-2–dependent, but not ALOX5–dependent, fashion. In addition, using IL-10–knockout mice colonized by E. faecalis, we show increased COX-2 expression in colonic macrophages in association with 4-HNE–protein adducts. These data suggest that colonic macrophages, when triggered by an intestinal commensal such as E. faecalis, can induce COX-2 and thereby increase the production of 4-HNE, a diffusible mutagen. These findings reinforce the role of COX-2 as a procarcinogenic enzyme that is able to endogenously initiate transforming events leading to CRC.

Materials and Methods

Cells, bacteria, and chemicals

Murine macrophages (RAW264.7 cells; American Type Culture Collection) were maintained in high-glucose Dulbecco’s Modified Eagle Medium (Life Technologies) supplemented with 10% FBS, penicillin G, and streptomycin at 37°C in 5% CO2. E. faecalis strain OG1RF, a human oral isolate, and the spectinomycin- and streptomycin-derivative strain OG1RFSS were grown in brain heart infusion (BD Diagnostics) and used for macrophage infection and to colonize mice, respectively, as previously described (15). In brief, RAW264.7 cells were treated with OG1RF at a multiplicity of infection of 1,000 in antibiotic- and serum-free Dulbecco’s for 2 hours at 37°C. After washing with PBS, cells were incubated in complete medium supplemented with 100 μg/mL gentamicin for 24 hours at 37°C. Supernatants were collected for 4-HNE analysis and cell pellets lysed for protein and RNA analyses. The COX-2–specific inhibitor celecoxib was kindly provided by C.V. Rao. The ALOX5–specific inhibitor AA861 [2-(12-hydroxy-dodecan-5,10-diylnyl)-3,5,6-trimethyl-p-benzoquinone] and COX and LOX inhibitor ETYA (eicosatetraynoic acid) were purchased from Enzo Life Sciences (25). Buthionine sulfoximine (BSO) was obtained from Sigma.

4-HNE and PGD2 analysis

Lipids were extracted from supernatants by the Folch procedure and reconstituted in 100% ethanol as previously described (15). Extracts were analyzed by high-performance liquid chromatography using reversed-phase chromatography coupled to a 4-cell electrochemical detector as previously described (15).

COX-2 catalysis of arachidonic acid

Reactions were conducted in a thermostatic cuvette (Gillon Medical Electronics) with 1 mL of 100 mmol/L KH2PO4 (pH 7.4) containing 100 μmol/L arachidonic acid (Cayman), 1 μmol/L hematin (Sigma), and 2 units of human recombinant COX-2 (Cayman) at 37°C. Reactions were
terminated at 5 minutes by adding butylated hydroxytoluene and depletion of oxygen. Mixtures were extracted and separated by reversed-phase high-performance liquid chromatography with 4-HNE and arachidonate metabolites determined by electrochemical detection as previously described (15). Enzyme activity was measured by O_2 uptake using an Oxygen Probe Amplifier equipped with a Clark-type electrode (Harvard Apparatus).

**siRNA and reverse transcriptase-(RT) PCR**

Cox1, Cox2, and Alox5 were silenced by RNA interference using siGENOME SMARTpool siRNAs or with siCONTROL Non-Targeting siRNA Pool (Dharmacon). Transient transfections were conducted using DharmaFECT 4 Transfection Reagent according to the manufacturer’s protocol and gene silencing confirmed by RT-PCR and Western blotting.

Total RNA was isolated from RAW264.7 cells using the NucleoSpin RNA II Kit (BD Biosciences). cDNA was synthesized at 37°C using TaqMan Reverse Transcription Reagents according to manufacturer’s instructions (Life Technologies). Primers and cycling parameters are shown in the Supplementary Table.

**Western blots**

Protein extraction, SDS-PAGE, and Western blotting were conducted as previously described (20). Goat polyclonal antibody for COX-2 (Santa Cruz Biotechnology) and anti-ALOX5 rabbit monoclonal antibody (Thermo Scientific) were used as primary antibodies. Rabbit anti-goat IgG horseradish peroxidase (HRP) conjugate (Millipore) and goat anti-rabbit IgG HRP conjugate (Cell Signaling Technology) were used for secondary antibodies. Signals were generated by Amersham ECL System (Bio-Rad).

**Colonization of Il10⁻/⁻ mice**

Conventionally housed Il10⁻/⁻ mice (C57Bl/6, Jackson Laboratory) were orogastrically inoculated with 1 x 10^9 colony-forming units of *E. faecalis* OG1RFSS or PBS as sham as previously described (26). Colonization was confirmed by plating stools on enterococcal agar (Becton Dickinson) supplemented with streptomycin and spectinomycin. After 9 months, mice were sacrificed and colons fixed for immunostaining. Animal protocols were approved by the University of Oklahoma Health Sciences Center IACUC and Oklahoma City Department of Veterans Affairs Animal Studies Committee.

**Immunohistochemical and immunofluorescent staining**

Immunohistochemical staining was conducted as previously described. For COX-2 staining, slides were blocked with 5% normal rabbit serum, incubated with goat anti-COX-2 polyclonal antibody (Santa Cruz), and stained with rabbit anti-goat IgG HRP conjugate (Millipore). Slides were developed with DAB Enhanced liquid substrate and counterstained with Mayer’s hematoxylin solution (Sigma).

Immunofluorescent staining for macrophages (F4/80) and COX-2 or 4-HNE was conducted as previously described (27). Goat anti-COX-2 antibody (Santa Cruz), rabbit anti-4-HNE protein adduct antibody (Alpha Diagnostic International), and rat anti-F4/80 antibody (ebioscience) were used as primary antibodies. Anti-goat IgG fluorescein isothiocyanate (FITC), anti-rabbit IgG FITC, and anti-rat IgG Texas Red (Santa Cruz) were used as secondary antibodies. Cells were counterstained with 4′-6-diamidino-2-phenylindole (DAPI) and images collected using a fluorescent microscope (Nikon Instruments).

**Statistical analysis**

Data are shown as means with SDs. Groups were compared using Student t test with *P* < 0.05 considered significant.

**Results**

**LOX and COX inhibitors decrease 4-HNE production by macrophages**

*E. faecalis*-infected macrophages produce increased 4-HNE, a lipid peroxidation byproduct of arachidonic acid (15). As COX and lipoxygenases catalyze reactions using this ω-6 polyunsaturated fatty acid and 4-HNE is a known breakdown product, we explored the role of these enzymes in increased 4-HNE production from infected murine macrophages. Initially, we treated uninfected macrophages with an ALOX5-specific inhibitor and a dual COX/LOX inhibitor. Both inhibitors decreased background levels of 4-HNE in supernatants from 252 ± 35 to 85 ± 18 and 87 ± 16 nmol/L for AA861 and ETYA, respectively (Fig. 1A, *P* < 0.01). These inhibitors also decreased 4-HNE production when macrophages were infected with *E. faecalis* (439 ± 104 to 105 ± 28 and 128 ± 34 nmol/L for AA861 and ETYA, respectively, *P* = 0.01). Of note, COX-2, which is not measurably expressed by these macrophages under ordinary culture conditions, is strongly induced by *E. faecalis* (19) and was less strongly induced in the presence of these inhibitors following infection (Fig. 1B). In contrast, COX-1 and ALOX5 were constitutively expressed by uninfected RAW264.7 cells and each decreased following *E. faecalis* infection (Fig. 1C–F). These changes made it difficult to determine whether the reduction in 4-HNE production caused by these inhibitors was due to enzyme inhibition versus changes in enzyme expression in infected macrophages.

**COX-1 and ALOX5 are not sources of 4-HNE for infected macrophages**

To assess potential contributions of COX-1 or ALOX5 to 4-HNE production in *E. faecalis*-infected macrophages, Cox1 and Alox5 were silenced using siRNA and 4-HNE production measured. RT-PCR showed a 38% decrease in *Cox1* expression for cells transfected with *Cox1*-specific siRNA compared with cells transfected with nontargeting siRNA (Fig. 2A and B). Similarly, a 60% reduction was noted in *Alox5* expression using *Alox5*-specific siRNA compared with control (Fig. 2C and D). Although the expression of
Cox1 and Alox5 decreased slightly in E. faecalis–infected macrophages transfected with gene-specific siRNAs compared with nontargeting siRNA, these effects were largely overwhelmed by the strong inhibition of gene expression for these enzymes in infected macrophages (Fig. 2A–D). No decrease was observed in 4-HNE production for Cox1–silenced macrophages compared with controls ($P = 0.24$ and 0.22, for either uninfected or E. faecalis–infected macrophages, respectively). Similarly, no change was noted in 4-HNE production for Alox5–silenced macrophages following E. faecalis infection compared with control. Of note, 4-HNE production modestly increased in Alox5–silenced uninfected macrophages (open bar; NS, not significant; *, $P < 0.05$; **, $P < 0.01$ compared with untreated control at zero time point for D and F). Data represent mean ± SD for 3 independent experiments.

Figure 1. Inhibitors for ALOX5 and COX decrease 4-HNE production from macrophages. A, AA861 (ALOX5 inhibitor) and ETYA (inhibitor for ALOX5 and COX) significantly decrease 4-HNE production in supernatants from uninfected (open bar) and E. faecalis–infected macrophages (solid bar; **, $P < 0.01$; #, $P = 0.01$ compared with control). B, Western blotting shows decreased COX-2 in macrophages by AA861 and ETYA following treatment with E. faecalis. C, RT-PCR shows decreased Cox1 expression in E. faecalis–infected macrophages (bottom) compared to uninfected macrophages (top). D, normalized Cox1 expression increases in uninfected macrophages (open bar) while decreases following E. faecalis infection (solid bar). E, Western blotting for ALOX5 in uninfected macrophages (top) and E. faecalis–infected macrophages (bottom). F, normalized ALOX5 production decreases at 24 to 72 hours following E. faecalis infection (solid bar) compared with uninfected macrophages (open bar; NS, not significant; *, $P < 0.05$; **, $P < 0.01$ compared with untreated control at zero time point for D and F). Data represent mean ± SD for 3 independent experiments.

Cox1 and Alox5 decreased slightly in E. faecalis–infected macrophages transfected with gene-specific siRNAs compared with nontargeting siRNA, these effects were largely overwhelmed by the strong inhibition of gene expression for these enzymes in infected macrophages (Fig. 2A–D). No decrease was observed in 4-HNE production for Cox1-silenced macrophages compared with controls ($P = 0.24$ and 0.22, for either uninfected or E. faecalis–infected macrophages, respectively). Similarly, no change was noted in 4-HNE production for Alox5-silenced macrophages following E. faecalis infection compared with control. Of note, 4-HNE production modestly increased in Alox5-silenced uninfected macrophages compared with nontransfected controls ($P = 0.04$, Fig. 2E). Although ALOX5 expression is typically associated with increased lipid peroxidation (28), this observation may represent a perturbation and increase in nonenzymatic lipid peroxidation from the loss of ALOX5 end products.

We tested the hypothesis that 4-HNE produced by macrophages derives, in part, from nonenzymatic lipid peroxidation using α-tocopherol, a potent terminator of lipid peroxidation chain reactions with no effect on COX-2 expression (19). We found that α-tocopherol significantly decreased 4-HNE production from both uninfected and E. faecalis–infected macrophages, suggesting that nonenzymatic lipid peroxidation was, in part, a source for the basal production of 4-HNE by these cells (Fig. 2F). In summary, the expression of Cox1 and Alox5 decreased in E. faecalis–infected macrophages as these cells otherwise were producing increased amounts of 4-HNE. Partial silencing of these genes resulted in either no change or a trend toward increased 4-HNE production. These observations indicate that Cox1 and Alox5 were not significant sources for 4-HNE in E. faecalis–infected macrophages.

**COX-2 produces 4-HNE**

The lack of association of Cox1 or Alox5 with 4-HNE production led us to more closely investigate the role of Cox2. Initially, we pretreated macrophages with celecoxib—a COX-2–specific inhibitor—before infection with E. faecalis. RT-PCR showed a 94% decrease in Cox2 expression compared with untreated E. faecalis–infected controls ($P = 0.01$, Fig. 3A). These findings suggest that celecoxib not only
blocks the active site of COX-2 but also suppresses Cox2 transcription. Western blotting showed a 46% decrease in COX-2 expression in E. faecalis–infected macrophages that were treated with celecoxib (Fig. 3B). This observation is consistent with previous reports (29). Of note, the inhibition of COX-2 by celecoxib was associated with a 45% decrease in 4-HNE production following E. faecalis infection (P < 0.001, Fig. 3C). As celecoxib may have off-target effects, we transfected macrophages with Cox2-specific siRNA and observed a 35% decrease in Cox2 expression and 67% decrease in COX-2 protein after E. faecalis infection (P = 0.001, Fig. 3D and E). This was associated with a 68% decrease in 4-HNE production compared with infected macrophages that were transfected with nontargeting siRNA (P < 0.001, Fig. 3F). These findings confirm COX-2 as the predominant source for increased 4-HNE in E. faecalis–infected macrophages.

To directly assess the ability of COX-2 to generate 4-HNE, we tested recombinant human COX-2 in vitro using arachidonic acid as substrate. We discovered that 4-HNE was produced in a concentration-dependent manner as a significant byproduct of catalysis. When the reaction was allowed to go to completion using 50, 100, or 2000 μmol arachidionate as substrate, 18, 36, and 1,560 μmol/L 4-HNE were detected, respectively, in final reaction mixtures. Controls using buffer and substrate, but no enzyme, did not produce detectable 4-HNE. Celecoxib decreased 4-HNE production by 71% when 50 μmol arachidonic acid was used as the substrate (Fig. 3G). This was associated with a 27% decrease in PGD2 production—a decomposition product of PGH2 (Fig. 3H). These observations offer additional support for COX-2 as the primary source of 4-HNE from infected macrophages.

Glutathione depletion increases 4-HNE production by E. faecalis–infected macrophages

We previously showed that 4-HNE mediates genotoxicity through macrophage-induced BSE (15). As glutathione is required for enzymatic scavenging of 4-HNE by glutathione S-transferase, we investigated the effect of BSO, a γ-glutamylcysteine synthetase inhibitor that depletes intracellular glutathione and enhances genotoxicity (20, 30), on 4-HNE production. In comparison to E. faecalis–infected controls, a 2.5-fold increase in 4-HNE production was seen for BSO-treated macrophages that were infected with E. faecalis (P = 0.003, Fig. 4A). A 1.3-fold increase in Cox2 expression was evident in these same macrophages (P = 0.03, Fig. 4B). As 4-HNE can specifically induce COX-2, this response might represent positive feedback by this electrophile (12). Although Cox2 expression appeared slightly suppressed by BSO in uninfected macrophages (Fig. 4B), this was not confirmed at the protein level (Fig. 4C). Finally, COX-2 activity, as measured by production of PGD2, increased 5.7-fold...
following BSO treatment of *E. faecalis*–infected macrophages compared with 3.3-fold for untreated macrophages infected with *E. faecalis* (*P* = 0.03, Fig. 4D).

**Co-localization of COX-2 and 4-HNE in colonized Il10−/− mice**

II10−/− mice colonized with *E. faecalis* develop colon inflammation and CRC (31). These pathologic changes are associated with increased 4-HNE–protein adducts in colon mucosal cells and stroma (15). To further examine the mechanism for these events, we stained colon biopsies from *E. faecalis*–colonized II10−/− mice for COX-2 and 4-HNE–protein adducts. Immunohistochemistry showed intense staining for COX-2 in macrophages in areas of inflammation (Fig. 5A). In comparison, we saw little to no staining for sham-colonized mice. Immunofluorescence showed similarly increased COX-2 expression in colonic macrophages (Fig. 5B) with markedly increased 4-HNE–protein adduct staining in macrophages and, compared with shams, associated intercellular spaces (Fig. 5C).

**Discussion**

These data show COX-2 as a predominant source for 4-HNE following macrophage infection by *E. faecalis*. In addition, staining for 4-HNE–protein adducts was noted in the colonic stroma and tissue macrophages of II10−/− mice colonized by *E. faecalis*. This intestinal commensal is a trigger for colitis and CRC in this model (15, 31). By comparison, COX-1 and ALOX5 did not contribute to 4-HNE production. These findings help link COX-2 to clastogenesis caused by BSE (19, 24). In the context of associations between this enzyme and carcinogenesis (1, 3–5, 32),
COX-2 should be considered an autochthonous source for mutagenesis leading to cellular transformation. The role of COX-2 in CRC has been recognized for many years (2, 9, 33). Several lines of evidence derive from animal models where tumorigenesis is curtailed after Cox is deleted or inhibited (4, 5). Although major products downstream from COX-2—PGE\(_2\) and PGD\(_2\)—are diffusible and promote cellular growth (9), these lipid mediators are not mutagenic and seem unlikely to initiate cellular transformation. COX-2, however, can produce less well-recognized byproducts that include 4-oxo-2-nonenal, 4-hydroperoxy-2-nonenal, 4-hydroxy-2E,6Z-dodecadienal, and 4-HNE (1). As amphiphilic aldehydes, these compounds can readily form adducts with proteins, phospholipids, and DNA (1, 10, 11, 14).

4-HNE and related carbonyl compounds are most often thought of as being derived from \(\omega-6\) polyunsaturated fatty acids due to oxidative stress (10, 11). These reactive aldehydes can form in cellular membranes from fatty acids via nonenzymatic pathways and potentially represent a major source of 4-HNE in tissue culture for cells not expressing COX-2 (28). Our findings, however, describe COX-2 as another important source for 4-HNE. We propose that the induction of this enzyme in macrophages, and subsequent increase in 4-HNE production, overwhelms scavenging mechanisms for this aldehyde in epithelial cells, namely, glutathione S-transferases (15, 34), and results in ongoing genomic damage that leads to cellular transformation.

Any plausible theory for sporadic CRC should address the role of COX-2 in carcinogenesis, describe the origin of CIN, and elucidate the relationship between reactive stromal cells and colonic epithelial cells. Our proposed model for autochthonous mutagenesis mechanically links these characteristics for inflammation-associated and sporadic CRC (Fig. 6). The lack of COX-2 expression in normal colonic mucosa, with strong expression in tissue macrophages in human adenomas, is consistent with a theory for human CRC that is caused by commensal-triggered and macrophage-induced BSE (7). We propose that specific commensals such as \(E.\ faecalis\) serve as triggers for innate immune responses that drive mutagenesis through BSE.
This hypothesis joins the oxidative physiology of *E. faecalis* to the expression of COX-2 in colonic macrophages. We postulate that extracellular superoxide from *E. faecalis* generates clastogens by chronically activating macrophages through ongoing infection. *E. faecalis* must translocate the intact intestinal epithelium for this to occur. This is a trait that has been previously described for this genus (35). In addition, enterococci must persist in macrophages despite the otherwise lethal effects of phagocytosis—again, this is a recognized phenotype for *E. faecalis* (36). Macrophages respond to *E. faecalis* infection by activating NF-κB and inducing COX-2 (37). Following this sequence of signaling events, as shown here, COX-2 is induced and generates mutagenic 4-HNE (and possibly related congeners although they were not specifically investigated in this study). As an amphiphilic aldehyde, 4-HNE can readily diffuse into nearby epithelial cells to stochastically damage DNA that, over long periods, leads to CIN.

The phenomenon of BSE is readily shown using supernatants from irradiated cells to induce CIN in nonirradiated cells (22). Congenic sex-mismatch bone marrow transplant studies in mice have confirmed that BSE occurs in vivo (38). BSE is not exclusively induced by irradiation. Superoxide also triggers this phenomenon with DNA damage prevented by superoxide dismutase (19, 23). Mediators for radiation- or superoxide-induced clastogenesis, however, have remained elusive. Potential candidates include long-lived lipid radicals, byproducts of lipid peroxidation, inositol, and TNF-α (16, 23, 39). A clue into these mediators was reported by Zhou and colleagues who described their dependence on COX-2 (24). Our laboratory similarly implicated this enzyme using infected macrophages (19, 20). In this article, we expand on these earlier observations by showing that COX-2 is a source for 4-HNE. Further evaluation of this using COX-2 inhibitors in IL-10–knockout mice would be difficult, as these drugs paradoxically cause severe colitis (40).
Genetically inactivating COX-2 in tissue macrophages using Cre mice is a potential alternative approach. In sum, these results link an intestinal commensal to BSE and provide additional evidence for 4-HNE as a bona fide mediator of clastogenesis (15, 16).

A focus on *E. faecalis* in the *Il10*−/− model should not be construed to mean that other intestinal commensals do not have the potential to serve as triggers for BSE and clastogenesis. Conversely, it is unlikely that any member of the colonic microbiota can cause CRC. Indeed, the vast majority of commensals co-exist as symbiotes and promote health while excluding potentially pathogenic exogenous bacteria (41). Commensals may even lower the risk for CRC. Strains of *Escherichia coli* express enterotoxins with antiproliferative properties (42). Despite these caveats, accumulating evidence provides a strong rationale for considering colonic commensals as sources for endogenous DNA damage in colorectal carcinogenesis. Similar to *E. faecalis*, *E. coli* can generate double-strand DNA breaks in mammalian cells and CRC in mono-associated *Il10*−/− mice when expressing an unusual hybrid peptide–polyketide toxin (43). Strains of *Bacteroides fragilis* can produce an enterotoxin that induces colonic tumors in *Apc*Min/+ mice (44). Finally, a role of commensals in colorectal carcinogenesis is found in diverse murine models for CRC where, in nearly every instance, including the IL-10−/− knockout model, germ-free or pathogen-free derivatives have a reduced tumor burden or fail to develop CRC (31, 45–49).

Collectively, these studies suggest that certain commensals or, more accurately, pathobioants can damage epithelial cell DNA and help drive colorectal carcinogenesis. Notwithstanding, these models have weaknesses. Commensals do not cause inflammation or CRC in healthy wild-type mice. Deficiencies in host immunity (e.g., *Il10*−/−) are typically necessary for cellular transformation by *E. faecalis*.

Despite this caveat, the *Il10*−/− model is useful for studying mechanisms that may contribute to human carcinogenesis.

In sum, our findings support a novel mechanism of inflammation-associated and sporadic CRC. This theory involves specific pathobioants (e.g., *E. faecalis*) that trigger abnormal innate immune responses that induce BSE, cause DNA damage in epithelial cells, and initiate CIN. The key effector in this scheme is COX-2 with its ability to generate 4-HNE. This theory, if confirmed in human tissue, will open new avenues for preventing these cancers.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: X. Wang, T.D. Allen, M.M. Huycke
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Figure 6. *E. faecalis*−infected macrophages. Translocating *E. faecalis* are phagocyted by tissue macrophages with superoxide contributing to peroxidation, oxidative stress, NF-κB signaling, and COX-2 expression that generates clastogenic lipid peroxidation byproducts [e.g., trans-4-hydroxy-2-nonenal (4-HNE)]. 4-HNE diffuses to nearby colonic epithelial cells to cause BSE. 4-HNE reacts with DNA to form mutagenic adducts and to cause CIN (1, 11, 15); prostanoids are derived from COX-2 and limit apoptosis, enhance angiogenesis, and promote proliferation in epithelial cells but are not mediators of BSE.
References


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