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Lipoxin A_4 and Aspirin-Triggered 15-Epi-Lipoxin A_4 Antagonize TNF-α-Stimulated Neutrophil-Enterocyte Interactions In Vitro and Attenuate TNF-α-Induced Chemokine Release and Colonocyte Apoptosis in Human Intestinal Mucosa Ex Vivo[1]

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Lipoxins (LXs) are lipoxygenase-derived eicosanoids and putative endogenous braking signals for inflammation in the gastrointestinal tract and other organs. Aspirin triggers the production of 15-epimers during cell-cell interaction in a cytokine-primed milieu, and aspirin-triggered 15-epi-5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-trans-11-*cis*-eicosatetraenoic acid (15-epi-LXA_4) may contribute to the bioactivity profile of this prototype nonsteroidal anti-inflammatory drug in vivo. We determined the effect of LXA_4, 15-(*R/S*)-methyl-11,12-dehydro-LXA_4 methyl ester (15-(*R/S*)-methyl-LXA_4), and stable analogs of LXA_4 on TNF-α-stimulated neutrophil-enterocyte interaction in vitro and TNF-α-stimulated chemokine release, changes in mucosal architecture, and enterocyte apoptosis in cytokine-activated intact human colonic mucosa ex vivo. LXA_4, 15-(*R/S*)-epi-LXA_4, and 16-phenoxy-11,12-dehydro-17,18,19,20-tetranor-LXA_4 methyl ester (16-phenoxy-LXA_4) inhibited TNF-α-stimulated neutrophil adherence to epithelial monolayers at nanomolar concentrations. In parallel experiments involving human colonic mucosa ex vivo, LXA_4potently attenuated TNF-α-stimulated release of the C-X-C chemokine IL-8, and the C-C chemokines monocyte-chemoattractant protein-1 (MCP-1) and RANTES. Exposure of strips of normal human colonic mucosa to TNF-α induced disruption of mucosa architecture and enhanced colonocyte apoptosis via a caspase-3-independent mechanism. Prior exposure of the mucosa strips to 15-(*R/S*)-methyl-LXA_4 attenuated TNF-α-stimulated colonocyte apoptosis and protected the mucosa against TNF-α-induced mucosal damage. In aggregate, our data demonstrate that lipoxins and aspirin-triggered 15-epi-LXA_4 are potent antagonists of TNF-α-mediated neutrophil-enterocyte interactions in vitro, attenuate TNF-α-triggered chemokine release and colonocyte apoptosis, and are protective against TNF-α-induced morphological disruption in human colonic strips ex vivo. Our observations further expand the anti-inflammatory profile of these lipoxygenase-derived eicosanoids and suggest new therapeutic approaches for the treatment of inflammatory bowel disease. ***The Journal of Immunology,*** **2001, 167: 2772–2780.**

Human intestinal epithelial cells are increasingly recognized as key components of the unique intestinal immune microenvironment. The multifaceted interaction between luminal Ags, intestinal epithelial cells, and the resident intestinal lymphocytes modulate the intensity of the state of controlled inflammation within the intestinal mucosa (1–4). Amplification of this system is appropriate in the fight against enteric pathogens, but when inappropriate results in debilitating illnesses such as inflammatory bowel disease (IBD)[3] (3, 4). Neutrophil infiltration of the human intestinal wall is a hallmark of intestinal inflammation and a central event in the pathogenesis of IBD. Transmigration of neutrophils across the human intestinal epithelial cells leads to compromised epithelial integrity, alteration in mucosal electrolyte transport and barrier function, and superoxide and free radical production, all of which contribute to the clinical syndrome of diarrhea and malabsorption in IBD (2, 3, 5). Histologically, this is characterized by mucosal edema, cryptitis, crypt abscess formation, and ulceration. Pioneering studies by Madara and coworkers (2, 6–8) using inverted model human intestinal epithelial monolayers enabled in vitro modeling of the neutrophil transepithelial migration process, which has, in turn, advanced the understanding of neutrophil-intestinal epithelial cell interaction and gut epithelial cell biology.

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[3] Abbreviations used in this paper: IBD, inflammatory bowel disease; LX, lipoxin; LXA_4, 5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-trans-11-*cis*-eicosatetraenoic acid; ATL, aspirin-triggered 15-epi-LX; 15-(*R/S*)-methyl-LXA_4, 15-(*R/S*)-methyl-11,12-dehydro-LXA_4 methyl ester; 16-phenoxy-LXA_4, 16-phenoxy-11,12-dehydro-17,18,19,20-tetranor-LXA_4 methyl ester; MCP-1, monocyte-chemoattractant protein-1; TER, transepithelial resistance.

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Recruitment of neutrophils to the gut is a complex process coordinated by cytokines, chemokines, and adhesion molecules. Chemokines are 8- to 10-kDa peptides whose principal actions are to serve as chemotactic cytokines for leukocytes (9). In vitro studies indicate that transformed human colonic adenocarcinoma cell lines secrete most chemokines upon cytokine activation or bacterial infection (10). Freshly isolated intestinal epithelial cells are also a source of chemokines (11). Potential cellular sources of chemokines in this environment include colonocytes, and resident and recruited leukocytes (9, 12, 13).

The relapsing-remitting course and frequent spontaneous remission observed in IBD imply the existence of endogenous anti-inflammatory mechanisms. Adding to the known endogenous anti-inflammatory armamentarium (which includes Th1/Th2 imbalance, IL-1 receptor antagonist) are a class of eicosanoids known as lipoxins (lipoxygenase interaction products; LXs). The major bioactive LXs in mammalian systems, 5(*S*),6(*R*),15(*S*)-trihydroxy-7,9,13-trans-11-*cis*-eicosatetraenoic acid (LXA_4) and 5(*S*),14(*R*),15(*S*)-trihydroxy-6,10,12-trans-8-*cis*-eicosatetraenoic acid ($LXB)_4$, are formed via pathways initiated by the dual lipoxygenation of arachidonic acid during cell-cell interactions (14, 15). They are rapidly metabolized in vitro, and stable analogs have been designed that retain the bioactivity of the native compounds (16).

LXs are endogenous eicosanoids, generated during cell-cell interactions, that appear to serve as braking signals for neutrophil-mediated tissue damage (14, 15, 17). LXs inhibit recruitment of neutrophils by attenuating their chemotaxis, adhesion, and transmigration across vascular endothelial (18, 19) and epithelial cells (7, 8) and by down-regulating chemokine production (20, 21). LXs may further contribute to the resolution of inflammation by stimulating nonphlogistic phagocytosis of apoptotic neutrophils by macrophages (22).

Recently, a seven-transmembrane G protein-coupled LXA_4 receptor has been cloned from T84 cells, a human colonic adenocarcinoma cell line (21). This is identical with the previously described LXA_4 receptor (termed ALXR) cloned from cells of myeloid lineage. LXs attenuate TNF-α-induced IL-8 secretion by these cells in vitro (21). Furthermore, LXs also inhibit *Salmonella typhimurium*-induced secretion of IL-8 and pathogen-elicited epithelial chemoattractant by model intestinal epithelial cells (23). Intriguingly, aspirin-acetylated cyclooxygenase-2 generates 15(*R*)-hydroxyeicosatetraenoic acid which, in the context of cell-cell interactions, can be converted by neutrophils to a series of 15-epimers (aspirin-triggered 15-epi-LXs; ATLs) that may share many anti-inflammatory activities with the native LXs (14, 15, 17). ATLs may contribute to the bioactivity profile of the prototype nonsteroidal anti-inflammatory drugs in vivo. Taken together, these data suggest that LXs may play important anti-inflammatory roles in intestinal inflammation that include neutrophil-independent modulation of cell function.

Here we assessed the influence of LXs, stable LX analogs, and aspirin-triggered 15-epi-LXA_4 on TNF-α-stimulated neutrophil-intestinal epithelial cell interaction in vitro and TNF-α-stimulated chemokine release by intestinal mucosa ex vivo. In addition, we monitored the effects of LXs on TNF-α-induced changes in colonocyte apoptosis and tissue architecture in normal human intestine ex vivo.

Materials and Methods

In vitro neutrophil-enterocyte interaction

T84 cell culture. T84, a human colonic adenocarcinoma cell line with a well characterized epithelial phenotype and polarity (24), was obtained from American Type Culture Collection (Manassas, VA) and grown to confluence in T75 tissue culture flasks in a 1:1 mixture of DMEM and Ham's F-12 medium supplemented with 15 mM HEPES buffer (pH 7.5), 10% FCS, 1% penicillin/streptomycin, and 1% L-glutamine. To facilitate study of neutrophil trafficking in a physiological (basolateral-to-apical) direction, T84 epithelial monolayers were grown on the undersurface of polycarbonate filters according to methods described by Colgan et al. (8). Briefly, the inverted monolayers were grown on the undersurface of ring-supported 0.33-cm^2 polycarbonate filters (Corning Costar, Cambridge, MA), and confluence was assessed electrophysiologically by measurement of transepithelial resistance (TER) with Endohm-6 and the EVOM resistance meter (World Precision Instruments, Sarasota, FL). Consistent with the known ability of T84 to form tight intercellular junctions, mean TER of the monolayers on filters at time of confluence was $1367.7 \pm 50.4\ \Omega.cm^2$ ($n = 45$). The monolayer on the filter effectively divided the well into an upper (basolateral) compartment and a lower (apical) compartment.

Neutrophil isolation and transmigration/adherence assays. Human neutrophils from healthy volunteers were isolated by density gradient centrifugation and dextran sedimentation and labeled with a fluorescent dye, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxy-fluorescein ester (Calbiochem, San Diego, CA) as described previously (19). Viability was determined by trypan blue exclusion test. One million fluorescence-labeled neutrophils suspended in 100 μl Dulbecco's PBS and 1% bovine calf serum were added to the upper (basolateral) compartment of the filter inserts. In all experiments, a positive control for neutrophil transmigration comprising the chemotactic peptide fMLP (6 μM; Sigma, St. Louis, MO) in the lower apical compartment was included. At the end of the 120-min experimental period at 37°C, the nonadherent neutrophils were removed by washing twice with Dulbecco's PBS. The number of neutrophils that adhered to the epithelial monolayers or transmigrated into the lower (apical) compartment were determined fluorometrically on the Cytoflour II (Perspective Biosystems, Framingham, MA).

For studies of the influence of cytokine activation of epithelium on neutrophil trafficking in vitro, both sides of the confluent epithelial monolayers were preincubated with TNF-α, IL-1β (100 ng/ml; R&D Systems, Minneapolis, MN), or vehicle control for 24 h at 37°C before initiation of transmigration experiments, and washed before addition of neutrophils. TER of the cytokine-treated monolayers was determined before and after 24 h of cytokine preincubation.

LXA_4 was purchased from Cascade Biologicals (Berkshire, U.K.). The synthetic LXA_4 analog 15-(*R/S*)-methyl-11,12-dehydro-LXA_4 methyl ester (15-(*R/S*)-methyl-LXA_4) covers the biological activity of 15-epi-LXA_4. This and the synthetic stable LXA_4 analog 16-phenoxy-11,12-dehydro-17,18,19,20-tetranor-LXA_4 methyl ester (16-phenoxy-LXA_4) were gifts from Dr. Nicos Petasis (University of Southern California, Los Angeles, CA) and prepared by total organic synthesis as previously described (16). Structures were confirmed by reversed phase-HPLC, nuclear magnetic resonance, and mass spectral analysis. The solutions were stored at −70°C in 99% ethanol. The modulatory effects of LXs on neutrophil-epithelial cell interaction were assessed by preincubation of human neutrophils and epithelial monolayers, respectively, with LX (0.1–100 nM, 37°C). Neutrophils were preincubated with LX for 15 min before addition to the epithelial monolayers. T84 monolayers were treated with LX for 20 min before incubation with cytokine. Vehicle controls comprised dilutions of the solvent (absolute ethanol) to the highest concentration of LXs used in the experiments (maximal ethanol concentration 0.01%). All individual experiments were performed in duplicate.

To determine the role of the C-X-C chemokine, IL-8, and the adhesion molecule, ICAM-1, in TNF-α-stimulated neutrophil-epithelial cell interactions, the blocking mAbs to the respective chemokine and adhesion molecule were added to both the basolateral and apical compartments at the beginning of transmigration assays (both mAbs, 50 μg/ml; R&D Systems).

Assessment of T84 viability and apoptosis in vitro. In experiments assessing TNF-α-induced changes in viability and apoptosis, T84 monolayers were grown to confluence on 12- and 24-well tissue culture plates. Monolayers were incubated with TNF-α (20–100 ng/ml) or vehicle control for 24 h. Gross morphological assessment of the monolayers was performed by standard light microscopy. The monolayers were subsequently trypsinized. Viability was assessed by flow cytometry following incorporation with propidium iodide. Apoptosis was quantified by flow cytometry as the percentage of cells with hypodiploid DNA. Cell suspensions were centrifuged at $200 \times g$ for 10 min. The cell pellets were resuspended in 500 μl of hypotonic fluorochrome solution (50 μg/ml propidium iodide, 3.4 nM sodium citrate, 1 mM EDTA, and 0.1% Triton X-100) and kept in the dark at 4°C before they were analyzed using a Coulter Elite cytofluorometer (Coulter, Bedfordshire, U.K.). A human prostate adenocarcinoma cell line, LNCaP androgen-dependent prostate cancer cells, treated with etoposide was used as positive control.

Studies of human intestinal mucosa ex vivo

Harvesting of human intestinal mucosa. Normal human colonic mucosa was harvested from colonic resection specimens for colorectal cancer following informed consent from patients preoperatively. Patients with known IBD, or ischemic bowel or familial colon cancer syndromes were excluded. The resected bowel specimen was transported to the pathology laboratory for processing within 30 min of resection. Twelve ~1-cm^2 strips of macroscopically normal colonic mucosa at least 10 cm away from the tumor margin were harvested and transported to the laboratory in prewarmed, preoxygenated Kreb's physiological solution (Sigma) at 37°C. The resection specimen was immersed in 10% formalin and processed routinely.

A purpose-built oxygenation manifold comprising six oxygenation ports was arranged in a longitudinal 2 × 3 fashion. Oxygenation of the colonic mucosa strips was delivered via the oxygenation ports fitted through perforations on the lid of a 12-well culture plate in the water bath at 37°C. A duplicate of the oxygenation manifold and culture well enabled six conditions in duplicate at one experiment. Two milliliters of prewarmed Kreb's physiological solution was added to each of the 12 wells and preoxygenated for 10 min. The 12 colonic mucosa strips harvested as described above were removed from the transport medium, placed in each well individually containing the physiological solution, and allowed to equilibrate for 10 min and maintained ex vivo at 37°C for up to 8 h.

Treatment of colonic mucosa with cytokine or LXs. Following equilibration, the mucosal strips were preincubated with LX at doses ranging from 0.1, 1, 10, and 100 nM in 2 ml physiological solution for 20 min. The vehicle control was 0.1% ethanol. At the end of the preincubation period, the experiment was started with activation by TNF-α (20 μg/ml; R&D Systems) or vehicle control (Dulbecco's PBS-BSA). Each well was wrapped in cling film and allowed to incubate for 8 h. To determine the time course of chemokine production and modulation by LX, two 100-μl aliquots were removed from each well at times 0, 3, and 8 h, snap-frozen, and stored at −70°C. The volume sampled was replaced with the appropriate physiological solution containing TNF-α.

At the end of the experimental period, the colonic mucosa strips were snap-frozen and stored at −70°C for protein assay. In separate experiments, the strips were stored in 10% neutral formalin, paraffin-mounted, and processed for H&E staining, or fixed in glutaraldehyde and processed for electron microscopy.

Chemokine assay in colonic mucosa. TNF-α-stimulated chemokines IL-8, monocyte-chemoattractant protein-1 (MCP-1), and RANTES release by the colonic mucosa ex vivo in the supernatant were assayed separately by ELISA according to manufacturer's protocol (Quantikine; R&D Systems). To account for individual variation in the sampling size of the colonic strips, chemokine release was expressed as picograms of chemokine per milligram of protein, the latter being determined by the standard Bradford protein assay (Bio-Rad, Hertfordshire, U.K.).

Histological assessment and apoptosis in colonic mucosa. At the end of the cytokine stimulation experiments, the colonic strips were processed for 1) H&E staining of paraffin-embedded sections or 2) electron microscopy on glutaraldehyde-fixed sections. The architectural integrity of the epithelial cells and crypt cells was assessed on H&E-stained slides.

Apoptosis of the surface colonocytes was defined as cells displaying the classical stigmata of cytoplasmic condensation and nuclear fragmentation on H&E-stained sections. The apoptotic count was taken as the average number of apoptotic epithelial cells per five high-power fields under light microscopy. The final figure was the average of two apoptotic counts assessed by an experienced pathologist blinded to the experimental conditions.

For electron microscopy, colonic strips were fixed in glutaraldehyde followed by osmium tetroxide and embedded in epoxy resin using routine methods. Sections (50-nm thick) were stained with uranyl acetate and lead citrate and examined in a JEOL 2000 transmission electron microscope.

Caspase 3 immunohistochemistry. To study the potential role of caspase 3, a cell death protease involved in apoptosis, on the observed TNF-α-induced colonocyte apoptosis, immunohistochemistry was performed on paraffin-embedded sections of colonic mucosa as previously described (25). Briefly, 4- to 5-μm thin sections of paraffin-embedded sections of colonic mucosa were cut and placed on polylysine coated slides. Following deparaffinization, endogenous peroxidase activity was quenched by 3% hydrogen peroxide in methanol. Nonspecific staining was reduced by preincubation with normal blocking serum. The sections were then incubated with the primary Ab CPP32 p20 (N19), a goat polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA), for 60 min at room temperature. After washing, sections were incubated with a biotinylated secondary Ab for 30 min, followed by Avidin Biotin enzyme reagent and DAB chromogen, a peroxidase substrate (ABC staining system; Santa Cruz Biotechnology). The sections were then counterstained with methyl green and mounted with DPX mountant (neutral solution of polystyrene and plasticizers in xylene) for microscopic assessment. Sections of human prostate gland with benign prostatic hypertrophy served as positive control for caspase 3 staining.

Ethics approval

This study was approved by the Ethics Committee of the Mater Misericordiae Hospital, Dublin, Ireland. Written informed consent for the harvesting of colonic mucosa was obtained from all patients preoperatively.

Statistical analysis

Data are expressed as mean ± SEM and were compared using Student's *t* test. Statistical significance was considered when $p < 0.05$.

Results

Effect of intestinal epithelial activation with TNF-α on neutrophil adherence and transmigration

Incubation of the T84 monolayers with TNF-α (100 ng/ml; 24 h at 37°C) enhanced neutrophil adherence (69.5 ± 4.6 vs 9.6 ± 2.8%, control; $p < 0.001$; $n = 6$) via an IL-8- and ICAM-1-independent mechanism (Fig. 1). Conditioning of T84 with TNF-α was not associated with alteration in TER (1467 ± 77.9 vs 1535 ± 132.3 Ω.cm^2; TNF vs control; $p = 0.67$; $n = 6$), epithelial cell viability, or apoptosis. Similarly, neutrophil adherence to TNF-α-activated T84 monolayers did not affect TER (1465 ± 72.1 vs 1247.4 ± 120.8 Ω.cm^2; pre- and postneutrophil adherence; $p = 0.63$; $n = 4$).

The addition of fMLP (6 μM) to the apical compartment induced a rapid and robust transmigration response (40.2 ± 2.9 vs 0 ± 0% at 2 h; $p < 0.001$; $n = 9$; Fig. 2) in keeping with previous reports (6–8). Incubation of T84 with TNF-α did not induce neutrophil transmigration, but did prime monolayers such that the fMLP response was enhanced (40.2 ± 2.9 vs 59.5 ± 3.7%, respectively, $n = 4$; $p < 0.05$). Transmigration was not further observed with TNF-α in studies examining extended experimental periods up to 4 and 6 h. Incubation of epithelial monolayers with IL-1β (100 ng/ml; 24 h) failed to induce neutrophil adherence or transmigration and was not studied further (data not shown).

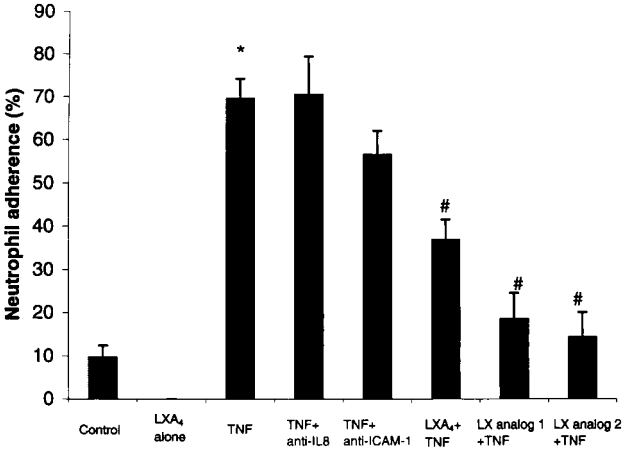

FIGURE 1. Effect of TNF-α on neutrophil adherence: modulation by LX. T84 monolayers cultured on polycarbonate filters were exposed to TNF-α (100 ng/ml, 24 h, 37°C) and adherence of 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein ester-labeled neutrophils were determined. Monolayers were pretreated with LXA_4, 15-(*R/S*)-methyl-LXA_4 (LX analog 1) and 16-phenoxy-LXA_4 (LX analog 2) (all compounds: 10 nM, 20 min, 37°C) before TNF-α activation. The potential role of ICAM-1 and IL-8 was investigated using mAbs (anti-ICAM-1 and anti-IL-8 Abs, 50 μg/ml, respectively). *, $p < 0.001$ vs control; #, $p < 0.05$ vs TNF-α.

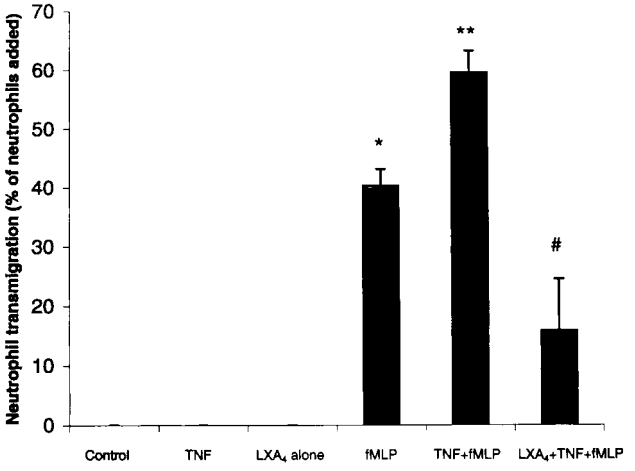

FIGURE 2. Effect of TNF-α on neutrophil transmigration: modulation by LX. Basolateral-to-apical neutrophil transmigration was determined using fMLP (6 μM) as chemoattractant. The effect of pretreatment with TNF-α (100 ng/ml, 24 h, 37°C) and LXA_4 (10 nM, 20 min, 37°C) on fMLP-directed neutrophil transmigration was examined. *, $p < 0.001$ vs control; **, $p < 0.05$ vs fMLP alone; #, $p < 0.05$ vs TNF-α + fMLP.

Effect of LXs on TNF-α-induced neutrophil adherence: in vitro evidence for direct LX-epithelial cell interaction

LXA_4 did not affect basal neutrophil adherence or transmigration (Fig. 1). Prior exposure of both the basolateral and apical aspects of T84 epithelial monolayers to LXA_4 (10 nM for 20 min) significantly attenuated TNF-α-induced neutrophil adherence during subsequent coincubations (percent inhibition = 53.5 ± 4.5%; $p = 0.003$; $n = 3$; Fig. 1) and blunted fMLP-induced transmigration across TNF-α-activated monolayers (59.5 ± 3.7 vs 15.8 ± 8.7% + LXA_4; $p < 0.05$; $n = 4$). These actions were shared by 15-(*R/S*)-methyl-LXA_4 and 16-phenoxy-LXA_4 at nanomolar concentrations (Fig. 1). In parallel experiments, pretreatment of human neutrophils with 15-(*R/S*)-methyl-LXA_4 (10 nM; 15 min) significantly attenuated TNF-α-induced neutrophil adherence (76.6 ± 6.2% control vs 45.2 ± 5.4% + LX; $p < 0.005$; $n = 3$).

The dose-response relationship among the three LX compounds (0.1–100 nM dose range) and TNF-α-induced neutrophil adherence was determined (Fig. 3). Maximal inhibition of neutrophil adherence by native LXA_4 was observed at 10 nM (53.5 ± 4.5%; $p < 0.005$; $n = 3$). At 10 nM, inhibition of neutrophil adherence by 15-(*R/S*)-methyl-LXA_4 and 16-phenoxy-LXA_4 was 58.2 ± 7.4 and 52.5 ± 7.5%, respectively; $p < 0.005$ and $p < 0.05$, respectively; $n = 3$). For the dose range studied, the most potent analog was 15-(*R/S*)-methyl-LXA_4 with maximal inhibition of neutrophil adherence at 100 nM (65.3 ± 3.6%; $n = 3$; $p < 0.001$) and statistically significant inhibition from 1 to 100 nM. At 0.1 nM, no statistically significant effect on TNF-α-induced neutrophil adherence was observed for all three compounds.

Influence of TNF-α on chemokine release by intestinal mucosa ex vivo: modulation by LXs

TNF-α (20 ng/ml) significantly enhanced release of MCP-1, IL-8, and RANTES by colonic strips ex vivo (Fig. 4). RANTES was detected in much smaller quantities relative to the other two chemokines but was also induced by TNF-α. The influence of LXs on TNF-α-stimulated chemokine release was examined by pretreatment of the colonic mucosa strips with LX for 20 min before the addition of cytokine. At a 10-nM concentration, 15-(*R/S*)-methyl-LXA_4 and 16-phenoxy-LXA_4 significantly attenuated TNF-α-stimulated IL-8 production from 5102.8 ± 528.3 to 2617.9 ± 124.4 and 1783.8 ± 241.4 pg/mg, respectively ($p < 0.005$ and $p < 0.05$, respectively, $n = 4$) (Fig. 5). Native LXA_4 at 10 nM attenuated IL-8 production, although this did not reach statistical significance (5102.8 ± 528.3 vs 1728.9 ± 711.1 pg/mg; $p = 0.07$; $n = 3$) (Fig. 5).

MCP-1 release was similarly attenuated by LXs. At 10 nM, effect of LXs on MCP-1 production were as follows: 1) LXA_4: 881.37 ± 374.3 vs 2563.6 ± 474.5 pg/mg (control) ($n = 3$; $p < 0.05$), 2) 15-(*R/S*)-methyl-LXA_4: 600 ± 328.3 vs 5602.9 ± 804.5 pg/mg (control) ($n = 3$; $p < 0.005$), and 3) 16-phenoxy-LXA_4: 58.7 ± 4.6 vs 6186.1 ± 485.4 pg/mg (control) ($n = 3$; $p < 0.005$). Maximal inhibition by all three LX compounds was observed at 100 nM.

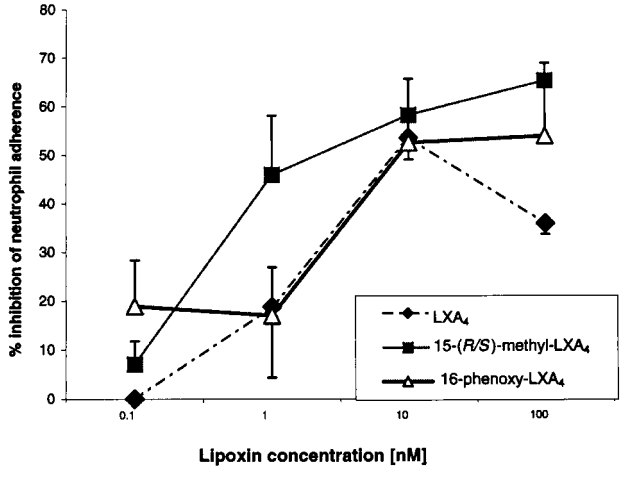

FIGURE 3. Dose-response relationship between LX and inhibition of TNF-α-induced neutrophil adherence. Graph shows the relationship between the three LX analogs (0.1–100 nM) and inhibition of TNF-α-induced neutrophil adherence.

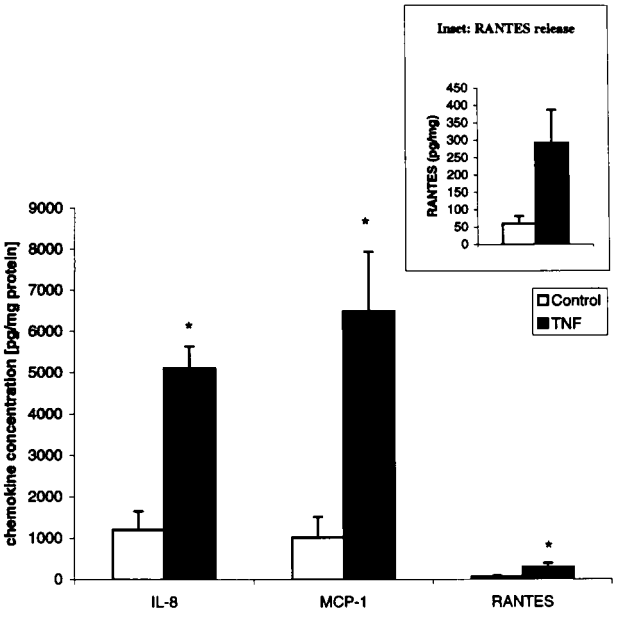

FIGURE 4. TNF-α-stimulated chemokine release by human colonic mucosa ex vivo. Normal human colonic mucosa was maintained ex vivo in oxygenated physiological solution at 37°C. Chemokine release by TNF-α-stimulated colonic mucosa was determined (TNF-α, 20 ng/ml, 8 h). Open bars represent control conditions and filled bars represent TNF-α stimulation. *, Statistical significance vs control. Graph inset shows RANTES release at a different scale.

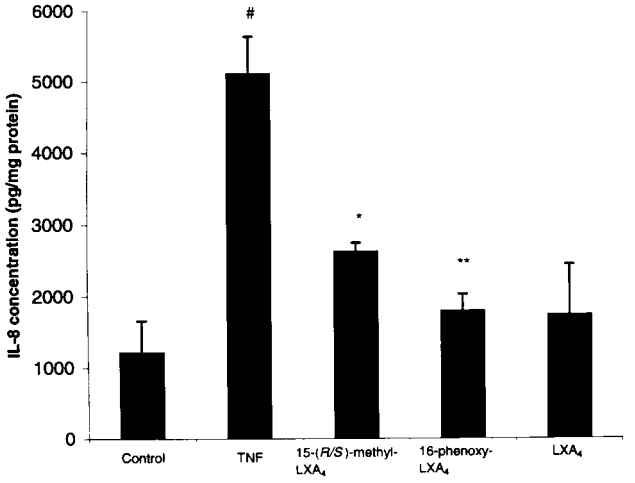

FIGURE 5. Effect of LXs on TNF-α-stimulated IL-8 release by human colonic mucosa. The effect of LXs on TNF-α-stimulated IL-8 release by human colonic mucosa ex vivo was determined by exposure of the colonic strips to LXA_4, 15-(*R/S*)-methyl-LXA_4, and 16-phenoxy-LXA_4 (10 nM, 20 min, 37°C) before TNF-α activation (100 ng/ml, 24 h). #, $p < 0.001$ vs control; ∗, $p < 0.005$; ∗∗, $p < 0.05$ vs TNF-α.

Although lower levels of RANTES were produced relative to the other chemokines, a trend toward attenuation by LX was observed. Statistically significant attenuation was only seen with the stable analogs at maximal dose, i.e., 100 nM. The effect of LXs on RANTES production was as follows: 1) LXA_4: 91.4 ± 24.4 vs 292.5 ± 95.8 pg/mg (control) ($n = 3$; $p = 0.16$), 2) 15-(*R/S*)-methyl-LXA_4: 128.4 ± 32.1 vs 389 ± 63.3 pg/mg (control) ($n = 3$; $p < 0.05$), and 3) 16-phenoxy-LXA_4: 175.7 ± 37.8 vs 389 ± 63.3 pg/mg (control) ($n = 3$, $p < 0.05$).

A representative dose-response relationship between LX concentration and inhibition of TNF-α-stimulated chemokine release (RANTES) is shown in Fig. 6. A similar relationship was observed for the other chemokines.

The effect of TNF-α on colonic mucosal architecture and modulation by LX

In separate experiments, the colonic mucosal strips were fixed, following exposure to TNF-α ± LXs, in 10% formalin, paraffin-embedded, and stained with H&E. At the end of the 8-h experimental period, colonic mucosa under basal unstimulated condition (vehicle control) exhibited relative preservation of the surface epithelial, crypt, and lamina propria architecture (Fig. 7*A*).

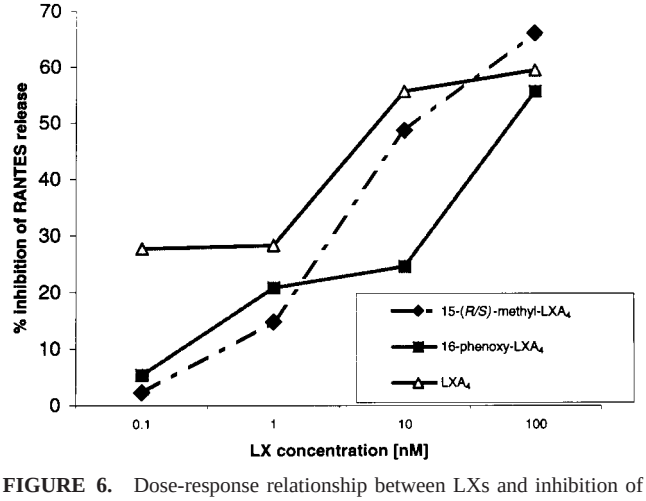

FIGURE 6. Dose-response relationship between LXs and inhibition of TNF-α-stimulated chemokine (RANTES) release. The relationship between LXA_4, 15-(*R/S*)-methyl-LXA_4, and 16-phenoxy-LXA_4 (0.1–100 nM dose range, 20 min, 37°C) on TNF-α-stimulated RANTES release is shown here. The colonic strips were stimulated with TNF-α (20 ng/ml) for 8 h. Similar dose-response curves were observed for IL-8 and MCP-1.

Incubation with TNF-α (20 ng/ml; 8 h) induced significant distortion in mucosa architecture of the surface colonocytes, crypt cells, and the lamina propria (Fig. 7*B*). Some of the surface enterocytes have begun to slough off the basement membrane and appeared necrotic. Apoptosis was also increased (see below). Treatment with the LXA_4 analog 15-(*R/S*)-methyl-LXA_4 (10 nM for 20 min) before the addition of TNF-α conferred cytoprotection against TNF-α-induced structural disruption (Fig. 7*C*). This protective effect was similarly observed with 16-phenoxy-LXA_4 and native LXA_4 (10 nM) (data not shown).

Effect of TNF-α on apoptosis of surface enterocytes and modulation by LX

Incubation of the colonic strips with TNF-α (20 ng/ml; 8 h) was associated with increased colonocyte apoptosis (apoptotic count of surface colonocytes: 38 ± 4 TNF-α vs 5 ± 1 control; $n = 3$; $p < 0.005$). To investigate whether the observed TNF-α-stimulated apoptosis of surface enterocytes was caspase 3-dependent, paraffin-embedded sections of TNF-α-stimulated colonic mucosa was processed for caspase 3 immunohistochemistry (described in *Materials and Methods*). In contrast to the strong (grade 4) staining seen in human prostate with benign prostatic hypertrophy, which served as positive control, cytoplasmic or nuclear caspase 3 immunostaining was not observed in the TNF-α-stimulated colonic mucosa.

Prior exposure of colonic strips to 15-(*R/S*)-methyl-LXA_4 (10 nM; 20 min) significantly attenuated this TNF-α-stimulated apoptotic response (apoptotic count 18 ± 3 LX vs 38 ± 4; TNF-α; $p < 0.005$; $n = 3$; Fig. 8*A*).

Apoptosis was confirmed ultrastructurally by electron microscopical examination of sections of the TNF-α-stimulated intestinal mucosa. The classical appearance of TNF-α-stimulated apoptotic colonocytes is shown in Fig. 8*B*. Pretreatment with 15-(*R/S*)-methyl-LXA_4 at 10 nM for 20 min conferred cytoprotection and relative ultrastructural preservation that approximated that of control tissue.

Discussion

The identification of the LXA_4 receptor in a human colon cancer cell line (21) and recent in vitro studies support an anti-inflammatory role for LXs in intestinal inflammation (8, 15, 21, 23; reviewed in Ref. 16). There is emerging evidence to suggest that the repertoire of anti-inflammatory mechanisms of LXs extend beyond their relatively well characterized antineutrophil activities to a direct LX-epithelial cell interaction (20, 21, 23; reviewed in Ref. 17). The current study further supports these data by demonstrating that LXs antagonized 1) TNF-α-mediated neutrophil adherence and transmigration in an in vitro model of neutrophil trafficking across intestinal epithelia and 2) TNF-α-induced chemokine production, architectural disruption, and colonocyte apoptosis in an ex vivo model of intestinal inflammation involving human colonic mucosa.

Using the well-characterized T84 cell line in an in vitro model of intestinal inflammation, we have demonstrated that TNF-α activation of these cells promotes adherence of neutrophils via an IL-8 and ICAM-1-independent mechanism and further enhanced fMLP-directed neutrophil transmigration. The neutrophil β_2 integrin CD11b/CD18 is required for neutrophil transepithelial migration (26). However, its ligand mediating neutrophil-endothelial adhesion, ICAM-1, is apically expressed in the intestinal epithelium (26) and therefore does not interact with CD11b/CD18 during the

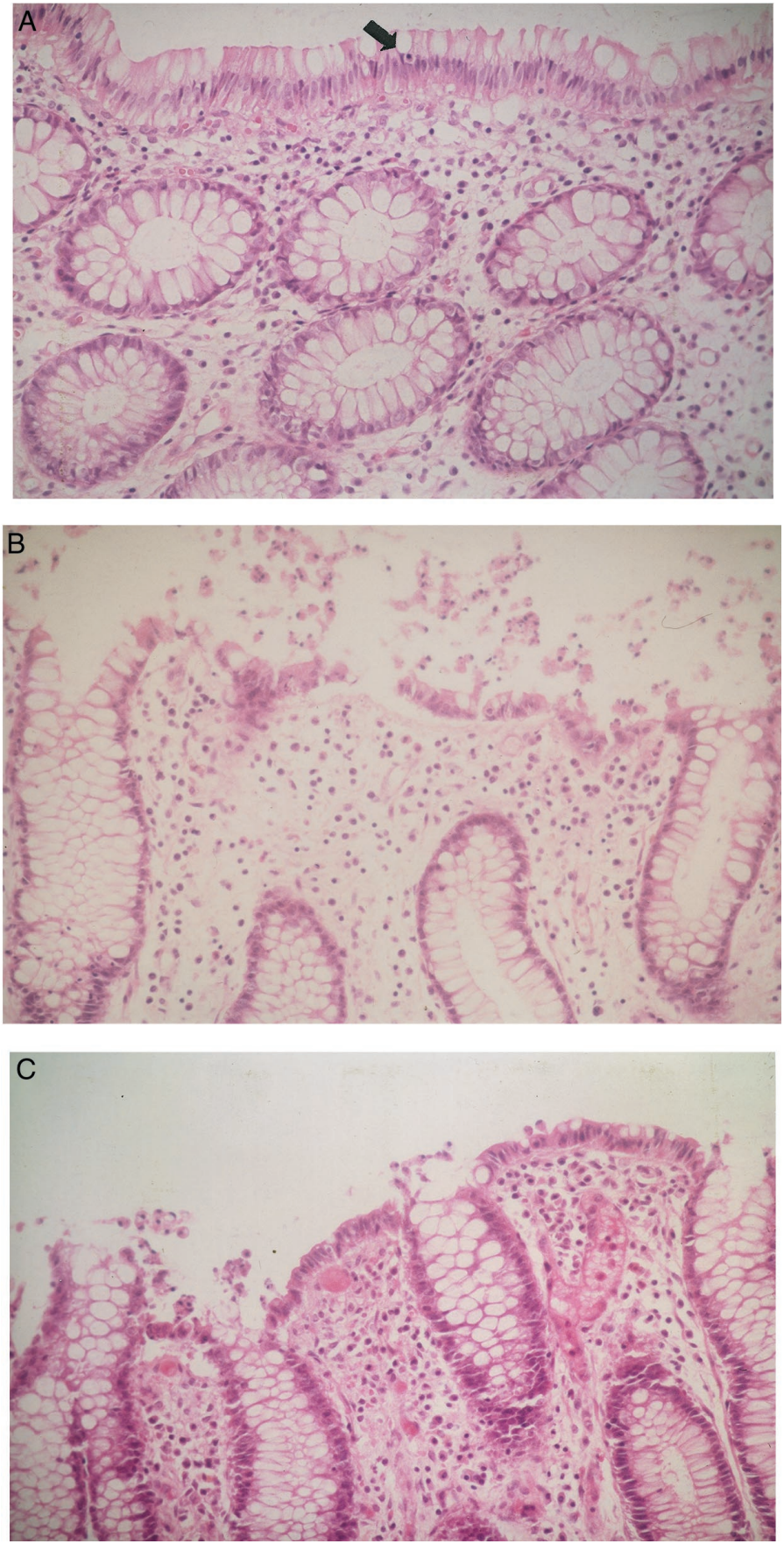

FIGURE 7. Assessment of colonic mucosa architecture by H&E staining. *A*, Colonic mucosa incubated with vehicle control at $t = 8$ h. There was almost complete preservation of mucosa architecture with normal surface enterocytes and goblet cells (original magnification, ×400). Only rare apoptotic bodies were identified (arrow). *B*, Mucosa treated with TNF-α (20 ng/ml, 8 h, 37°C) exhibiting extensive surface epithelial disruption and numerous apoptotic bodies, singly and in clusters (original magnification, ×400). *C*, Amelioration of TNF-α-induced mucosal damage by pretreatment with 15-(*R*/*S*)-methyl-LXA_4 (10 nM, 20 min, 37°C). Mild surface disarray with significantly less colonocyte apoptosis and relative preservation of surface and crypt architecture was noted (original magnification, ×400).

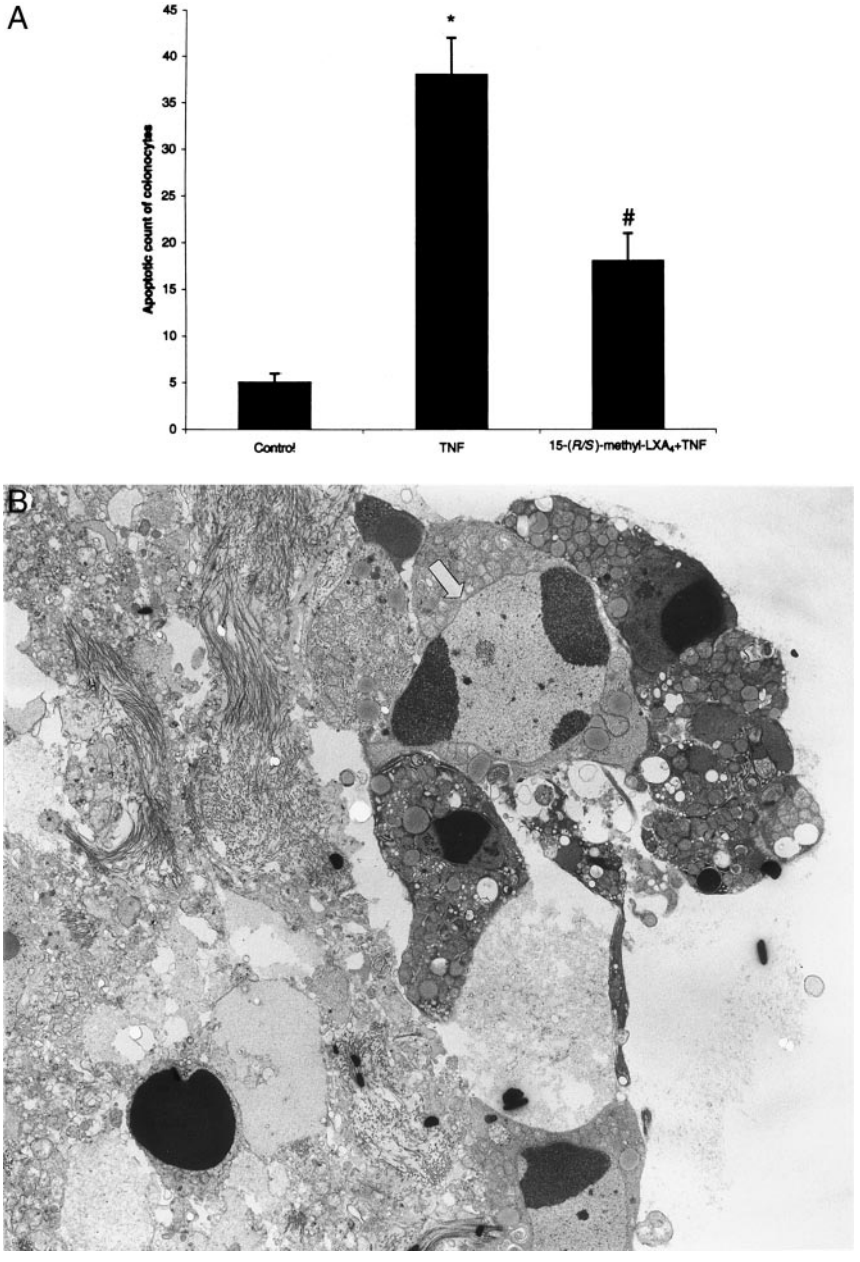

FIGURE 8. Effect of TNF-α on apoptosis of surface enterocytes and modulation by LX. *A*, Apoptotic count defined as the total number of apoptotic surface enterocytes per five high-power fields under light microscopy. *A*, Effect of TNF-α stimulation (20 ng/ml, 8 h, 37°C) on apoptotic count of surface colonocytes ($n = 3$; *, $p < 0.005$ vs control). The effect of prior exposure to 15-(*R/S*)-LXA_4 (10 nM, 20 min, 37°C) on TNF-α-stimulated colonocyte apoptosis was also determined. #, $p < 0.005$ vs TNF-α. *B*, Electron microscopical features of TNF-α-treated colonic mucosa demonstrating colonocyte apoptosis characterized by nuclear fragmentation and cytoplasmic condensation (white arrow; original magnification, ×7500).

transmigration process (2, 5, 26). Indeed, neutralizing Ab to ICAM-1 failed to inhibit fMLP-directed neutrophil transepithelial migration (2) and TNF-α-stimulated neutrophil adherence in vitro. The basolaterally expressed IL-8 may act as chemotactic signal for neutrophil trafficking across the vascular endothelium and subepithelial matrix (2, 27), and is probably not crucial for neutrophil-epithelial interaction. The precise molecular mechanisms underlying TNF-α-promoted neutrophil adherence remain to be elucidated, and candidate molecules include the neutrophilic CD11b/CD18 and epithelial CD47 (2, 5, 26). In contrast to alteration of epithelial barrier function following neutrophil transmigration (6, 28), TER was not affected by TNF-α-promoted neutrophil adherence. This may infer that the initial neutrophil adherence process elicited by TNF-α in the absence of a chemotactic gradient may involve interaction between neutrophils with the basement membrane or basolateral membrane of the enterocytes. Further transit of the neutrophils across the paracellular space cannot take place unless other cofactors are present, such as chemoattractants in the lumen. Indeed, fMLP-directed neutrophil transmigration was more efficient following TNF-α conditioning of the epithelia. TNF-α, at the dose and duration studied, was not associated with alteration in T84 epithelial barrier function, basal viability, and apoptosis rates.

Dynamic cross-talk between intestinal epithelial cells and immune cells of the lamina propria results in the production of chemokines that serve to recruit inflammatory cells, a feature central to the pathogenesis of IBD (3, 4, 9). Increased levels of chemokines have been detected in IBD tissues and in vitro models of intestinal inflammation (9, 29–31). It has previously been shown that IL-1β, TNF-α, and IFN-γ stimulated IL-8, MCP-1, and RANTES mRNA expression and secretion from HT29 and Caco-2 cells, both human colonic epithelial cell lines (11, 29–31). Furthermore, T84 cells also secrete a wide array of chemokines in response to cytokine stimulation and bacterial infection (10). In vivo studies showed that cellular sources of MCP-1 include lamina propria macrophages, endothelial cells, spindle cells, and intestinal epithelial cells (11). Colonic mucosa constitutively express MCP-1

and the C-X-C chemokine, ENA-78, which are up-regulated during inflammation (11, 32). However, this model system did not distinguish the cells of origin of the chemokines studied. We demonstrated that TNF-α stimulated the release of IL-8, MCP-1, and RANTES over an 8-h period from normal colonic mucosa. The low level of RANTES detected in the time course studied relative to IL-8 and MCP-1 suggested a differential effect of cytokine stimulation on chemokine release.

Absence of autolysis and preservation of normal colonic architecture and cellular ultrastructure (electron microscopy data not shown) were demonstrable in the tissue maintained under vehicle control conditions. The observed architectural and structural damage of intestinal mucosa induced by TNF-α did not resemble that seen classically in infective colitis or IBD. Importantly, apoptosis of surface colonocytes appeared to be a cardinal feature. The influence of cytokines on colonocyte apoptosis has been examined in in vitro studies involving intestinal cell lines. For instance, TNF-α induces apoptosis in certain intestinal epithelial cell lines such as HT-29 (33, 34), but not in others such as T84 cells. In addition, antagonism of TNF-α and NO inhibit apoptosis of HT-29 cells following infection with *Salmonella* (34). In the presence of TNF-α alone or in synergy with IFN-γ, HIEC, a nontransformed human intestinal epithelial cell line, become highly susceptible to Fas-induced apoptosis (35). This enhanced sensitivity is mediated via TNF-α- and IFN-γ-induced up-regulation of Fas expression, and the effect of TNF-α on Fas is mediated via the p55 TNFR. Similarly, in colonic organ cultures, IFN-γ and TNF-α also enhance colonocyte Fas expression, resulting in markedly enhanced apoptotic response to stimulation of this receptor (35).

Because TNF-α-induced chemokine production is NF-κB-mediated (36, 37) and NF-κB is generally considered anti-apoptotic (38, 39), the concurrent finding of TNF-α-mediated apoptosis and chemokine production is surprising. In this regard, we postulate that TNF-α induced enterocyte apoptosis via activation of the classical TNFR death domain mechanism, but the influence of NF-κB remains unclear. Although it is intriguing that this form of apoptosis is not associated with activation of caspase-3, one of the key enzymes involved in the lethal proteolytic cascade of apoptosis execution, our finding complemented findings by Ruemmele et al. (40) that caspase 3 is not activated in TNF-α-induced apoptosis in another intestinal cell line, IEC-6.

The anti-inflammatory properties of LXs have been most extensively described with reference to neutrophil function. These include attenuation of neutrophil chemotaxis, adhesion to endothelial cells, transendothelial and transepithelial migration, degranulation, superoxide anion generation, and neutrophilic expression of L-selectin and CD11/CD18 expression (8, 15–18, 41). In this context, Hachicha et al. (41) provided the first in vivo example of LX-induced cytokine regulation by demonstrating that LXA_4, 15-epi-LXA_4, and its analogs are potent regulators for gene expression of individual cytokines relevant in inflammation. Godson et al. (22) recently showed that LXs also stimulate human macrophages to phagocytose apoptotic neutrophils. The identification and cloning of human nonmyeloid LXA_4 receptors on intestinal epithelial cells (21), synovial fibroblasts (20), and mesangial cells (42) infer a potential local immunoregulatory role of LXs in these organ systems. In the gastrointestinal tract, direct incubation of intestinal epithelial cells with LXs results in attenuation of TNF-α-induced IL-8 secretion by T84 cells (21) and inhibition of *S. typhimurium*-induced secretion of IL-8 and pathogen-elicited epithelial chemoattractant by model intestinal epithelial cells (23). In the current study, we show that LX-epithelial interactions are potent inhibitors of TNF-α-stimulated neutrophil adherence and blunt fMLP-directed neutrophil transmigration across TNF-α-primed epithelium. There are few agents that display potency in neutrophil-epithelial cell interactions within the 1- to 10-nM range. In addition, this potency range confirms and is consistent with observations in the literature (8, 16, 19).

In addition to the available in vitro data, we have shown that incubation of human colonic mucosa with LXs results in dose-dependent attenuation of TNF-α-induced release of IL-8, MCP-1, and RANTES by human colonic mucosa ex vivo. This bioactivity was shared by LXA_4, 15-epi-LXA_4, and LX analog and observed at nanomolar doses. Although this model of intestinal inflammation excludes recruitable circulating leukocytes, participation from the resident inflammatory cells in the lamina propria cannot be ruled out. Our results from the present study with human colonic mucosal tissue ex vivo demonstrate that these compounds, namely LXA_4 and the 15-epi-LXA_4 stable analogs, are potent inhibitors of key responses of interest in mucosal inflammation in an explant tissue milieu that is biologically relevant. Our data enhance earlier findings with isolated cell types in vitro (21, 23) and confirm and extend these earlier results. Furthermore, the observation that LXA_4 and the 15-epi-LXA_4 are potent inhibitors of RANTES release is an important addition to the bioactivity profile of these compounds and suggests a role for LXs as potential therapeutic modifiers of other diseases in which RANTES dysregulation plays an important role, such as allograft rejection (43), atopy (44), and viral infections (45). The net effect of LX-mediated attenuation of neutrophil adherence and chemokine production may translate into a reduction in the recruitment of inflammatory cells and down-regulation of intestinal inflammation.

Aspirin and nonsteroidal anti-inflammatory drugs cause upper gastrointestinal pathology including inflammation and ulceration on one hand while may be protective against lower gastrointestinal carcinogenesis on the other (46, 47). Aspirin acetylation of cyclooxygenase-2 stimulates the production of 15(*R*)-hydroxyeicosatetraenoic acid from arachidonic acid in endothelial cells, which may then be converted to ATLs (including 15-epi-LXA_4 and 15-epi LXB_4) by neutrophil 5-lipoxygenase in the context of cell-cell interaction (14). ATLs share many anti-inflammatory properties of LXs and may account for much of the favorable bioactivity profile of aspirin. In this context, we have demonstrated that LXA_4 and 15-epi-LXA_4 ameliorated TNF-α-induced intestinal mucosal structural disruption and colonocyte apoptosis. This observed cytoprotective effect of 15-epi-LXA_4 may seem at variance with reports of apparent exacerbation of IBD in patients ingesting nonsteroidal anti-inflammatory drugs (48). A potential cytoprotective effect of LXA_4 and 15-epi-LXA_4 may be due to combination of inhibition of 1) neutrophil-mediated tissue destruction through attenuation of neutrophil recruitment and 2) cytokine-stimulated architectural destruction and enterocyte apoptosis through mechanisms as yet unknown. The ability of LXs to antagonize a wide array of TNF-α-mediated inflammation as diverse as neutrophil activation (41), chemokine release, and noninflammatory events such as apoptosis may infer interaction with TNF-α at an upstream level. Further studies are needed to examine whether this interaction occurs at the receptor binding, early signal transduction pathway, or nuclear transcription level.

In summary, we have shown that LXA_4 and 15-epi-LXA_4 inhibited TNF-α-induced neutrophil-intestinal epithelial cell interaction in vitro, chemokine release and enterocyte apoptosis, and conferred cytoprotection in human intestinal mucosa ex vivo. We also provided further evidence in support of anti-inflammatory mechanisms of LXs that are mediated through their direct interaction with epithelial cells independent of their antineutrophil property.

Furthermore, the counterregulatory role of LXs in intestinal inflammation may in part be mediated through antagonism of the proinflammatory actions of TNF-α. Collectively, our data support the important roles of the intestinal epithelial cells and LXs in the regulation of immune function within the intestinal mucosa. The accumulating evidence in favor of LXs as anti-inflammatory eicosanoids in intestinal inflammation should provide the rationale for the evaluation of their efficacy and safety as potential therapeutic agent in IBD.

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