NO-mesalamine protects colonic epithelial cells against apoptotic damage induced by proinflammatory cytokines

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Fiorucci, Stefano, Eleonora Distrutti, Maureen N. Ajuebor, Andrea Mencarelli, Roberta Mannucci, Barbara Palazzetti, Piero Del Soldato, Antonio Morelli, and John L. Wallace. NO-mesalamine protects colonic epithelial cells against apoptotic damage induced by proinflammatory cytokines. Am J Physiol Gastrointest Liver Physiol 281: G654–G665, 2001.—The activation of a self-amplifying cascade of caspases, of which caspase-8 is the apical protease, mediates Fas-, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-, and TNF-α-induced apoptosis in colon cell lines. Nitric oxide (NO) protects from apoptosis induced by Fas and TNF-α. We examined whether NCX-456, an NO-releasing derivative of mesalamine, protects colonic epithelial cells from cytokine-induced apoptosis. Caco-2 and HT-29 cell lines express death factor receptors and are driven to apoptosis in response to incubation with Fas-agonistic antibody, TNF-α/interferon-γ, and TRAIL. The two novel observations reported here are that 1) cotreatment of cells with NCX-456, but not mesalamine, resulted in concentration-dependent protection against death factor-induced apoptosis and inhibition of caspase activity, and 2) exposure to dithiothreitol, an agent that effectively removes NO from thiol groups, resulted in a 76% recovery of caspase activity, which is consistent with S-nitrosation as a major mechanism for caspase inactivation. These data suggest that caspase S-nitrosation represents a mechanism for protection of colonic mucosal epithelial cells from death factor-induced death.

CROHN’S DISEASE and ulcerative colitis, the two main forms of inflammatory bowel disease (IBD), are chronic, spontaneously relapsing disorders that appear to be immunologically mediated (13). Although the underlying genetic and environmental causes remain to be elucidated, CD4+ T helper 1 (Th1) lymphocytes play a pivotal role in the pathogenesis of these diseases. A Th1-like phenotype, with its signature cytokines interferon (IFN)-γ and tumor necrosis factor (TNF)-α, is shared among many colitis models and is found in patients with active Crohn’s disease (6, 8, 13). The role of T cell-derived cytokines seems to be critical because IFN-γ and TNF-α drive colonic epithelial cells to apoptosis and injection of anti-TNF-α antibodies in patients with active Crohn’s disease leads to a transient improvement of the disease and attenuates the development of colitis in some experimental mouse models (13). Moreover, IFN-γ not only cooperates with TNF-α to cause colonic cell death, it also modulates epithelial Fas expression and sensitizes colonic epithelial cells to Fas-induced apoptosis (13).

Apoptosis, or programmed cell death, is regulated by tightly controlled intracellular signaling events in response to pathological cytotoxic stimuli including TNF-α, TNF-related apoptosis-inducing ligand (TRAIL), and Fas (3, 4, 37). Fas, a transmembrane receptor that belongs to the TNF-α receptor family, is constitutively expressed by the basolateral membrane of normal colon and small intestinal epithelium (6, 8, 16, 22, 36, 38). Fas ligation induces apoptosis in colonic epithelial cells and is implicated in the epithelial damage seen in ulcerative colitis (6, 8, 16, 22, 36, 38). The ligand for Fas (FasL) is expressed by intraepithelial and lamina propria lymphocytes, and its expression is increased in the lamina propria of ulcerative colitis patients, suggesting that Fas-FasL-induced apoptosis participates in the mucosal damage of ulcerative colitis (6, 8). Inhibition of cytokine-regulated apoptosis may therefore be useful in preventing or treating intestinal lesions in patients with IBD.

One way of interfering with the progression of disease is to reduce intracellular events that lead to apoptotic cell death. The intracellular domains of Fas, TRAIL receptors 1 and 2 (TRAIL-R1 and -R2), and TNF-α receptor 1 (TNF-R1) contain a region termed the death domain (DD), which is required for induction of apoptosis (4, 37). Binding of Fas by FasL, TRAIL-R1 or -R2 by TRAIL, and TNF-R1 by TNF-α leads to caspase activation and death of the cell.

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the association of Fas-associated DD (FADD) and TNF-R1-associated DD (TRADD), respectively, with Fas, TRAIL-R1 and R2, or TNF-R1 via their homologous DD (4, 37). In their turn, FADD and TRADD recruit the zymogen form of the apoptosis-initiating protease, caspase-8, a member of the interleukin 1 (IL)-β-converting enzyme (ICE) family (3, 37), through homophilic interaction of “death effector domains” leading to the assembly of a death-inducing signaling complex (DISC) at the cytoplasmic DD of Fas, TRAIL-R1, TRAIL-R2, or TNF-R1 (4). The proximity of caspase-8 zymogens facilitates activation through self-processing, leading to cleavage of downstream caspases including caspase-3, which is largely responsible, directly and indirectly, for dismantling the apoptotic cells from within (37). Inhibition of proapoptotic caspases is therefore a new pharmacological means of protection from death factor-induced apoptosis (11).

Mesalamine (5-aminosalicylic acid) is one of the most commonly used drugs for the treatment of active IBD and for maintenance of remission (34). However, the mechanism by which mesalamine reduces mucosal injury in colitis is not clear. Mesalamine exerts many effects that could contribute to its anti-inflammatory activity. These include inhibition of leukotriene synthesis, scavenging of oxygen-derived free radicals, scavenging of peroxynitrite, and inhibition of IL-1β synthesis (34). Mesalamine has also been demonstrated to reduce peroxynitrite-induced apoptosis in Caco-2 cells, but whether it is also active in protecting from cytokine-induced apoptosis is unknown (33).

In recent years, we (14, 15) and others (12, 39) have demonstrated that the addition of a nitric oxide (NO)-releasing moiety to conventional drugs, such as aspirin and other anti-inflammatory analgesic drugs, and more recently to acetaminophen, results in new chemical entities that share the property of releasing small amounts of NO at target tissues. An increasing body of evidence demonstrates that the addition of the NO-releasing moiety to these compounds markedly enhances their anti-inflammatory, antipyretic, and/or analgesic effects (14, 15, 39, 40). Although the molecular mechanisms underlying the enhanced anti-inflammatory properties of NO-releasing compounds are poorly defined, we demonstrated previously (14, 15) that, similarly to NO, they cause the nitrosation/inhibition of cysteine proteases that mediate inflammation (ICE/caspase-1) and apoptosis (caspases-9 and -3). On the basis of these findings we hypothesized that adding an NO-releasing moiety to mesalamine would increase its effectiveness in reducing colonic inflammation. Indeed, we reported (40) that an NO-mesalamine derivative (hereafter referred to as NCX-456) exhibited improved efficacy over mesalamine in a rat model of chemically induced colitis. Compared with mesalamine, NCX-456 was more effective in reducing inflammation and chemotaxin-induced leukocyte adherence and released NO when incubated with colonic mucosa fragments (40). However, NCX-456 failed to inhibit IFN-γ release in the inflamed colon, suggesting that it may protect colon cells by acting downstream to this cytokine. Because protection against cytokine-induced apoptosis/necrosis contributes to increased drug efficacy, the present study was designed to investigate whether NCX-456 protects colonic epithelial cells against apoptosis induced by Fas, IFN-γ, TRAIL, and TNF-α and to define the intracellular mechanism involved in this effect. Moreover, because it is still unknown whether colon cell lines express functionally active TRAIL receptors, we have evaluated the presence and functional activity of receptors for this death factor.

MATERIALS AND METHODS

Materials. RPMI-1640 medium, l-glutamine, EGTA, FCS, concanavalin A, aprotonin, leupeptin, dithiothreitol (DTT), HEPES, propidium iodide, BSA fraction V, phenylethylsulfonyl fluoride (PMSF), and mesalamine were from Sigma Chemical (St. Louis, MO). Recombinant human IFN-γ and TNF-α were from ICN Flow (Milan, Italy), whereas other media and serums were purchased from GIBCO (Paisley, UK). The anti-human Fas antibody (APO-1–3) and 2-phenyl-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO) were from Alexis (San Diego, CA). The anti-human caspase-8 antibody (p20 subunit) was from New England Biolab (Beverly, MA), and anti-human polyclonal cytochrome c (Cyt) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). TRAIL peptide was from Biomol (Plymouth Meeting, PA), and 4,5-diaminofluorescein diacetate (DAF-DA) was synthesized by Nicox (Nice, France).

Cell culture. HT-29 and Caco-2, two human colon adenocarcinoma cell lines, were obtained from American Type Culture Collection (Rockville, MD). HT-29 cells were cultured in RPMI-1640 medium containing 10% FCS, glutamine (2 mM), and antibiotics (50 U/ml penicillin, 50 μg/ml streptomycin) (1, 2). Caco-2 cells were cultured in MEM containing 10% FCS, glutamine, and antibiotics and supplemented with nonessential amino acids and 1 mM sodium pyruvate. Both types of cells were grown in 75-cm² culture flasks, and the medium was changed every other day. The cells were passaged weekly and, for experiments, were aliquoted into 96-well plates (10⁴ cells/well) and allowed to adhere overnight before cytokine stimulation in medium without serum.

Induction and detection of apoptosis. To test whether cytokines and death factors drive colon cells to apoptosis, HT-29 and Caco-2 cells were incubated with medium alone or IFN-γ (1,000 U/ml) and TNF-α (100 ng/ml) or 10 μg/ml of APO-1–3 monoclonal anti-human Fas antibody or human recombinant TRAIL (100 ng/ml) alone or in combination for 8 h. In experiments in which IFN-γ and Fas monoclonal antibody were used to induce apoptosis, cells were preincubated for 3 h with IFN-γ and then Fas monoclonal antibody was added. To test whether NCX-456 and mesalamine modulate cytokine-induced apoptosis, cells were incubated with the cytokine mixture (i.e., IFN-γ + Fas agonistic monoclonal antibody or IFN-γ + TNF-α at the concentration described) with or without 1–100 μM NCX-456 or mesalamine for 8 h. In some experiments, cells were incubated with the NO scavenger PTIO (100 μM) to assess whether NO scavenging reversed the protective effect of NCX-456 on cytokine-induced cell death (3, 4). At the end of the culture period, adherent cells were detached and mixed with floating cells by gentle centrifugation. Apoptosis was detected by staining the cells with propidium iodide (PI). Briefly, cell pellets were
washed twice in PBS, resuspended in hypotonic fluorochrome solution (50 μg/ml PI in 0.1% sodium citrate + 0.1% Triton X-100), kept 4–8 h at 4°C in the dark, and analyzed using a Epics XL flow cytometer (Beckman-Coulter, Miami, FL). The percentage of apoptotic cells was determined by evaluating hypodiploid nuclei after proper gating on DNA content. Alternatively, cells were recovered from cultures (both floating and adherent), fixed with 3.7% paraformaldehyde-PBS, and stained with 0.1 μg/ml 4,6-diamino-2-phenylindole (DAPI). The percentage of cells with condensed chromatin and fragmented nuclei was determined by ultraviolet (UV) microscopy (14). Each experiment was performed in triplicate on separate days.

Detection of apoptosis by ELISA assay. ELISA is based on the photometric sandwich-enzyme-immunoassay principle and uses mouse monoclonal antibodies directed against cytosolic DNA fragments and histones. Briefly, three incubation steps were performed. First, the antihistone antibody was fixed on the wall of a microtiter plate. Second, the nucleosomes contained in the sample were bound via their histones to the antihistone antibody. Third, the anti-DNA-proteinase was added to react with the DNA part of the nucleosome. The amount of peroxidase retained in the sample was determined photometrically (absorbance at 405/490 nm) with some. The amount of peroxidase retained in the sample was determined by ultraviolet (UV) microspectrophotometer.

DNA fragmentation assay. In some experiments apoptosis was also assessed by DNA ladder assay, as previously described (14). Briefly, the floating and adherent cells collected and sedimented by centrifugation. Washed cell pellets were resuspended in cell lysis buffer [10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0), 0.5% Triton X-100] and incubated. RNase A (0.5 mg/ml) and proteinase K (5 mg/ml) were added, respectively, and incubated for 2 h. DNA was precipitated with ethanol, and 3 μg (or 106 cells) of water-diluted sample was run on a 2% agarose gel. Gels were stained with ethidium bromide, and DNA was visualized by UV transilluminator. RT-PCR. Total RNA was isolated from human biopsies using TRIzol reagent (Life Technologies, Milan, Italy). First-strand cDNA was synthesized from total cellular RNA (1 μg) with 200 U of SuperScript II (Life Technologies) in 50 μl of oligo(dT)12 primer (Sigma Genosys, Gallarate, Italy) and dNTPs (200 μM; Promega). Ten microliters of sample and microtiter plate was added to the DNA part of the nucleosome. The amount of peroxidase retained in the sample was determined photometrically (absorbance at 405/490 nm) with some. The amount of peroxidase retained in the sample was determined by ultraviolet (UV) microspectrophotometer.

Analysis of caspase activity. After incubation with appropriate agents (see Fig. 2), HT-29 and Caco-2 cells were recovered into lysis buffer [100 mM Tris-HCl (pH 7.3), 25 mM NaCl, 0.2% Triton X-100, 1 mM EDTA]. After centrifugation at 10,000 g for 30 min, the resulting supernatants were adjusted to 1 mg/ml with lysis buffer and 25 μg total protein was incubated in 100 μl of caspase buffer [50 mM HEPES (pH 7.2), 100 mM NaCl, 1 mM EDTA (pH 8.0), 10% sucrose, 0.1% 3-(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), and 1 mM DTT] with various fluorogenic substrate peptides (100 μM) including acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethyl-coumarin) (Ac-DEVD-AFC) for caspase-3, Ac-Asp-Glu-Thr-Asp-AFC (Ac-IETD-AFC) for caspase-8, and Ac-Leu-Glu-His-Asp-AFC (Ac-LEHD-AFC) for caspase-9. Caspase-8, -3, and -9 activity was assayed for caspase-8, Ac-DEVD-AFC substrate peptides (100 μM) including acetyl-Asp-Glu-Val-Asp-(7-amino-4-trifluoromethyl-coumarin) (Ac-DEVD-AFC) for caspase-3, Ac-Asp-Glu-Thr-Asp-AFC (Ac-IETD-AFC) for caspase-8, and Ac-Leu-Glu-His-Asp-AFC (Ac-LEHD-AFC) for caspase-9. Caspase-8, -3, -9 activity was assayed using a fluorimeter plate reader in kinetic mode with excitation and emission wavelengths of 405 and 519 nm, respectively. Continuously measuring release of AFC from substrate peptides was as described (14, 15).

Western blot analysis of caspase-8 cleavage. Cells (1×107) were lysed in 100 μl of lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 40 μg/ml aprotinin, 20 μg/ml leupeptin] at 4°C. Ten microliters of sample were run on 8–16% linear gradient polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA). Gels were transferred to nitrocellulose membrane (Hybond-C Extra, Amersham). Membranes were blocked in 5% milk powder in PBS and probed with either anti-Flice antibody (M2, Sigma Chemical) or a rabbit polyclonal antiboxase 8 antibody, both at dilutions of 1:1,000. The secondary antibody was goat anti-mouse IgG horseradish peroxidase-conjugated antibody (PharMingen, San Diego, CA) used at a dilution of 1:1,000.

Analysis of Cyt c release. Caco-2 cells were scraped off in isotonic isolation buffer (in mM: 1 EDTA, 10 HEPES, 250 sucrose, pH 7.6), collected by centrifugation at 2,500 g for 5 min at 4°C, and resuspended in hypotonic isolation buffer (in mM: 1 EDTA, 10 HEPES, 50 sucrose, pH 7.6). Cells were then incubated at 37°C for 6 h, and homogenized under a Teflon pestle. Hypertonic isolation buffer (in mM: 1 EDTA, 10 HEPES, 450 sucrose, pH 7.6) was added to balance the buffer’s toxicity. Samples were centrifuged at 2,000 g for 5
min at 4°C. Supernatants were recovered and centrifuged again at 10,000 g for 10 min. The pellet contained the mitochondrial fraction, which was resuspended in isotonic isolation buffer, whereas the supernatant contained cytosolic proteins. Protein concentrations in pellets and supernatants were determined using the Bio-Rad protein assay kit according to the manufacturer’s specifications. After electrophoresis separation of 50 μg of protein in sodium dodecyl sulfate-12% polyacrylamide, gels were transferred by semidyey transfer (Bio-Rad) to nitrocellulose membranes. Immunoblots were blocked in TTBS (10 mM Tris·HCl, 150 mM NaCl (pH 7.5), 0.05% Tween 20) containing 5% nonfat dried milk and incubated overnight with the anti-Cyt c antibody (diluted 1:1,000 in TTBS with 0.5% nonfat dried milk). After being washed, membranes were incubated with peroxidase-conjugated secondary antibody (1:5,000 in TTBS with 0.5% nonfat dried milk) for 2 h, and the blot was developed with the ECL system (ECL Western blotting kit, Amersham International).

Detection of intracellular NO formation. To assess whether colon cells generate intracellular NO or NO-derived compounds from NCX-456, adherent Caco-2 cells were incubated with 100 μM NCX-456, mesalamine, or S-nitroso-N-acetylpenicillamine (SNAP) and intracellular NO formation was assessed according to the method of Nakatsubo et al. (30) with DAF-DA. Briefly, adherent cells (1 × 10^6/ml) were preincubated with 1 mM L-N6-(1-iminoethyl)lysine (L-NIL) for 30 min to suppress endogenous NO generation and then loaded by incubating them in PBS in the presence of 10 μM DAF-DA at 37°C for 30 min. Cells were then washed three times with PBS and placed on a confocal microscope (Bio-Rad 1024, 37°C with a Hitachi 2000 (Hitachi, Milan, Italy) fluorescence stirring and temperature thermostatically maintained at 37°C with a Hitachi 2000 (Hitachi, Milan, Italy) fluorescence spectrophotometer. SNAP, NCX-456, or mesalamine (100 μM) was then added to the cell suspension. Cells were excited at 395-nm wavelengths with a 10-nm slit, and the intensity of fluorescence emitted at 515 nm was recorded. NO generation was expressed in arbitrary units of absorbance (16).

Nitrite/nitrate assay. Nitrite/nitrate concentrations in cell supernatants were measured by a fluorometric detection kit (Cayman Chemical, Ann Arbor, MI). The lower detection limit, as reported by the manufacturer, was ~4 pM/well (16). Assessment of caspase S-nitrosation. To investigate whether the inhibition of caspase-like proteases exerted by NCX-456 was caused by protein S-nitrosation, cell lysates obtained from Caco-2 cells were incubated alone or with a cytokine mixture in combination with 100 μM NCX-456, mesalamine, or SNAP and were exposed to DTT (20 mM) to remove thiol-bound NO. The DTT and excess of NO were then removed by passing the sample through a Sephadex G-25 column preequilibrated with the lysis buffer and enzyme activity was assessed (14–16, 24–28). In another set of experiments, lysates obtained from Caco-2 cells incubated with Fas or IFN-γTNF-α alone or in combination with NCX-456 were exposed to DTT (20 mM) and/or HgCl2 (5 mM) for 40 min on ice and caspase-8-like activity was measured (14–16, 24–28).

Data analysis. All values are expressed as means ± SE of n observations. Groups of data were compared using a one-way analysis of variance followed by a Student-Newman-Keuls test. With all analyses, an associated P value of <5% was considered significant (5).

RESULTS

Death receptor expression on colon cancer lines. We first investigated whether HT-29 and Caco-2 cells express death receptors. As illustrated in Fig. 1, RT-PCR analysis demonstrated that Fas and TNF-R1 are expressed in both cell lines, although to a different extent, whereas TRAIL was not expressed. Both cell lines also expressed FLICE (caspase-8). As illustrated in Fig. 1B, both colon cancer cell lines expressed TRAIL receptors (R1–R4). Incubation of the cells with IFN-γ markedly upregulated Fas mRNA expression in both cell lines, although it had no effect on TNF and TRAIL receptors (data not shown). Thus colon cell lines express death receptors.

Cytokines and Fas drive colon epithelial cells to apoptosis and require caspase activation. Incubation of growth-arrested HT-29 and Caco-2 monolayers with a combination of IFN-γ (1000 U/ml) and TNF-α (100 ng/ml) or Fas-agonistic monoclonal antibody for 8 h resulted in a 30–35% increase in the expression of

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**Fig. 1.** RT-PCR analysis of death factor receptor expression on colon cancer cell lines. A: multiple RT-PCR demonstrating the expression of Fas, FLICE (caspase-8), tumor necrosis factor (TNF)-α receptor 1 (TNF-R1), and TNF-related apoptosis-inducing ligand (TRAIL) on Caco-2 and HT-29 colon cancer cell lines. Lane 1, negative control; lane 2, positive control; lane 3, HT-29 cells; lane 4, Caco-2 cells. The blot is representative of at least 3 RT-PCR analyses. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. B: Caco-2 cells express TRAIL receptors. For each blot: lane 1, molecular markers; lane 2, negative control; lane 3, positive control; lane 4, Caco-2 cells. Each blot is representative of at least 3 RT-PCR analyses.
apoptotic markers, as assessed by PI incorporation, nuclei staining with DAPI, DNA fragmentation, and DNA-histone association (Figs. 2 and 3). In contrast, treatment with individual cytokines or a Fas-agonistic monoclonal antibody alone did not increase cell death above basal levels (Fig. 2). However, there were differences in the sensitivity of the two cell lines to apoptosis induced by the cytokine mixture, because HT-29 cells were sensitive to Fas- and TNF-α-induced apoptosis and Caco-2 cells were resistant to apoptosis induced by the Fas-agonistic monoclonal antibody. In contrast, both cell lines were sensitive to TRAIL-induced apoptosis (Fig. 2, B and C). In both cell lines morphological changes suggestive of apoptosis were associated with an increased activity of caspase-8 like proteases (Fig. 2C) as well as caspase-3- and -9-like proteases (data not shown). As shown in Fig. 2D, pretreatment of the cells with 100 μM Z-VAD-FMK, a pancaspase inhibitor, rescued both cell lines from apoptosis induced by IFN-γ/TNF-α, IFN-γ/Fas-agonistic antibody, and TRAIL and caused an ~80% reduction of caspase-8 activation caused by proapoptotic agents. Thus caspase activation is required for apoptotic signal transduction in colon cell lines challenged with TNF-α or Fas in the presence of IFN-γ and TRAIL.

NCX-456 protects colon epithelial cells against cytokine-induced apoptosis. Exposure of HT-29 and Caco-2 cells to NCX-456, but not mesalamine, protected against cytokine-induced cell death (Figs. 3 and 4). Indeed, at a concentration of 100 μM, the NO-mesalamine derivative caused ~80% reduction of death factor-induced lethality (*P < 0.001 vs. cytokine alone) as measured by PI incorporation. Mesalamine (100 μM) also caused ~30% reduction of apoptosis caused by a combination of IFN-γ with Fas or TNF-α, although it was significantly less effective than NCX-456 (*P < 0.001). As shown in Fig. 4, the protective effect exerted by NCX-456 was concentration dependent and lasted for at least 24 h. Protection exerted by NCX-456 was detectable at a concentration of 1 μM, half-maximal at 6 μM, and maximal at 100 μM. Coincubation of Caco-2 cells with NCX-456 (100 μM) was also effective in preventing changes of nuclear morphology as evaluated by DAPI staining and DNA fragmentation as assessed by DNA ladder assay and DNA-histone formation (Fig. 3). Moreover, as shown in Fig. 3E, NCX-
NCX-456 modulates caspase activity. The association between the suppression of cytokine-induced apoptosis and caspase inhibition suggests that NCX-456 inhibits apoptosis by blocking caspase activation. Because there is evidence that NO-releasing compounds inhibit caspase activities by causing enzyme nitrosation, we next investigated biochemically whether exposure to NCX-456 results in caspase-8 cleavage (Fig. 6D). Thus NCX-456 protects colon epithelial cell against cytokine-induced apoptosis by inhibiting proapoptotic caspases.

NCX-456 inhibits caspase activity by nitrosation. After treatment with IFN-γ/TNF-α, procaspase-8 was cleaved in Caco-2 and HT-29 cells. Cotreatment of the cells with NCX-456, but not with mesalamine, almost completely abolished procaspase-8 cleavage (Fig. 6D). This NCX-456 protects colon epithelial cell against cytokine-induced apoptosis by inhibiting proapoptotic caspases.

456 inhibited cytokine-induced Cyt c release from mitochondria. Mesalamine was significantly less effective than NCX-456 in reducing these markers of apoptosis (Fig. 3), and at a concentration of 100 μM it had no effect on DNA fragmentation or Cyt c release.

Inhibition of apoptosis caused by NCX-456 is mediated by an NO-dependent mechanism. To gain insight on the mechanism responsible for apoptosis inhibition, we then evaluated whether incubating the cells with an NO scavenger reversed the protection exerted by NCX-456. As shown in Fig. 5, the ability of NCX-456 to rescue cells from death induced by IFN-γ/TNF-α, IFN-γ/FasL, and TRAIL was significantly reduced when the NO scavenger PTIO (100 μM) was added to the incubation medium. Thus NCX-456 acts through an NO-dependent pathway.

NCX-456 inhibits caspase activity. Because activation of the caspase cascade is pivotal to the death execution phase of apoptosis, we tested the effect of NCX-456 on proapoptotic caspases. As illustrated in Fig. 6, incubation of Caco-2 (Fig. 6, A–C) and HT-29 (data not shown) cells with 100 μM NCX-456 resulted in a 70–80% reduction of cytokine-induced caspase-8, -9, and -3 activity. To confirm that NCX-456 directly inhibits caspase-8 activation, we examined the cleavage of the procaspase-8 zymogen by Western blotting with an antibody that specifically detects the p20 subunit of this caspase. After treatment with IFN-γ/TNF-α, procaspase-8 was cleaved in Caco-2 and HT-29 cells. Cotreatment of the cells with NCX-456, but not with mesalamine, almost completely abolished procaspase-8 cleavage (Fig. 6D). Thus NCX-456 protects colon epithelial cell against cytokine-induced apoptosis by inhibiting proapoptotic caspases.

NCX-456 inhibits caspase activity by nitrosation.
from cytokine-treated cells with HgCl₂, an agent that binds thiol groups and removes NO. Indeed, at a concentration of 5 mM, HgCl₂ caused a 90% loss of proteolytic activity (Fig. 7B). Inhibition induced by HgCl₂ was partially reversed by DTT. Thus NCX-456 causes caspase nitrosation.

NCX-456 causes intracellular NO formation. Because these data indicate that NCX-456-derived NO was responsible for the antiapoptotic effect exerted by NCX-456, we next investigated whether incubation of Caco-2 cells with this compound results in intracellular NO formation. As shown in Fig. 8, exposure of DAF-DA-loaded cells to NCX-456 (100 μM) resulted in a time-dependent increase in intracellular fluorescence, suggesting that NCX-456 penetrates colon cell membranes and is metabolized to release NO. The cell metabolism, however, is slow, because maximal fluorescence was evident after 3 h of incubation. In contrast to NCX-456, the addition of SNAP caused a rapid increase in intracellular fluorescence (Fig. 8D). No changes in intracellular fluorescence were documented in cells incubated with mesalamine alone (data not shown). To confirm these findings by another means, we measured intracellular NO fluorescence in nonadherent Caco-2 cells incubated with 100 μM NCX-456, SNAP, or mesalamine. As illustrated in Fig. 8E, although exposure of the DAF-DA-loaded cells to SNAP resulted in a rapid rise of intracellular fluorescence, NCX-456 caused a slow but sustained increase of intracellular fluorescence. Again, no changes were measured in cells loaded with mesalamine. In confirmation of these findings, incubation of the cells with SNAP or NCX-456 resulted in a significant increase in nitrite/nitrate release (Fig. 8F). Exposure of Caco-2 cells to 100 μM NCX-456 resulted in a high output of nitrite/nitrate in cell supernatants (~150 μM/10⁷ cells at 3 h of incubation). No changes in nitrite/nitrate production were observed in cells incubated with mesalamine alone (P < 0.001 compared with NCX-456). Thus NCX-456 is metabolized by colon epithelial cells to release intracellular NO.

**DISCUSSION**

T lymphocyte-derived cytokines not only recruit inflammatory cells at the site of inflammation but directly kill colonic epithelial cells or sensitize them to death induced by death factors such as TNF-α and FasL. TNF-α and FasL are expressed by intraepithelial and lamina propria lymphocytes, and their expression is increased in the lamina propria of ulcerative colitis patients (6, 8, 13). The effects of T cell-derived
cytokines on cultured intestinal epithelial cells have also been well documented because human lamina propria T lymphocytes produce IFN-γ and TNF-α, which act in concert to kill colon epithelial cells. The effects of IFN-γ on colon epithelial cells may have clinical relevance, as this cytokine has been implicated in a variety of inflammatory conditions including ulcerative colitis, Crohn’s disease, and human immunodeficiency virus-related enterocolitis (13). Indeed, biological agents that inhibit TNF-α and/or IFN-γ production (IL-10 and thalidomide) or TNF-α activity (infliximab) effectively reduce inflammation in IBD patients (31, 34). In the present study we demonstrated that exposure of two cell lines, which express different stages of epithelial cell maturation, to IFN-γ sensitizes them to the proapoptotic/necrotic effect of a Fas-agonistic monoclonal antibody and TNF-α and that this effect is mediated by activation of the caspase cascade. However, our results demonstrated that Caco-2 and HT-29 cells have different sensitivities to the apoptosis induced by death factors, because Caco-2 cells, in contrast to HT-29 cells, are resistant to Fas-mediated apoptosis (1, 2). In line with this finding, it was demonstrated previously (1, 2) that Caco-2 cells not only express very low amounts of Fas but lack one or more presently unidentified factor(s) necessary to activate the chain of reactions that leads to caspase-8 recruitment. In the present study we also demonstrated for the first time that colon cancer lines express TRAIL receptors and are sensitive to TRAIL-induced apoptosis (4). These cells are therefore a useful model for the study of the effect of drugs on cytokine-regulated apoptosis in colonic mucosa.

NCX-456 is a derivative of mesalamine that consists of the parent drug linked to an NO-releasing moiety through an ester linkage. In a previous study (40) we demonstrated that this compound was significantly more effective than mesalamine in reducing colonic inflammation in a rat model of colitis. In the present study we have extended these observations by demonstrating that NCX-456 directly protects IFN-γ-sensitized colon epithelial cells from apoptotic/necrotic death induced by TNF-α or Fas-agonistic monoclonal antibodies. In 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), a NO scavenger, reverses protection exerted by NCX-456.

**Fig. 5.** 2-Phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (PTIO), a NO scavenger, reverses protection exerted by NCX-456. A: effect of PTIO on protection exerted by NCX-456 against cell death induced by FasL and TNF-α in IFN-γ-treated cells. Cell death was assessed by PI staining (see MATERIALS AND METHODS). Data are means ± SE of 6 experiments. *P < 0.01 vs. control cells; **P < 0.01 vs. cells treated with cytokine alone; ***P < 0.01 vs. cells treated with cytokine + NCX-456. B: PTIO reverses protection exerted by NCX-456 against TRAIL-induced apoptosis. Cell death was assessed by PI staining. Data are means ± SE of 6 experiments. *P < 0.01 vs. control cells; **P < 0.01 vs. cells treated with TRAIL alone; ***P < 0.01 vs. cells treated with TRAIL + NCX-456.

NCX-456 inhibits caspase activation. A–C: NCX-456 (100 μM) inhibits caspase activation in HT-29 cells. Data are means ± SE of 6 experiments. *P < 0.01 vs. control cells; **P < 0.01 vs. cells treated with cytokine mixture. D: NCX-456, but not mesalamine, inhibits procaspase-8 cleavage. Western blot analysis of procaspase-8 cleavage induced by IFN-γ and Fas is shown. lane 1, control cells; lane 2, cells treated with cytokine mixture; lane 3, cells treated with cytokine + NCX-456; lane 4, cells treated with cytokine + mesalamine. Figure is representative of 3 others.
NCX-456 MODULATES CASPASE ACTIVITY

Fig. 7. NCX-456 causes caspase-8 S-nitrosation. A: dithiothreitol (DTT), an agent that removes thiol-bound nitric oxide (NO), causes a 70% recovery of caspase-8 activity in HT-29 cell lysates. Cells were treated with the cytokine mixture alone or cytokine + 100 μM NCX-456, mesalamine, or S-nitroso-DL-penicillamine (SNAP). After 8 h of incubation, cell lysates were prepared and incubated with 20 mM DTT. Data are means ± SE of 6 experiments. **P < 0.01 vs. control; ***P < 0.01 vs. cytokine mixture + NCX-456; ****P < 0.01. NCX-456 or SNAP vs. cells treated with cytokine mixture.

B: SNAP causes caspase-8 S-nitrosation. Data are means ± SE of 6 experiments. **P < 0.01 vs. control cells; ***P < 0.01, DTT vs. cytokine mixture + SNAP; ****P < 0.01, SNAP-treated cells vs. cells treated with cytokine mixture.

antibody by causing the S-nitrosation/inhibition of pro-apoptotic caspases. Activation of the caspase cascade appears to be essential to cytokine-induced apoptosis of HT-29 and Caco-2 cells, in light of our observation that pretreatment with the pan caspase inhibitor Z-VAD-FMK completely prevents apoptosis induced by TNF-α and Fas (Fig. 2). Our current understanding of caspase involvement in Fas/TNF-α-induced apoptosis indicates that Fas and/or TNF-R1 cross-linking leads to the activation of a self-amplifying cascade of caspases, of which caspase-8 is the apical protease (37). This particular caspase is recruited and probably auto-proteolytically activated through protein-protein interaction with FADD among a complex of proteins (DISC) that dock onto the cytoplasmic DD of oligomerized Fas. Activated caspase-8 releases active caspase-8 subunits that can then cleave other caspases (e.g., caspase-3), which can in turn cleave a large number of intracellular death substrates. In the present study, we demonstrated that, in contrast to mesalamine, NCX-456 prevents caspase activation in Fas- and TNF-α-challenged cells and that this effect is mainly mediated by protein nitrosation/inhibition. Supporting this view, we demonstrated that 1) caspases-8,-3, and -9 are inhibited in NCX-456-treated cells but not in cells treated with mesalamine, which suggests that the NO group of NCX-456 is responsible for this effect; 2) exposure to PTIO, an NO scavenger, reversed protection exerted by NCX-456 on cytokine-induced apoptosis (Fig. 5); 3) exposure of NCX-456-treated cells to DTT, an agent that effectively removes NO from thiol groups, resulted in a 70% recovery of caspase activity, which is consistent with nitrosation as a major mechanism for caspase inhibition; 4) HgCl2, which binds thiol groups, caused a DTT-reversible inhibition of caspase activity, again suggesting a role for protein nitrosation in caspase inhibition (14–16, 24–26); 5) similarly to NCX-456, the NO donor SNAP inhibited caspase activity and rescued cells from death induced by Fas and TNF-α; and 6) incubation of nontreated Caco-2 cells with NCX-456 resulted in a time- and concentration-dependent increase in intracellular NO as detected by intracellular fluorescence in adherent and nonadherent cells and nitrite/nitrate in cell supernatants. In contrast to NCX-456, mesalamine did not cause NO formation and was significantly less effective than NCX-456 in protecting from apoptosis caused by Fas and TNF-α (33). Together these data suggest that NO released from the nitroxy moiety of NCX-456 is responsible for caspase inhibition in this experimental setting (7, 29).

NO has emerged as a potent inhibitor of apoptosis in many cell types, and we and others (9, 15, 24–26) have previously demonstrated that a dominant mechanism for apoptosis prevention is the inhibition of caspase activity by nitrosation. NO-dependent caspase inhibition was demonstrated previously to prevent Bel-2 cleavage and Cyt c release as well as activation of proteases that become activated after the release of Cyt c in hepatocytes and other cell types (24–27). Because of the capacity of NO to rapidly diffuse intra-cellularly and from cell to cell, this would represent an efficient mechanism to guard against the consequences of the activation of caspases, which might result from cell injury or exposure to other activators such as IFN-γ, TNF-α, and FasL in the local environment (6, 7, 10, 13, 18, 21). Caspases are likely targets for nitrosation because their catalytic mechanisms require a redox-sensitive cysteine residue in the catalytic core (9). Previous studies carried out with purified subunits of caspases-1 and -3 demonstrated that the p17 subunit of caspase-3 and the p20 subunit of caspase-1 are selective targets for NO compounds and that the nitrosation of these subunits leads to a concentration-dependent inhibition of enzyme activity (9, 27, 28). More generally, there is now evidence that nitrosation is a mechanism that is extensively involved in caspase regulation (10, 19, 24–28). A recent report from Mannick et al. (27) indicates that in resting human cell lines caspase-3 zymogens are nitrosated and denitrosated on Fas/FasL cross-linking, indicating that caspase activa-
tion requires both denitrosation and zymogen cleavage. Although the reversal of NO-mediated inhibition by DTT is consistent with nitrosation being the main mechanism for caspase inhibition by NCX-456 and our results demonstrated NO formation in colon cell lines incubated with this compound, NO radical does not effectively nitrosate thiol groups, suggesting that a NO reaction product is implicated in caspase nitrosation (10, 17). NO\textsuperscript{1} equivalent nitrosates thiols and mimics the effect of NO donors when added to cells (10). Reactive nitrogen oxide species, including NO\textsuperscript{+} and its equivalents, can be generated by the reaction of NO with O\textsubscript{2} or iron-sulfur clusters (17). These complexes have been shown to carry out transnitrosative reactions (10). In our in vitro experiments, the cells were grown under aerobic conditions and the interaction of NO with O\textsubscript{2} could form N\textsubscript{2}O\textsubscript{3}, which has been shown to cause nitrosation via the formation of NO\textsuperscript{+} equivalents.

The failure of DTT treatment to induce a full recovery of all caspase-8 activity, however, raises the possibility that NO may also suppress caspase-8 activation (27). Supporting this concept, NCX-456 markedly reduced the amount of the caspase-8 p17 subunit released under Fas cross-linking (Fig. 6). Indeed, because caspase-8 activation is partially due to the autocatalytic cleavage of the inactive proenzyme, it cannot be excluded that S-nitrosation/inhibition of caspase-8 reduces the amount of active enzyme that is further generated through this pathway (19, 37). However, because caspase-8 may be activated via caspase-6 and NCX-456 behaves as a pancaspase inhibitor, it cannot be excluded that the NO-mesalamine derivative acts on multiple points of the caspase pathway (26).

In summary, we demonstrated that in contrast to mesalamine, NCX-456, an NO-mesalamine derivative, protects IFN-\gamma-sensitized colon epithelial cells from death induced by TNF-\alpha and Fas. The ability of NCX-456 to interfere with TNF-\alpha-mediated signals may be of clinical relevance, considering that anti-TNF-\alpha therapies effectively reduce inflammation in IBD patients. The ability of NCX-456 to prevent caspase activation...
may contribute to further downregulation of TNF-α signals in the inflamed mucosa and may hold some promise for biological therapies to attenuate T cell-dependent inflammation in IBD patients (11).

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