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

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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 to the mesenteric endothelium 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, aprotinin, leupeptin, dithiothreitol (DTT), HEPES, propidium iodide, BSA fraction V, phenylmethylsulfonyl 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 mediased serum 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 cytokrome c (Cyt c) 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 from Calbiochem (Darmstadt, Germany). NO-mesalamine (NCX-456, 5-amino-2-hydroxybenzoic acid 4-(nitroxybutyl)ester) 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 20% 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 an 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-diamidino-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 histone binding to the anti-histone antibody. Third, anti-DNA-precipitating antibody 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 2,2-azino-di-(3-ethylbenzthiazoline) sulfonate) as a substrate (14, 15). Assays were performed using a cell death detection ELISA kit following the recommendations of the manufacturer (Boehringer Mannheim, Indianapolis, IN). DNA fragmentation assay. In some experiments apoptosis was also assessed by DNA laddering assay, as previously described (14). Briefly, the floating cells and detached cells were 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 μg/ml) and proteinase K (0.5 μg/ml) were added, were resuspended, and were incubated for 2 h. DNA was precipitated by ethanol, and 3 μg (or 1-106 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), 500 μM of oligo(dT)18 primer (Sigma Genosys, Gallarate, Italy), and dNTPs (200 μM, Promega). Ten microliters of sample and two microliters of oligo(dT) primer were heated to 94°C for 2 min and cooled on ice for 5 min. To this mixture was added 4 two microliters of oligo(dT) primer were heated to 94°C for 2 min. One microliter of SuperScript II then added, and the reaction was incubated at 42°C for 2 min, heated at 70°C for 10 min, followed by 30 cycles of 94°C for 15 s, 55°C for 30 s, and 72°C for 30 s. One microliter of SuperScript II then added, and the reaction was incubated at 42°C for 2 min, heated at 70°C for 10 min, inactivate the enzyme, and cooled at 4°C. Multiplex PCR was performed with Human Apoptosis Genes Set-3 kit (Maxim Biotech, San Francisco, CA) using human Fas gene, human Fas ligand gene, human TRAIL gene, human FLICE gene (caspase-8), and human GAPDH gene as a control. PCR was performed using specific primers (Sigma Genosys). For human TRAIL R1 the sense primer was 5'-GGCACGACATCGCAAACAGATT-3' and antisense was 5'-TTTCCACAGTGGGATTGGCACC-3'; for human TRAIL R2 the sense primer was 5'-CAAATACACCGCAGTTGCGCC-3' and antisense was 5'-GTCGAGGCACGCCAGAAGAACAG-3'; for human TRAIL R3 the sense primer was 5'-GGCACGATCTCGAAACAGAGT-3' and antisense was 5'-GGCAGGATCGACGACGACTT-3'; for human TRAIL R4 the sense primer was 5'-TGGGACTTTCGGGCGAACAAAGGGT-3' and antisense was 5'-GCCGACACCGCCGACGATG-3'. The cDNA was amplified with a “hot start” reaction in a 20-μl reaction containing 5 μl of cDNA product, 2 μl of PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 200 μM dNTPs, 1 μM sense and antisense primers, 1.5 mM MgCl2, 1 U of Platinum Taq polymerase (Life Technologies), and water in a Hybaid PCR Sprint thermocycler (Celbio, Milan, Italy). PCR was carried out for 35 cycles (30 min for amplification of β-actin) as follows: 94°C for 30 s, 60°C for 15 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. Multiplex PCR was carried out for 35 cycles as follows: 94°C for 1 min and 60°C for 1 min with a final extension at 72°C for 10 min. PCR products were then separated on 1.5% agarose gel and stained with 0.5 μg/ml ethidium bromide. The size of PCR products was assessed by comparison with 1 μg of 100-bp DNA ladder (Life Technologies). The gel was photographed under UV transillumination with a Kodak Digital Science ID Image Analysis Software (Kodak, Rochester, NY); images were then digitalized and a semiquantitative densitometric analysis performed. Each PCR assay was carried out in triplicate. The β-actin primers were used as a control for both reverse transcription and the PCR reaction itself and also for comparing the amount of products from samples obtained with the same primer. Assessment of caspase activity. After incubation with appropriate agents (see Fig. 2) HT-29 and Caco-2 cells were recovered into lysis buffer [10 mM Tris-HCl (pH 7.3), 25 mM NaCl, 0.25% Triton X-100, 1 mM EDTA]. After centrifugation at 16,000 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) 10 mM NaCl, 1 mM EDTA (pH 8.0), 10% sucrose, 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate (CHAPS), and 1 mM DTT] with various fluorogenic substrate peptides (100 μM) including acetyl-Asp-Glu-Val-Asp (z-VAD-fmk) and acetyl-Ile-Glu-Val-Asp (z-IETD-fmk) for caspase-8, and Ac-Asp-Glu-Thr-Asp-AFC (Ac-DEVD-AFC) for caspase-9, Ac-Asp-Glu-Thr-Asp-AFC (Ac-LEHD-AFC) for caspase-8, -3, -9 and -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 as previously 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 then 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 anti-caspase-8 antibody, both at dilutions of 1:1000. 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 scratched 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 5 min 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 tonicity. Samples were centrifuged at 2,000 g for 5
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-acetylpralamin (SNAP) and intracellular NO formation was assessed according to the method of Nakatsubo et al. (30) with DAF-DA. Briefly, adherent cells (1 × 10⁶/ml) were preincubated with 1 mM L-Nω-(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, Bio-Rad, Milan, Italy), and images were taken every 5 s 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 fluorimetric 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-γ/TNF-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...
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-\(\alpha\)-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 \(\mu\)M Z-VAD-FMK, a pancaspase inhibitor, rescued both cell lines from apoptosis induced by IFN-\(\gamma\)/TNF-\(\alpha\), IFN-\(\gamma\)/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-\(\alpha\) or Fas in the presence of IFN-\(\gamma\) 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 \(\mu\)M, 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 \(\mu\)M) also caused \(~30\%\) reduction of apoptosis caused by a combination of IFN-\(\gamma\) with Fas or TNF-\(\alpha\), 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 \(\mu\)M, half-maximal at 6 \(\mu\)M, and maximal at 100 \(\mu\)M. Coincubation of Caco-2 cells with NCX-456 (100 \(\mu\)M) 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

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. 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 nitrosation/inhibition. To ascertain this point, lysates obtained from cytokine-treated cells were incubated with 20 mM DTT, an agent that effectively displaces thiol-bound group from proteins. As shown in Fig. 7, exposure to DTT resulted in ~70% recovery of protease activity in lysates obtained from cells incubated with cytokine plus NCX-456. Confirming the role of NO in the inhibition of caspase-8, SNAP (100 μM) caused 80% reduction of caspase-8-like activity induced by TNF-α and IFN-γ (Fig. 7A), an effect that was significantly reduced by DTT (P < 0.01). Caspase-8-like activity was also reduced by incubating lysates obtained...
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

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**Fig. 5.** 2-Pheny1-4,4,5,5-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 cytokine mixture.

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**Fig. 6.** 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 pro-caspase-8 cleavage. Western blot analysis of pro-caspase-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 + 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, DTT 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) H2ClO2, 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 nitrate/nitrite 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 Bcl-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 intracellularly 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-3zymogens are nitrosated and denitrosated on Fas/FasL cross-linking, indicating that caspase activation...
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 (17). NO\textsuperscript{+} equivalent nitrosates thiols and mimics the effect of NO donors when added to cells (10).

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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