NF-κB activation and susceptibility to apoptosis after polyamine depletion in intestinal epithelial cells

Li, Li, Jaladanki N. Rao, Barbara L. Bass, and Jian-Ying Wang. NF-κB activation and susceptibility to apoptosis after polyamine depletion in intestinal epithelial cells. Am J Physiol Gastrointest Liver Physiol 280: G992–G1004, 2001.—The maintenance of intestinal mucosal integrity depends on a balance between cell renewal and cell death, including apoptosis. The natural polyamines, putrescine, spermidine, and spermine, are essential for mucosal growth, and decreasing polyamine levels cause G1 phase growth arrest in intestinal epithelial (IEC-6) cells. The present study was done to determine changes in susceptibility of IEC-6 cells to apoptosis after depletion of cellular polyamines and to further elucidate the role of nuclear factor-κB (NF-κB) in this process. Although depletion of polyamines by α-difluoromethylornithine (DFMO) did not directly induce apoptosis, the susceptibility of polyamine-deficient cells to staurosporine (STS)-induced apoptosis increased significantly as measured by changes in morphological features and internucleosomal DNA fragmentation. In contrast, polyamine depletion by DFMO promoted resistance to apoptotic cell death induced by the combination of tumor necrosis factor-α (TNF-α) and cycloheximide. Depletion of cellular polyamines also increased the basal level of NF-κB proteins, induced NF-κB nuclear translocation, and activated the sequence-specific DNA binding activity. Inhibition of NF-κB binding activity by sulfasalazine or MG-132 not only prevented the increased susceptibility to STS-induced apoptosis but also blocked the resistance to cell death induced by TNF-α in combination with cycloheximide in polyamine-deficient cells. These results indicate that 1) polyamine depletion sensitizes intestinal epithelial cells to STS-induced apoptosis but promotes the resistance to TNF-α-induced cell death, 2) polyamine depletion induces NF-κB activation, and 3) disruption of NF-κB function is associated with altered susceptibility to apoptosis induced by STS or TNF-α. These findings suggest that increased NF-κB activity after polyamine depletion has a proapoptotic or antiapoptotic effect on intestinal epithelial cells determined by the nature of the death stimulus. programmed cell death; ornithine decarboxylase; growth arrest; IκB; intestinal epithelium

THE EPITHELIUM OF THE INTESTINAL mucosa is continuously renewed from the proliferative zone within the crypts and has the most rapid turnover rate of any tissue in the body (21, 38). These undifferentiated epithelial cells divide and differentiate as they migrate to the luminal surface of the colon or up the villous surface in the small intestine. To maintain stable numbers of enterocytes, cell division is counterbalanced by apoptosis, a fundamental biological regulatory mechanism involving selective cell deletion to maintain tissue homeostasis (21, 22). Apoptosis is a genetically regulated form of programmed cell death defined by distinct morphological and biochemical features, including chromatin condensation, DNA fragmentation, and membrane blebbing (12, 14, 23, 53, 57). Apoptosis occurs in epithelial cells in the crypt area, where it maintains the critical balance in cell number between newly divided and surviving cells, and at the luminal surface of the colon and villous tips in the small intestine, where differentiated cells are lost (14, 23).

Normal physiological processes and pathological stimuli induce apoptosis via different signal transduction cascades (12, 14, 23, 53, 57). Many of the genes that are activated in the initiation of apoptosis are target genes of nuclear factor-κB (NF-κB) (3, 4). The NF-κB family of transcription factors consists of five different subunits in mammalian cells, including p50, p52, p65/Rel A, c-Rel, and Rel-B, which are able to form homodimers or heterodimers (3). Under nonstress conditions, NF-κB dimers are sequestered in most cells in the cytoplasm by a member of the IκB family of proteins: IκBo, IκBβ, or IκBe (11, 40). Binding of IκB to NF-κB blocks nuclear localization signals on NF-κB and prevents its translocation to the nucleus (6, 26, 29, 54). In response to a host of stimuli, IκB is phosphorylated and then degraded by the ubiquitin-proteasome pathway (26, 39), which allows free NF-κB to translocate to the nucleus to activate transcription of specific genes involved in apoptosis. Accumulating evidence has revealed that NF-κB has a proapoptotic or antiapoptotic function, depending on cell type and the death stimulus (5, 29, 30, 46, 56). Overexpression of NF-κB in chick bone marrow cells leads to apoptosis (1), and suppression of NF-κB activity by increasing IκB expression inhibits Sindbis virus-induced cell death in AT-3 prostate carcinoma cells (29, 30), indicating that NF-κB acts as a proapoptotic tran-
scription factor. Consistently, several proapoptotic genes such as c-myc, p53, Fas ligand, and the interleukin-1β-converting enzyme caspase-1 have NF-κB binding sequences in their promoter regions (3, 24, 45, 69). In contrast, NF-κB activity appears to be necessary for the activation of genes that suppress some type of apoptosis. For example, inhibition of NF-κB activity in immature B cells after addition of anti-IgM or in liver during development markedly enhances apoptotic cell death (7, 68). Antiapoptotic genes that are regulated by NF-κB include manganese superoxide dismutase (63) and the zinc finger protein A20 (40). However, the precise factors that determine the ability of NF-κB to regulate these divergent biological actions are unknown.

Our previous studies (60–62) and others (20, 33, 38) have shown that the polyamines spermine and spermidine and their diamine precursor putrescine are absolutely required for the maintenance of normal intestinal mucosal homeostasis and that intracellular polyamine levels are highly regulated by the cell according to the state of growth. Decreasing cellular polyamines by inhibition of ornithine decarboxylase (ODC), the rate-limiting step in polyamine biosynthesis, with α-difluoromethylornithine (DFMO) suppresses mucosal growth in vivo (33, 62) as well as in vitro (27, 63). Because polyamines not only play a critical role in cell proliferation (20, 38) but also are involved in apoptosis (16, 36), inhibition of the intestinal mucosal growth after polyamine depletion could be due to a decrease in cell renewal and/or an increase in apoptosis. We (27) recently demonstrated that depletion of cellular polyamines by DFMO results in a significant increase in G1 phase growth arrest in intestinal epithelial (IEC-6) cells but does not directly induce apoptosis.

The present studies asked whether inhibition of polyamine synthesis alters susceptibility of intestinal epithelial cells to apoptotic stimuli and further investigated the involvement of NF-κB in this process. First, we wanted to examine changes in susceptibility of IEC-6 cells to staurosporine (STS)- and tumor necrosis factor-α (TNF-α)-induced apoptosis after depletion of cellular polyamines. Second, we examined whether polyamine depletion increased NF-κB activity. Third, we wished to elucidate whether the observed increase in NF-κB activity played a role in the alteration of epithelial cell susceptibility to apoptotic stimuli.

MATERIALS AND METHODS

Chemicals and supplies. Disposable culture ware was purchased from Corning Glass Works (Corning, NY). Tissue culture media and dialyzed fetal bovine serum (FBS) were purchased from GIBCO BRL (Gaithersburg, MD), and biochemicals were obtained from Sigma Chemical (St. Louis, MO). The double-stranded oligonucleotides used in electrophoretic mobility shift assays and antibodies against NF-κB were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-32P]ATP (3,000 Ci/mmole) was purchased from Amersham (Arlington Heights, IL). DFMO was a gift from Merrell Dow Research Institute of Marion Merrell Dow (Cincinnati, OH).

Cell culture and experimental design. The IEC-6 cell line was purchased from the American Type Culture Collection at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (47). Originating from intestinal crypt cells as judged by morphological and immunological criteria, the IEC-6 cells are nontumorigenic and retain the undifferentiated character of epithelial crypt cells. Stock cells were maintained as previously described (42), and passages 15–20 were used in the experiments.

In the first series of studies, we examined whether depletion of cellular polyamines by treatment with DFMO could alter susceptibility to STS- and TNF-α-induced apoptosis in IEC-6 cells. The general protocol of the experiments and methods were similar to those described previously (63). Briefly, IEC-6 cells were plated at 6.25 × 104 cells/cm2 in 100-mm dishes or two-well chamber slides. The cells were grown in control medium (DMEM + 5% dialyzed FBS + 10 μg insulin and 50 μg gentamicin sulfate per milliliter) or DMEM containing 5 mM DFMO or DFMO + 5 μM spermidine for 5 days. After cells were washed with serum-free medium, they were exposed to STS or TNF-α in combination with cycloheximide. Apoptosis was examined by changes in morphology and DNA fragmentation.

In the second series of studies, we examined whether polyamine depletion increases NF-κB activity in IEC-6 cells. Cells were grown in control cultures, cultures in which ODC was inhibited with 5 mM DFMO, and cultures inhibited with DFMO and supplemented with 5 μM spermidine for 4, 6, and 8 days. The NF-κB protein levels, cellular distribution, and sequence-specific DNA binding activity were measured by Western blotting analysis, immunohistochemical staining, and electrophoretic mobility shift assays.

In the third series of studies, we determined whether decreased NF-κB activity by treatment with specific NF-κB inhibitors, sulfasalazine (58) and MG-132 (18), prevented the altered susceptibility to STS- and TNF-α-induced apoptosis in polyamine-deficient cells. IEC-6 cells were grown in the presence or absence of 5 mM DFMO for 6 days and then exposed to sulfasalazine or MG-132 for 90 min before administration of STS or TNF-α. Apoptosis was measured 4 h after treatments.

Assessment of morphology. After various experimental treatments, cells were photographed with a Nikon inverted microscope before fixation. Cells then were fixed with D-PBS containing Nonidet P-40, Hoechst-33342, and 4% formaldehyde as described previously (16). Hoechst-stained cells were visualized and photographed under ultraviolet excitation with a Nikon microscope, and the percentage of “apoptotic" cells was determined.

DNA fragmentation. DNA from treated cells was assayed using a modification of the method described by Armstrong et al. (2). Briefly, cells were lysed with 1.0 ml of digestion buffer and incubated at 50°C for 18 h. Samples were extracted twice with 1 vol of phenol-chloroform-isooamyl alcohol, precipitated with 7.5 M ammonium acetate and 100% ethanol, and resuspended in 10 mM Tris-HCl. Samples were then treated with RNase (40 μg/ml) in the presence of 0.1% SDS for 1 h at 37°C. Samples were reextracted, precipitated, and resuspended a second time as described above. Ten micrograms of DNA were loaded into each well and electrophoresed in 1.5% agarose gel. Gels were visualized by ultraviolet fluorescence and photographed with a Polaroid camera system.

Preparation of nuclear protein and electrophoretic mobility shift assays. Nuclear proteins were prepared by the procedure described previously (70), and the protein contents in nuclear preparation were determined by the de-
scribed by Bradford (10). The double-stranded oligonucleotides used in these experiments included 5'-AGTTGAGGG-
GACTTTCCAGGC-3', which contains a consensus NF-κB binding site that is underscored. These oligonucleotides were
radioactively end-labeled with [γ-32P]ATP and T4 polynucleotide kinase (Promega, Madison, WI). For mobility shift as-
says, 0.035 pmol of 32P-labeled oligonucleotides (~30,000
cpm) and 10 μg of nuclear protein were incubated in a total
volume of 25 μl in the presence of 10 mM Tris-HCl (pH 7.5),
50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol,
and 1 μg of poly(dI-dC). The binding reactions were allowed
to proceed at room temperature for 20 min. Thereafter, 2 μl
of bromphenol blue (0.1% in water) were added, and protein-
DNA complexes were resolved by electrophoresis on nonde-
naturating 5% polyacrylamide gels and visualized by auto-
radiography. The specificity of binding interactions was
assessed by competition with an excess of unlabeled double-
stranded oligonucleotide of identical sequence.

**Western blotting analysis.** Ten micrograms of cytoplasmic protein extracts were dissolved in SDS sample buffer, boiled
for 5 min, and then subjected to electrophoresis on 10%
acrylamide gels according to Laemmli (25). After SDS-PAGE,
the gels were transferred to nitrocellulose membranes for 1 h
at 4°C. The blots were blocked with 5% nonfat dry milk in
PBS-0.1% Tween 20 (PBS-T) overnight at 4°C. Immunologi-
cal evaluation was then performed for 1 h in PBS-T contain-
ing 0.2 μg/ml affinity-purified polyclonal antibodies against
NF-κB subunits p50 and p65 (50) or IκB protein. The blots
were subsequently washed with PBS-T and incubated for 1 h
with goat anti-rabbit IgG antibody conjugated to peroxidase
at a dilution of 1:3,000 in PBS-T. After extensive washing
with PBS-T, the blots were developed for 30 or 60 s with
enhanced chemiluminescence reagents (Amersham).

**Immunohistochemical staining.** Immunohistochemical stain-
ing for NF-κB protein was performed in IEC-6 cells by the
indirect immunoperoxidase method as described previously
(27). The cells were incubated with rabbit polyclonal antibody
against the p65 subunit of NF-κB at a dilution of 1:100 in PBS
containing 1% BSA for 1 h at room temperature and then 1 h
of incubation with biotinylated goat anti-rabbit IgG at a dilution
of 1:500. Nonspecific slides were incubated without antibody
against NF-κB. The bound antibody was visualized with avidin-
biotin complexes. The slides were counterstained with hema-
toxalin, mounted, and viewed with an Olympus microscope.

**Polyamine analysis.** The cellular polyamine content was
analyzed by HPLC as described previously (62). After the
monolayers were washed three times with ice-cold D-PBS,
0.5 M perchloric acid was added, and the monolayers were
frozen at ~80°C until ready for extraction, dansylation, and
HPLC. The standard curve encompassed 0.31–10 μM. Val-
ues that fell 25% below the curve were considered not detect-
able. Protein was determined by the Bradford method (10).
The results are expressed as nanomoles of polyamines per milligram of protein.

**Statistics.** Values are means ± SE from six dishes. Auto-
radiographic results were repeated three times. The signifi-
cance of the difference between means was determined by
ANOVA. The level of significance was determined using
Duncan’s multiple range test (17).

**RESULTS**

**Effects of polyamine depletion on STS- and TNF-α-
induced apoptosis.** Administration of 5 mM DFMO,
which totally inhibited ODC activity (27, 63), almost
completely depleted cellular polyamines in IEC-6 cells.
The levels of putrescine and spermidine were undetect-
able at 4, 6, and 8 days after DFMO treatment. Spermi-
dine was less sensitive to the inhibition of ODC but was
decreased by >60% in cells exposed to DFMO for 4, 6,
and 8 days (data not shown). Similar results have been
published previously (42).

Our previous studies demonstrated that depletion of
acellular polyamine does not directly induce pro-
grammed cell death in IEC-6 cells (27). To further
determine the involvement of cellular polyamines in
the regulation of susceptibility to apoptotic stimuli,
STS and TNF-α, two widely accepted agents for induc-
programmed cell death in a variety of cell types (5,
9, 19), were used in this study. As can be seen in Fig. 1,
inhibition of polyamine synthesis by treatment with
DFMO significantly increased the susceptibility of in-
testinal epithelial cells to STS-induced apoptosis.
Exposure of normal IEC-6 cells (without DFMO) to 1 μM
STS did not induce apoptotic cell death as assessed by
morphological and biochemical methods (Fig. 1, A and
B, Control a vs. b, and C, left). However, polyamine-
deficient cells were more sensitive to STS-induced apop-
tosis. When exposed to 1 μM STS, typical morphologi-
cal features of programmed cell death were identified
(Fig. 1A, DFMO α vs. b). Staining of nuclei with the
DNA-binding dye Hoechst-33342 revealed a significant
increase in nuclear condensation and fragmentation in
polyamine-deficient cells treated with 1 μM STS (Fig. 1B,
DFMO α vs. b). Morphological assessments of apop-
totic cells were confirmed by measurement of internucleo-
osomal DNA fragmentation. The classic “ladder” of DNA
fragmentation was observed in DFMO-treated cells
exposed to 1 μM STS (Fig. 1C, left). The percentage of
apoptotic cells in DFMO-treated cells was ~45% com-
pared with ~2% in control cells after exposure to 1 μM
STS (Fig. 1D). Spermidine (5 μM) given together with
DFMO completely prevented the increased susceptibil-
ity to STS-induced apoptosis. Morphological features
and internucleosomal DNA levels in cells treated with
DFMO + spermidine were indistinguishable from those
in cells grown in control culture after exposure to 1 μM
STS (Fig. 1B). Treatment with 4 μM STS resulted in
programmed cell death in all three treatment groups (Fig.
1A, B, and C, left). These results clearly indicate that polyamine depletion dramatically
increases susceptibility of IEC-6 cells to STS-induced
apoptosis.

In contrast, polyamine depletion protected IEC-6
cells from TNF-α-induced apoptosis (Fig. 2). When
TNF-α (20 ng/ml) together with cycloheximide (25 μg/ ml) was added to control cultures, it induced typical
apoptotic cell death (Fig. 2A, a vs. b, and B, left). In
DFMO-treated cells, treatment with the same doses of
TNF-α and cycloheximide caused no apoptosis. There
were no differences in morphological features and per-
centage of apoptotic cells between cells treated with
DFMO alone and DFMO-treated cells exposed to TNF-
α + cycloheximide (Fig. 2A, c vs. d, and B, middle).
Interestingly, when spermidine was added concomi-
tantly with DFMO, the protective effect of polyamine
depression on TNF-α-induced apoptotic cell death was
Fig. 1. Apoptotic response of IEC-6 cells to staurosporine (STS) in the presence or absence of cellular polyamines. Cells were grown in DMEM containing 5% dialyzed fetal bovine serum (FBS) and 5 mM α-difluoromethylornithine (DFMO) with or without 5 μM spermidine (SPD) for 6 days and then exposed to 1 or 4 μM STS. A: STS-induced apoptosis in control cells (Control) and cells pretreated with DFMO alone or DFMO + SPD. a, Cells treated without STS; b, cells exposed to 1 μM STS for 4 h; c, cells exposed to 4 μM STS for 4 h. Original magnification ×150. B: STS-induced apoptotic nuclei in cells described in A. After all 3 groups of cells were exposed to 1 or 4 μM STS for 4 h, they were fixed and stained with Hoechst-33342. Original magnification ×350. C: induction of DNA fragmentation induced by 1 and 4 μM STS in cells described in A. Genomic DNA was isolated from cells exposed to STS for 4 h and analyzed on 1.5% agarose gel. Three experiments showed similar results. D: percentage of apoptotic cells after different treatments. Values are means ± SE of data from 3 experiments. *P < 0.01 compared with No-STS. *P < 0.05 compared with cells grown in the presence of DFMO or DFMO + SPD for 6 days and then exposed to 1 μM STS.
Values are means ± SE of data from 3 experiments. *P < 0.05 compared with cells that were not exposed to TNF-α and CHX (No-TNF-α). †P < 0.05 compared with cells grown in control medium and medium containing DFMO + SPD for 6 days and then exposed to TNF-α and CHX.

completely prevented (Fig. 2A, e vs. f, and B, right). In addition, neither TNF-α nor cycloheximide alone induced typical apoptotic cell death in any of the three groups (data not shown). These results indicate that polyamine depletion promotes the resistance of IEC-6 cells to apoptotic cell death induced by TNF-α in combination with cycloheximide.

To extend our findings, two other apoptotic stimuli, diclofenac and indomethacin (32), were used, and the effects of polyamine depletion on the susceptibility to apoptosis induced by diclofenac or indomethacin were examined. Our results demonstrated that decreased cellular polyamines sensitized IEC-6 cells to diclofenac- and indomethacin-induced apoptosis. The percentage of apoptotic cell death in polyamine-deficient cells was significantly increased (from 5 ± 0.4% in controls to 24.1 ± 1.8% in DFMO-treated cells, P < 0.05) after they were exposed to 100 μM diclofenac for 24 h. Similarly, polyamine-deficient cells were more sensitive to indomethacin-induced cell death. Apoptotic bodies induced by treatment with 100 μM indomethacin for 24 h were significantly increased from 8 ± 0.7% in control cells to 29.5 ± 2% in DFMO-treated cells (P < 0.05). Together, these results indicate that polyamine depletion alters susceptibility of intestinal epithelial cells to apoptosis in a death stimulus-dependent manner.

**Effect of polyamines on NF-κB protein levels and cellular distribution.** To elucidate the mechanism responsible for altered susceptibility of polyamine-deficient IEC-6 cells to STS- and TNF-α-induced apoptosis, the role of NF-κB in this process was investigated. Data presented in Fig. 3 clearly show that depletion of cellular polyamines by treatment with DFMO not only significantly increased NF-κB protein levels but also induced NF-κB nuclear translocation. The increase in protein levels for NF-κB (p50 and p65 subunits) was noted 4 days after DFMO exposure and remained elevated 6 and 8 days after exposure (Fig. 3, A and B). The levels of NF-κB p50 protein in cells exposed to DFMO were ~1.8, ~2.1, and ~2.4 times the normal values (without DFMO) 4, 6, and 8 days after DFMO, respectively. Protein levels for the NF-κB p65 subunit were almost twice the normal values 4, 6, and 8 days after DFMO treatment. Spermidine (5 μM) given together with DFMO completely prevented the increased levels of NF-κB proteins. Putrescine (10 μM) had an effect equal to spermidine on NF-κB activation when it was added to cultures that contained DFMO (data not shown).

Increased NF-κB proteins after polyamine depletion were visible and present just inside a defined nuclear area (Fig. 3Cb). These nuclear immunoreactivities for NF-κB subunits markedly increased in DFMO-treated cells as expected. Observation was facilitated for every experiment by heavily immunostained nuclei. In the presence of DFMO, spermidine eliminated the increased immunostaining levels for NF-κB and blocked NF-κB translocation to the nucleus (Fig. 3C, b vs. c). The appearance of NF-κB distribution in cells grown in the presence of DFMO and spermidine was indistinguishable from that in control cells (Fig. 3C, a vs. c).

Changes in NF-κB sequence-specific DNA binding activity in polyamine-deficient cells. Consistently, increased levels of NF-κB protein in cells exposed to
DFMO were paralleled by a marked increase in NF-κB binding activity as measured by electrophoretic mobility shift assay (Fig. 4A). The NF-κB binding activity was increased by nearly twofold 4 days after DFMO treatment and by approximately threefold 6 and 8 days after DFMO (Fig. 4B). Spermidine (5 μM) given together with DFMO completely prevented the increase in NF-κB binding activity. To evaluate the specificity of the binding reaction in Fig. 4A, competitive inhibition experiments were performed. As shown in Fig. 4C, a and b, NF-κB binding activities in control cells and cells exposed to DFMO were dose-dependently inhibited when various concentrations of the unlabeled NF-κB oligonucleotide were added to the binding reaction mixture. We also examined the effect of the unlabeled oligonucleotide containing a mutated NF-κB binding site on NF-κB binding activity and found that the NF-κB-mutated oligonucleotide did not inhibit NF-κB binding activity in IEC-6 cells (Fig. 4C). Figure 4Cd further shows that preincubation of nuclear extracts with a specific antibody against the NF-κB p65 subunit significantly inhibited the formation of NF-κB binding complex. In contrast, preincubation with the control (anti-Myc) antibody had no inhibitory effect on NF-κB binding activity (data not shown). These results indicate that altered susceptibility of IEC-6 cells to apoptotic stimuli after polyamine depletion is associated with a significant increase in the NF-κB activity.

**Effect of polyamine depletion on IκB.** To determine the involvement of IκB in the process of NF-κB nuclear translocation after polyamine depletion, expression of IκBα protein was examined in cells grown in the presence or absence of DFMO. As shown in Fig. 5, depletion of cellular polyamines by DFMO significantly inhibited the content of IκBα protein in IEC-6 cells. The level of IκBα protein was decreased by ~45% and ~65% 4 and 6 days after exposure to DFMO, respectively. The IκBα protein content returned to normal levels when DFMO was given together with spermidine. These results suggest that activation of NF-κB activity after depletion of cellular polyamines is mediated at least partially through the IκB pathway.

**Effects of NF-κB inhibitors on STS- and TNF-α-induced apoptosis in polyamine-depleted IEC-6 cells.** To investigate the role of induced NF-κB activation in the process of altered susceptibility to apoptosis after polyamine depletion, two potent and specific inhibitors of NF-κB, sulfasalazine (58) and MG-132 (18), were used in this study. Inhibition of NF-κB activity by treatment with sulfasalazine prevented the increased susceptibility of polyamine-deficient cells to STS-induced apoptosis (Fig. 6). Sulfasalazine at 1 mM slightly decreased the rate of apoptosis, but these differences were not statistically significant. However, sulfasalazine at 2 mM significantly blocked the increased sensitivity of polyamine-deficient cells to STS-induced apop-
tosis. Typical morphological features of programmed cell death (Fig. 6A) and internucleosomal DNA fragmentation (Fig. 6B, right) decreased markedly in DFMO-treated cells pretreated with 2 mM sulfasalazine. The percentage of STS-induced apoptotic cells was decreased from 46% in DFMO-treated cells to 18% when DFMO-treated cells were pretreated with 2 mM sulfasalazine (Fig. 6C). In addition, inhibition of NF-κB activity by sulfasalazine also prevented increased susceptibility of polyamine-deficient cells to apoptosis induced by diclofenac or indomethacin (data not shown). Sulfasalazine at 1 and 2 mM alone caused no apoptosis in the absence of STS (Fig. 6C, right).

On the other hand, inhibition of NF-κB activity by sulfasalazine blocked the protective effect of polyamine depletion against TNF-α-induced apoptosis in IEC-6 cells (Fig. 7). When DFMO-treated cells were pretreated with 1 mM sulfasalazine, TNF-α significantly induced cell death in the presence of cycloheximide (Fig. 7A, b vs. c). Pretreatment with 2 mM sulfasalazine completely prevented the tolerance of polyamine-deficient cells to TNF-α-induced apoptosis (Fig. 7Ad). The percentage of apoptotic cells (~27%) in DFMO-treated cells pretreated with 2 mM sulfasalazine was similar to that in control cells (~28%) and cells exposed to DFMO + spermidine (~25%) after exposure to TNF-α and cycloheximide.

Disruption of NF-κB function by treatment with MG-132 was also associated with altered susceptibility of DFMO-treated cells to apoptosis induced by STS or...
The increased susceptibility of polyamine-deficient cells to STS-induced apoptosis was remarkably prevented by pretreatment with MG-132. The percentage of STS-induced apoptotic cells was decreased from 43% in DFMO-treated cells to 22 and 16% when DFMO-treated cells were pretreated with 1 and 10 μM MG-132, respectively (Fig. 8A, b vs. c, and B). In contrast, pretreatment with MG-132 blocked the resistance of polyamine-deficient cells to apoptosis induced by TNF-α in combination with cycloheximide (Fig. 8A, d vs. e and f, and C). The percentage of TNF-α-induced apoptosis in DFMO-treated cells was increased by ~9 and ~20% when these cells were pretreated with 1 and 10 μM MG-132, respectively. Exposure to 1 or 10 μM MG-132 alone (without STS or TNF-α + cycloheximide) did not result in apoptotic cell death (Fig. 8, B and C, right). These findings suggest that increased NF-κB activity after polyamine depletion plays a critical role in the regulation of susceptibility of intestinal epithelial cells to apoptosis and that the function of NF-κB in this process depends on the nature of the death stimulus.

Effects of sulfasalazine and MG-132 on NF-κB activity in DFMO-treated cells. To confirm the inhibitory effects of sulfasalazine and MG-132 on NF-κB in polyamine-deficient cells, the activity of NF-κB was measured...
sured in DFMO-treated cells exposed to sulfasalazine or MG-132. Cells were grown in the presence of 5 mM DFMO for 6 days and then treated with different concentrations of sulfasalazine or MG-132. Exposure to sulfasalazine or MG-132 for 90 min decreased NF-κB binding activity in polyamine-deficient cells (Fig. 9). When various doses of sulfasalazine were tested, NF-κB binding activity was inhibited dose dependently, with concentrations ranging from 1 to 4 mM. Significant inhibition of NF-κB binding activity occurred at 2 mM, and the binding activities were decreased by ~80% (Fig. 9, left). In cells treated with MG-132, NF-κB activity was decreased by ~30 and ~80% at 1 and 10 μM, respectively (Fig. 9, right).

Fig. 7. Effect of administration of sulfasalazine on apoptosis induced by TNF-α in combination with CHX in polyamine-deficient IEC-6 cells. A: TNF-α-induced apoptosis after different treatments. a, Cells treated with DFMO alone; b, DFMO-treated cells exposed to TNF-α (20 ng/ml) and CHX (25 μg/ml); c, DFMO-treated cells pretreated with 1 mM sulfasalazine and then exposed to TNF-α and CHX; d, DFMO-treated cells pretreated with 2 mM sulfasalazine and then exposed to TNF-α and CHX. Cells were grown in DMEM containing 5% dialyzed FBS and 5 mM DFMO for 6 days, treated with 1 or 2 mM sulfasalazine for 90 min, and then exposed to TNF-α + CHX. Cells were photographed 4 h after administration of TNF-α and CHX. Original magnification ×200. B: percentage of apoptotic death in cells described in A. Values are means ± SE of data from 3 experiments. *P < 0.01 compared with the group treated without TNF-α and CHX.

Fig. 8. Effect of administration of MG-132 on the rates of apoptotic cell death induced by STS or TNF-α + CHX in polyamine-deficient cells. A: DFMO-treated cells after different treatments. a, Cell treated with DFMO alone; b, DFMO-treated cells exposed to 1 μM STS; c, DFMO-treated cells pretreated with 10 μM MG-132 and then exposed to 1 μM STS; d, DFMO-treated cells exposed to TNF-α (20 ng/ml) and CHX (25 μg/ml); e, DFMO-treated cells pretreated with 1 μM MG-132 and then exposed to TNF-α + CHX; f, DFMO-treated cells pretreated with 10 μM MG-132 and exposed to TNF-α + CHX. Cells were grown in the presence of 5 mM DFMO for 6 days, pretreated with MG-132 for 90 min, and then exposed to STS or TNF-α + CHX. Cells were photographed 4 h after treatment with STS or TNF-α. Original magnification ×200. B: percentage of apoptotic bodies in cells exposed to STS. Values are means ± SE of data from 3 experiments. *P < 0.05 compared with the groups without MG-132.
Sulfasalazine or MG-132 before treatment with STS or nuclear protein and 0.035 pmol of 32P-end-labeled oligonucleotides

A decrease in polyamine content by the super-

levels and abnormal polyamine metabolic enzyme ac-

sis in certain cell types and that decreasing cellular
demonstrated that polyamines protect against apopto-

tive enzymes in this process have been defined. The role of polyamines in apoptotic pathways has been rather controversial, depending on the
cell type and death stimulus. Numerous studies have demonstrated that polyamines protect against apoptosis in certain cell types and that decreasing cellular polyamines induces programmed cell death (15, 16, 31, 36, 37, 43). The deregulation of cellular polyamine levels and abnormal polyamine metabolic enzyme activities are observed in cells undergoing apoptosis (15, 16, 31). A decrease in polyamine content by the super-

induction of the polyamine catabolic enzyme spermi-
dine/spermine N1-acetyltransferase results in a typical
process of programmed cell death in non-small cell
lung carcinoma NCI H57 cells (16, 37). Exposure to the polyamine oxidase inhibitor MDL-72527 reduces the levels of putrescine and spermidine and induces apoptosis in transformed hematopoietic cells (13). Similar findings are also observed in breast cancer cell lines (36).

On the other hand, contrary evidence has been re-
ported, indicating that high levels of polyamines favor apoptosis and that inhibition of polyamine synthesis protects cells from apoptotic cell death. For example, excessive polyamine accumulation in ODC-overproducing L1210 cells induces characteristic features of apoptosis (44). Administration of the amino acid ornithine stimulates the accumulation of cellular putrescine and increases programmed cell death in ODC-overproducing mouse myeloma cells (55). Recently, Ray et al. (48) reported that depletion of cellular polyamines by treatment with DFMO decreased the apoptotic index induced by the DNA topoisomerase I inhibitor camptothecin in IEC-6 cells.

The findings reported here clearly indicate that poly-
amines are involved in the regulation of susceptibility of intestinal epithelial cells to apoptosis. The data also reveal that alteration in the tolerance or the sensitiza-
tion to apoptosis after polyamine depletion depends on
the death stimulus. For example, the susceptibility of intestinal epithelial cells to STS-induced apoptosis increased dramatically after polyamine depletion. In con-
trast, inhibition of polyamine synthesis by treatment with DFMO prevents TNF-α-induced apoptosis in IEC-6 cells. Results presented here further dem-
strate that altered susceptibility of intestinal epithelial
cells to apoptotic stimulus after polyamine depletion is
associated with a significant increase in NF-κB activity. Although the exact intracellular signaling pathway initiated by depletion of cellular polyamines leading to NF-κB activation is unclear, levels of IκBα protein decreased significantly in polyamine-deficient cells. Because NF-κB normally exists in an inactive form in
the cytoplasm bound to IκB (11), the downregulation of IκBα protein expression could be partially responsible for the increased activation and nuclear translocation of NF-κB in polyamine-depleted cells. It is not clear whether decreased IκBα levels after polyamine deple-
tion are due to an increase in IκBα degradation by
phosphorylation or a decrease in IκBα production.

Another study indicates that polyamines induce activa-
tion of NF-κB. Shah et al. (52) recently reported that natural spermine exerts a significant stimulatory effect on NF-κB binding activity in breast cancer (MCF-7) cells. Spermidine and putrescine are also ca-

B binding activity in breast cancer

Fig. 9. Effects of sulfasalazine and MG-132 on NF-κB binding activity in polyamine-deficient IEC-6 cells. A: representative autoradiograms from polyamine-deficient cells exposed to sulfasalazine (a) or MG-132 (b). Cells were grown in the presence of 5 mM DFMO for 6 days and then exposed to different concentrations of sulfasalazine or MG-132. Nuclear extracts were isolated 90 min after treatment with sulfasalazine or MG-132. EMSA was performed using 10 μg of nuclear protein and 0.035 pmol of 32P-end-labeled oligonucleotides containing a single NF-κB binding site. Positions of the specifically bound DNA-protein complex are indicated. B: quantitative analysis of EMSA by densitometry from cells described in A. Values are means ± SE of data from 3 separate experiments. *P < 0.05 compared with groups treated without sulfasalazine or MG-132.

There was no apparent loss of cell viability in cells treated with DFMO alone or DFMO + various doses of sulfasalazine or MG-132 before treatment with STS or TNF-α (data not shown).

DISCUSSION

Cellular polyamines have been essential for the maintenance of intestinal epithelial integrity, but few specific functions of polyamines in this process have been defined. The role of polyamines in apoptotic path-
ways has been rather controversial, depending on the
cell type and death stimulus. Numerous studies have demonstrated that polyamines protect against apoptosis in certain cell types and that decreasing cellular polyamines induces programmed cell death (15, 16, 31, 36, 37, 43). The deregulation of cellular polyamine levels and abnormal polyamine metabolic enzyme activities are observed in cells undergoing apoptosis (15, 16, 31). A decrease in polyamine content by the sup-

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the culture medium with DFMO and kept in the medium for 4, 6, and 8 days. In the studies with MCF-7 cells, polyamines were added directly to the reaction mixtures of gel shift assays. Second, there were significant differences in concentrations of polyamines between these two studies: IEC-6 cells were exposed to 5 μM spermidine, and cellular extracts from MCF-7 cells were incubated with polyamines ranging from 0.1 to 2 mM. Finally, the cell types are different: the IEC-6 line is derived from the normal small intestinal crypts, and the MCF-7 line is from breast tumor tissue.

Our results suggest that NF-κB activation plays a critical role in the process through which polyamine depletion alters the sensitivity of IEC-6 cells to apoptotic stimulus. Inhibition of NF-κB activity by treatment with sulfasalazine not only prevented the increased susceptibility to STS-induced apoptosis but also blocked the protection against apoptosis induced by TNF-α in combination with cycloheximide in polyamine-deficient cells. Although sulfasalazine may exert different effects on apoptosis through a process independent of NF-κB, data obtained from the utilization of another specific pharmacological inhibitor of NF-κB, MG-132, are similar to those from the application of sulfasalazine. These findings from using two different NF-κB inhibitors provide the strong evidence supporting our hypothesis.

The observation that NF-κB is involved in proapoptotic and antiapoptotic processes is not surprising, because the function of NF-κB in the regulation of apoptosis has been shown to depend on cell type and death stimulus (5, 29, 30, 46, 56). There is evidence indicating that NF-κB protects dividing cells against apoptotic cell death induced by TNF-α in certain cell types (5, 56). The NF-κB p65 subunit knockout animals are more sensitive to TNF-α-induced programmed cell death (68). Conversely, other studies have suggested that NF-κB promotes apoptosis in different cell injury models (29, 30, 46). For example, quinolinic acid-induced apoptotic cell death in striatal neurons is associated with a significant increase in NF-κB activity, and prevention of NF-κB nuclear translocation diminishes quinolinic acid-induced apoptosis (46). Similar results from HL-60 cells also show that activation of NF-κB activity by triphenyltin triggers apoptotic cell death (31). Although the precise factors that determine the ability of NF-κB to regulate these divergent biological outcomes are unknown, the interaction of NF-κB with other effectors or regulators of apoptosis may be involved in this process. It is possible that polyamine depletion may induce expression of inactive forms of one or more proapoptotic or antiapoptotic factors, which would be activated by a specific death stimulus. Cross talk between NF-κB and these activated factors could determine the function of NF-κB in the regulation of apoptosis in polyamine-deficient cells.

How might NF-κB mediate the increased susceptibility of intestinal epithelial cells to apoptosis after polyamine depletion? NF-κB modulates transcription of many genes and has transcriptional cross talk with p53 (66, 69). The product of the p53 gene serves as a critical regulator of the cell cycle and of apoptotic mechanisms in normal and malignant cells (51). The important contribution of p53 to the apoptotic process has been well documented in various cell types and under different conditions (45, 51, 69). We (27) recently reported that polyamine depletion by DFMO significantly increases expression of the p53 gene in IEC-6 cells. The remarkable parallelism that exists between the stimulation of p53 gene expression and the increased susceptibility to apoptosis elicited by NF-κB suggests the possibility that activation of NF-κB after polyamine depletion sensitizes intestinal epithelial cells to apoptosis in association with its ability to regulate p53 gene expression. This contention is supported by recent publications that indicate that activated NF-κB plays a role in p53-mediated programmed cell death (49) and that NF-κB mediates Bcl-2 suppression in hypoxia-induced endothelial apoptosis (35). In addition, other signaling pathways may also be involved in STS-induced apoptosis. Polyamines have been shown to regulate intracellular Ca2+ concentration ([Ca2+]cyt) through K+ channels, and depletion of cellular polyamines reduces [Ca2+]cyt in IEC-6 cells (65). Because [Ca2+]cyt has been implicated in apoptosis through the regulation of bcl-2 expression and protein kinase activity (8), the increased susceptibility of polyamine-deficient cells to STS-induced cell death may result, at least partially, from the decrease in [Ca2+]cyt. It is possible that STS induces apoptosis synergistically with the reduction of [Ca2+]cyt.

The findings that activation of NF-κB protects against apoptosis induced by TNF-α in combination with cycloheximide in polyamine-deficient cells are consistent with those from other investigators, who demonstrated that NF-κB is a cell survival factor (5, 56, 68). In general, NF-κB has been believed to inhibit apoptosis in the caspase-dependent pathways, because numerous reports documented the antiapoptotic action of NF-κB and apoptosis caused by inactivation of NF-κB in a variety of cell types (5, 56, 59, 68). Although the exact mechanism through which activation of NF-κB prevents TNF-α-induced apoptosis in DFMO-treated cells is unclear, it has been shown that polyamine depletion decreases caspase-3 activity, which is associated with delayed apoptosis after exposure to camptothecin (48).

In summary, these results indicate that cellular polyamines play a role in the regulation of apoptosis in intestinal epithelial cells and that inhibition of polyamine synthesis alters susceptibility to apoptotic stimuli. The present study also shows that depletion of cellular polyamines significantly increases NF-κB activity and induces NF-κB nuclear translocation in IEC-6 cells. Inactivation of NF-κB by treatment with sulfasalazine or MG-132 in polyamine-deficient cells is associated with altered susceptibility to apoptosis induced by STS or TNF-α + cycloheximide. These findings suggest that NF-κB can play a proapoptotic or antiapoptotic role in intestinal epithelial cells, which is dependent on the death stimulus.
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