Mesalamine induces manganese superoxide dismutase in rat intestinal epithelial cell lines and in vivo

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Received 31 July 2000; accepted in final form 9 May 2001.

Valentine, J. F. Mesalamine induces manganese superoxide dismutase in rat intestinal epithelial cell lines and in vivo. Am J Physiol Gastrointest Liver Physiol 281: G1044–G1050, 2001.—Mesalamine (5-ASA) is effective in the treatment of inflammatory bowel diseases. However, the mechanisms of action of 5-ASA remain unclear. IEC-6 and IRD-98, nontransformed rat small intestinal epithelial cell lines, were used to examine the effect of 5-ASA on the expression of manganese superoxide dismutase (MnSOD). Rats were given 5-ASA enemas to determine the effect on colonic MnSOD expression. Treatment with 5-ASA at 0.02 or 2 mg/ml induced MnSOD mRNA levels 2.67-fold or 5.66-fold, respectively. Inhibition of 5-lipoxygenase activating protein with MK-886 or cyclooxygenase with indomethacin did not influence the level of MnSOD mRNA. Nuclear run-on experiments demonstrated an increase in de novo transcription following treatment with 5-ASA. MnSOD protein levels were induced 2-fold at 24 h and 4.23-fold at 48 h following treatment with 1 mg/ml 5-ASA. 5-ASA increased MnSOD 1.7-fold in vivo. Pretreatment with 5-ASA significantly protected IRD-98 cells from tumor necrosis factor-α cytotoxicity. This is the first example of transcriptional gene regulation by 5-ASA. The induction of MnSOD by 5-ASA may contribute to the therapeutic mechanism of 5-ASA.

SULFASALAZINE AND MESALAMINE (5-ASA), the active ingredient in sulfasalazine (3), are effective first-line agents in the treatment of active inflammatory bowel disease (IBD) and in the maintenance of remission (19). Sulfasalazine has been used for over 50 years, but the mechanisms of action of sulfasalazine and 5-ASA have remained elusive. 5-ASA compounds are capable of multiple effects that may protect the colon from an inflammation-mediated damage. 5-ASA has been demonstrated to directly scavenge free radicals (1, 2), inhibit leukotriene production (43), inhibit the chemotactic response to leukotriene B4 (LTB4) (33), and inhibit cellular release of interleukin (IL)-1 in cultured mucosal biopsy specimens from ulcerative colitis patients (37). Sulfasalazine and 5-ASA inhibit the binding of formyl-methionyl-leucyl-phenylalanine to its receptor on neutrophils (15). Some authors believe that sulfasalazine and 5-ASA are effective because of the additive effects of their multiple actions on the immune system (15, 16).

The ability of 5-ASA and sulfasalazine to inhibit 5-lipoxygenase and LTB4 production (43), the chemotactic response to LTB4 (33), and the enhanced production of LTB4 in the inflamed colonic mucosa (42) resulted in clinical trials examining more potent inhibitors of LTB4 production. However, more potent 5-lipoxygenase inhibitors such as zileuton have not been effective (21). Although inhibition of leukotriene production may contribute to the mechanism of action of the 5-ASA compounds, the interest in this mechanism of action has waxed.

In this study, 5-ASA at therapeutically relevant concentrations is shown to induce manganese superoxide dismutase (MnSOD) in vitro and in vivo. The following data examine, in detail, the regulation of MnSOD mRNA and protein levels in the rat intestinal epithelial cells and in the rat colon in vivo.

METHODS

Cell culture and experimental treatments. All chemicals including 5-ASA were purchased from Sigma (St. Louis, MO) unless otherwise stated. IEC-6 (ATCC CRL-1592) is a nontransformed rat small intestinal crypt cell line developed by Quaroni et al. (36). IRD-98 is a fetal rat small intestinal epithelial cell line that was established by Negrel et al. (32) and was a gift from Patrick Rampal (Hôpital de Cimiez, Nice, France). The IEC-6 and IRD-98 cells were grown to confluence in DMEM (GIBCO, Gaithersburg, MD) with 10 μg/ml insulin, 0.6 mg/ml l-glutamine, antibiotic/antimycotic solution, and 5% fetal bovine serum at 37°C in 95% air and 5% CO2. The cells were treated with 5-ASA at concentrations ranging from 0.002 to 2.0 mg/ml, Escherichia coli endotoxin [lipopolysaccharide (LPS)] at 0.5 μg/ml, tumor necrosis factor (TNF)-α at 10 or 100 ng/ml (R&D Systems, Minneapolis, MN), MK-886 (an inhibitor of 5-lipoxygenase activating protein; Merck, Malvern, PA) at 10 ng/ml, or indomethacin (an inhibitor of cyclooxygenase) at 1 μM. The cells were also cotreated with 1 mg/ml 5-ASA and 4 μM actinomycin D or 20 μM cycloheximide.

RNA isolation and Northern blot analysis. After 8 h of treatment, total cellular RNA was isolated from the cultured cells by a protocol modified from the guanidinium thiocyanate-phenol-chloroform extraction method of Chomczynski.

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and Sacchi (8) as previously described (47). Twenty micro-
grains of total cellular RNA from each sample was subjected
s to Northern blot analysis as has previously been described
(47). The RNA was electrophoresed and cross-linked to
nylon membrane (CUNO, Meriden, CT). The membranes
were probed with a rat MnSOD, rat Cu/ZnSOD, and cat-
hepsin D or glyceraldehyde-3-phosphate dehydrogenase
(GAPDH) cDNA probes. All of the probes used were radiola-
beled by random primer extension. Following an overnight
hybridization at 62°C, the membranes were washed at 65°C
in a 40 mM sodium phosphate, 0.1% SDS, 1 mM EDTA
solution and exposed to X-ray film. The autoradiographs
were analyzed with a Millipore video image densitometer
(Millipore, Ann Arbor, MI).

Nuclear run-on experiments. Four plates each of control
IEC-6 cells and cells treated for 3 h with 2 mg/ml 5-ASA or
0.5 μg/ml LPS were washed with PBS, trypsinized, and
centrifuged for 5 min at 4°C and 300 g to pellet the cells. The
pellets were resuspended in 6 ml of lysis buffer (10 mM
Tris- HCl, 10 mM NaCl, 3 mM MgCl₂, and 0.5% NP-40, pH
7.4) and homogenized. To eliminate nuclei free of cell
membrane debris, the nuclei were pelleted by centrifugation
at 1,200g for 5 min. The nuclei were resuspended in 2 ml of
storage buffer (50 mM HEPES, 4 mM MnCl₂, 1 mM MgCl₂,
0.1 mM EDTA, 5 mM dithiothreitol (DTT), and 50% glycerol)
and snap frozen in liquid N₂.

Radiolabeled mRNA transcript analysis of control and
treated cells were performed according to a modified proce-
dure described by Laine et al. (26). The isolated nuclei were
thawed on ice and centrifuged at 1,200g. The pellet of each
sample was resuspended in 175 μl of incubation buffer (75
mM HEPES, 100 mM KCl, 2.5 mM MgCl₂, 0.05 mM EDTA,
25% glycerol, 5 mM DTT, 0.5 mM CTP, 0.5 mM GTP, 1.0 mM
ATP, 22 U/ml creatine kinase, and 8.8 mM creatine phos-
thepsin D or glyceraldehyde-3-phosphate dehydrogenase
(47). The RNA was electrotransferred to a nitrocellulose mem-
brane (CUNO, Meriden, CT). A 20-
gram of total cellular RNA from each sample was subjected
to Northern blot analysis as has previously been described
(47). The membranes were washed at 65°C in 40 mM
sodium phosphate, 0.1% SDS, 1 mM EDTA solution.

RESULTS

Effect of 5-ASA on MnSOD mRNA levels in cell
culture. Treatment of IEC-6 cells with 5-ASA results in
an induction of MnSOD mRNA levels. Figure 1A is a
Northern blot analysis of MnSOD and Cu/ZnSOD
mRNA levels at an 8-h time point, examining the effect
of a concentration curve of 5-ASA. MnSOD has five
mature mRNA transcripts that contain the complete
coding sequence. MnSOD mRNA levels are induced by
5-ASA beginning at 0.02 mg/ml and further increase
through 2 mg/ml. Cu/ZnSOD mRNA levels were not
affected by treatment with 5-ASA and are used as an
internal control. Figure 1B is the result of densitome-
try data from five experiments resulting in a 2.67-fold
induction of MnSOD mRNA at 0.02 mg/ml and reaching
a 5.66-fold induction at 2 mg/ml.

Actinomycin and cycloheximide experiments. To eluci-
date the mechanism underlying the increase in Mn-
SOD mRNA in response to treatment with 5-ASA, the
cells were cotreated with 5-ASA and actinomycin D, an
RNA synthesis inhibitor, or the protein synthesis
inhibitor cycloheximide. As shown in Fig. 2, treatment
with actinomycin D alone does not alter the basal level
of MnSOD mRNA, but cotreatment with 5-ASA and
actinomycin D inhibits the 5-ASA-dependent elevation
in the level of MnSOD mRNA. Similarly, treatment
with cycloheximide alone has little effect on the basal
level of MnSOD mRNA; however, the induction of
MnSOD mRNA by 5-ASA is not inhibited by cotreat-
ment with 5-ASA and cycloheximide.

Nuclear run-on experiments. Nuclear run-on exper-
iments are the gold standard for determining that the
induction of a gene is transcriptional (17). By this
method, newly synthesized RNA in isolated nuclei can
be labeled with a high specific activity. Figure 3 is a
representative nuclear run-on experiment from control
cells and cells treated for 3 h with 5-ASA or LPS. If

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Cu/ZnSOD levels are used for the internal control, 5-ASA induces MnSOD mRNA production by twofold and LPS induces MnSOD mRNA production by fourfold. If cathepsin signal is used as the control, 5-ASA induces MnSOD mRNA production by 2.8-fold and LPS induces MnSOD mRNA production by 6-fold. Similar data has been obtained from two additional experiments.

Effect of lipoxygenase and cyclooxygenase inhibition on MnSOD mRNA levels. A proposed mechanism for the therapeutic action of 5-ASA is the inhibition of 5-lipoxygenase (33). 5-ASA has also been reported to inhibit cyclooxygenase (15). To learn if inhibition of lipoxygenase and/or cyclooxygenase was involved in the mechanism for 5-ASA induction of MnSOD, the cells were treated with MK-886, an inhibitor of 5-li-

doxygenase activating protein (39), at 10 ng/ml, indomethacin, an inhibitor of cyclooxygenase, at 1 μM/l (20), or a combination of MK-886 and indomethacin. Figure 4 is a representative Northern blot analysis of MnSOD mRNA levels following treatment with 5-ASA, indomethacin, MK-886, or indomethacin plus MK-886. Ethanol was the solvent for indomethacin and MK-886, and therefore ethanol serves as an additional control. Inhibition of 5-lipoxygenase activating protein by treatment with MK-886 and/or inhibition of cyclooxygenase by treatment with indomethacin did not affect MnSOD mRNA levels compared with control.

5-ASA oxidation. 5-ASA readily oxidizes in most solutions. To attempt to determine if the 5-ASA oxidation leads to induction of MnSOD, IRD-98 cells were treated at a final concentration of 2 mg/ml 5-ASA in the form of Rowasa enema, a 5-ASA suspension in an antioxidant carrier containing carbomer 934P, edetate disodium, potassium acetate, potassium metabisulfite,
In vitro induction of MnSOD protein levels. To verify that the induction of MnSOD mRNA by 5-ASA is followed by an increase in MnSOD protein levels, IEC-6 cells were treated with 1 mg/ml 5-ASA and MnSOD protein levels were determined by Western blot analysis. In Fig. 6A, MnSOD protein levels were determined at 12, 24, and 48 h after treatment with 5-ASA. Densitometry revealed that MnSOD protein levels are induced 1.99-fold at 24 h and 4.23-fold at 48 h and are shown in Fig. 6B.

In vivo induction of MnSOD by 5-ASA. To confirm that the induction of MnSOD observed in cell culture reflects the in vivo effects, 150-g Sprague-Dawley rats were treated with a 2-ml enema of a 4 mg/60 ml 5-ASA suspension (Rowasa enema) on four consecutive days. The results of the Western blot analysis of the distal colonic mucosal MnSOD levels are shown in Fig. 7. Densitometry indicated that treatment with 5-ASA results in a 1.7-fold induction of MnSOD protein levels compared with controls.

Protection from TNF-α-mediated cytotoxicity. An assay using the conversion of WST was used to demonstrate protection from TNF-α-mediated cytotoxicity by 5-ASA. WST reagent is converted by functional mitochondrial dehydrogenase to tetrazolium and thus is a marker of viable cells. Figure 8 shows the results of an experiment in IRD-98 cells performed in triplicate. Treatment for 24 h with 2 mg/ml 5-ASA before the addition of 2 mg/ml 5-ASA plus 100 ng/ml TNF-α and 0.5 μg/ml cycloheximide resulted in 96.7% cell survival at 12 h and 88.7% survival at 24 h. However, only 32.7% of cells treated with TNF-α/cycloheximide alone survived at the 24-h time point. The addition of 5-ASA at the same time as the addition of TNF-α/cycloheximide did not afford protection from TNF-α-mediated cytotoxicity.
5-ASA, the active constituent in sulfasalazine, is poorly absorbed in the colon, and ~50% is metabolized to acetyl 5-ASA by the intestinal epithelium and luminal bacteria (16). However, acetyl 5-ASA is even more poorly absorbed and is therapeutically inactive (16). Although the mechanism of action of 5-ASA remains ill defined, it may be therapeutically effective due to multiple effects that may protect the colon from an inflammatory response (15, 16). The induction of MnSOD may contribute to the free radical scavenging activity of 5-ASA that has been observed in cellular systems and in vivo; however, 5-ASA also has free radical scavenging activity independent of MnSOD in cell-free systems (2).

The induction of MnSOD by 5-ASA is not limited to IEC-6 and IRD-98 cells. Similar results were obtained in rat lung pulmonary epithelial L2 cells and the rat intestinal epithelial cell line FRI-1 (unpublished data). 5-ASA did not induce MnSOD in the human colon carcinoma cell line T84 (unpublished data); however, we have not been able to induce MnSOD with any stimulus in this cell line, which is consistent with our findings of abnormal MnSOD regulation in many carcinoma cell lines (5, 9). Other investigators have shown that overexpression of MnSOD results in a reduction of the malignant phenotype in multiple cell lines, including breast cancer (28), prostate cancer (29), and melanoma cell lines (9). Therefore, the induction of MnSOD may contribute to the chemopreventive properties of 5-ASA (7, 38).

The induction of MnSOD by 5-ASA is eliminated by actinomycin D but is unaffected by cotreatment with cycloheximide, implicating de novo transcription but not translation as a requirement for the induction of MnSOD mRNA levels by 5-ASA. This finding is similar to the regulation of MnSOD in IEC-6 cells by LPS, TNF-α, and IL-1β (44). Nuclear run-on experiments confirmed the transcriptional nature of the regulation of MnSOD mRNA levels by 5-ASA. This procedure results in the elongation of the transcripts initiated at the time of nuclei isolation. Therefore, the rate of RNA synthesis can be compared between the control and treated cells.

Translation was confirmed by the 4.23-fold increase in MnSOD protein levels following treatment of IEC-6 cells with 5-ASA and the 1.7-fold increase in the colonic mucosa in vivo. The lesser degree of induction observed in vivo may be the result of differences between cell culture and the in vivo environment or poor retention and penetration of 5-ASA through the mucus layer in the colon. We are not aware of other publications reporting gene induction by 5-ASA. 5-ASA has been reported to enhance the induction of heat shock protein expression in intestinal epithelial cells, but 5-ASA alone did not affect heat shock protein expression (6). Stevens et al. (44) found that 5-ASA and sulfasalazine reduced IL-2 expression in cultured T cells by a largely posttranscriptional mechanism. However, sulfasala-
zine and 5-ASA treatment of these cells also resulted in 63 and 37% cytotoxicity, respectively.

We have hypothesized that MnSOD is functioning as a cytokine-inducible acute-phase protein that functions to protect the cell from cytokine toxicity and increased intracellular free radical production (46). Recently, gene array technology has identified MnSOD as a gene that is induced fivefold in surgical specimens from patients with active ulcerative colitis (10). In an animal model of acute colitis, we have found that MnSOD mRNA is induced as early as 4 h after the colonic insult (45). Other investigators have demonstrated that MnSOD is both induced by TNF-α and IL-1 and required for protection from TNF-α and IL-1 cytotoxicity (49, 22). Treatment with 5-ASA protected IEC-6 cells from TNF cytotoxicity. TNF-α results in a free radical leak from the ubisemiquinone step (complex III) of the mitochondrial electron transport chain (41), and thus the induction of MnSOD may protect the cell from TNF-induced free radical damage. We used the TNF/cycloheximide cytotoxicity system with which Wong et al. (49) had established a cytoprotective role for MnSOD; however, IRD-98 and IEC-6 cells appear more sensitive to the effects of cycloheximide (data not shown). For MnSOD to protect cells from TNF cytotoxicity, MnSOD levels must be elevated before exposure to TNF/cycloheximide, and our data is consistent with this finding.

The mechanism of 5-ASA induction of MnSOD is not clear but likely involves a redox-sensitive transcription factor. Neither inhibition of cyclooxygenase with indomethacin nor inhibition of the 5- lipoxigenase pathway with MK-886 resulted in changes in MnSOD mRNA levels, making this mechanism unlikely for the induction by 5-ASA. Several transcription factors, such as nuclear factor-κB (NF-κB) and AP-1 are activated by TNF-α, IL-1, or oxidants (40, 35). 5-ASA readily oxidizes; however, once oxidized, 5-ASA will not induce MnSOD (unpublished data). Treatment with 5-ASA in an antioxidant carrier still resulted in induction of MnSOD; however, we are unable to confirm that intracellular oxidation was prevented. 5-ASA, through radical scavenging activity and by inducing MnSOD, may prevent the activation of NF-κB. In cancer cell lines, overexpression of MnSOD has been reported to inhibit the activation of the transcription factors AP-1, NF-κB, and c-jun (25, 27). The data on inhibition of NF-κB activation by 5-ASA is conflicting. Two reports describe inhibition of NF-κB activation by sulfasalazine but not 5-ASA (30, 48), whereas others have reported that 5-ASA does inhibit the activation of NF-κB (11, 24, 51).

The induction of MnSOD by 5-ASA at therapeutically relevant concentrations is the first demonstration of transcriptional gene regulation by 5-ASA. Our results do not address the signal transduction mechanism that leads to the induction of MnSOD by this poorly absorbable compound. It remains to be determined whether increasing the intracellular concentration of 5-ASA is required for the induction of MnSOD. The induction of MnSOD by 5-ASA may contribute to the mechanism of action of 5-ASA by reducing the cytotoxicity of cytokines and oxygen free radicals generated in the gut and by altering the activity of redox-sensitive transcription factors.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO1 DK-54919 and by the Medical Research Service of the Department of Veterans Affairs.

REFERENCES


