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

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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.

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and Sacchi (8) as previously described (47). Twenty micrograms of total cellular RNA from each sample was subjected 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 cathepsin D or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. All of the probes used were radiolabeled 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. The nuclei were pelleted by centrifugation at 1,200 g 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 procedure described by Laine et al. (26). The isolated nuclei were thawed on ice and centrifuged at 1,200 g for 5 min. 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 phosphate) and 4 μl of RNase inhibitor (40 U; stock 10 U/ml) and 250 μCi [³²P]UTP. The reaction was incubated for 45 min at 30°C with agitation. RNA, including the radioactive nascent RNA, was then isolated according to our standard procedure (47).

Nyrol membranes were dot blotted with 2.5 μg of denatured MnSOD cDNA, and various cDNA fragments of constitutively expressed genes (Cu/ZnSOD and cathepsin B) were used to quantitate the assay. Linearized pUC19 DNA served as a negative control. One milliliter of hybridization solution was added to each radiolabeled RNA sample. A 2- to 5-μl aliquot was counted in a scintillation counter. The samples were normalized based on the total counts per million (cpm), and an equal volume of hybridization solution was added. The dot blot membranes were prehybridized for 1 h at 60°C. After a 48-h incubation at 60°C in 2 ml of hybridization solution containing [³²P]-labeled RNA (containing 1–4 × 10⁷ cpm) from control and treated cells, the membrane strips were washed and autoradiographed.

Protein isolation and Western blot analysis. Following a treatment period of 12–48 h, control and 5-ASA-treated cells were rinsed twice with sterile PBS and scraped off the plate in 2 ml ice-cold PBS. The cells were pelleted, and the cellular protein was isolated as previously described (47). In the in vivo experiments, after 4 days of 5-ASA enema administration (2 ml of a 4 g/60 ml 5-ASA suspension; Rowasa; Solvay Pharmaceuticals, Marietta, GA), the rats were killed and the colons were excised. The mucosa and submucosa were separated from the remaining distal colon, and protein was isolated as previously described (45). The protein concentrations of the samples were determined by a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). A 20-μg aliquot of each sample was denatured by boiling, loaded onto a stacking gel, and fractionated on a 15% acrylamide-SDS running gel and electrotransferred to a nitrocellulose membrane as has been previously described (45, 47). The membranes were probed by incubation for 1 h with a 1:1,000 dilution of rabbit anti-rat MnSOD polyclonal antibody (in blocking buffer). The bound MnSOD antibody was detected using a donkey anti-rabbit secondary antibody and an Amersham enhanced chemiluminescence detection system (Amersham, Arlington Heights IL). The autoradiographs were analyzed with a Millipore video image densitometer or with Scion Image software (Scion, Frederick, MD).

Protection from TNF-α-mediated cytotoxicity. IRD-98 cells were plated in 96-well plates. Some wells were pretreated with 2 mg/ml 5-ASA for 24 h before the addition of 5-ASA plus TNF-α (100 ng/ml) and cycloheximide (0.5 μg/ml). Other wells were treated with TNF-α (100 ng/ml) and cycloheximide (0.5 μg/ml) or 5-ASA (2 mg/ml) plus TNF-α (100 ng/ml) and cycloheximide (0.5 μg/ml). After 12–24 h, 10 μl/well of the WST reagent (Roche Molecular Biochemicals, Indianapolis) was added, and the plates were read after 3 h at 450 nm in an ELISA plate reader (STL Lab Instruments, Grodig, Austria).

Statistics. Statistical significance was determined by the t-test, with the value deemed significant at P ≤ 0.05. All data are expressed as means ± SE.

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 densitometry 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 elucidate the mechanism underlying the increase in MnSOD 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 cotreatment with 5-ASA and cycloheximide.

Nuclear run-on experiments. Nuclear run-on experiments 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.
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-lipoxygenase activating protein (39), at 10 ng/ml, indomethacin, an inhibitor of cyclooxygenase, at 1 μM/ml (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, and other antioxidants. The results showed no induction of MnSOD mRNA levels compared with control.

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**Fig. 1.** A: representative Northern blot analysis of manganese superoxide dismutase (MnSOD) and copper/zinc superoxide dismutase (Cu/ZnSOD) levels following treatment with increasing concentrations of mesalamine (5-ASA) for 8 h. Cu/ZnSOD levels were used as an internal control for densitometry. B: densitometry data plotted as the mean fold induction ± SE relative to control; n = 3–8 for each concentration; *P < 0.02; **P < 0.005.

**Fig. 2.** IEC-6 cells were cotreated with 5-ASA and actinomycin D or cycloheximide for 8 h, and MnSOD and Cu/ZnSOD levels were evaluated by Northern blot analysis. A representative study is shown.

**Fig. 3.** is a representative nuclear run-on experiment from control cells (C) and cells treated for 3 h with 5-ASA or lipopolysaccharide (LPS); n = 3. Nylon membranes were dot blotted with 2.5 μg of denatured MnSOD cDNA and various cDNA fragments of constitutively expressed genes (Cu/ZnSOD and cathepsin B) used to quantitate the assay, and linearized pUC19 DNA served as a negative control. The membranes were probed with newly synthesized RNA from isolated nuclei.
sodium benzoate, purified water, and xanthan gum. 5-ASA in the form of Rowasa has a shelf life of several months and thus is effective in inhibiting 5-ASA oxidation. Figure 5 shows that 5-ASA in the form of Rowasa induces MnSOD mRNA levels similar to that observed with nonproprietary 5-ASA. Similar results were observed in IEC-6 cells (data not shown).

**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.

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**Figures:**

- Fig. 4. IEC-6 cells were treated with MK-886, an inhibitor of 5-lipoxygenase activating protein, at 10 ng/ml, with indomethacin (Indo), an inhibitor of cyclooxygenase, at 1 μM/l, or with MK-886 plus indomethacin. Ethanol (EtOH) was the solvent for indomethacin and MK-886; therefore, ethanol-treated cells serve as an additional control. MnSOD and cathepsin mRNA levels were evaluated by Northern blot analysis.

- Fig. 5. IRD-98 cells were treated for 8 h. Total RNA was isolated, and MnSOD and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels were determined by Northern blot analysis. The cells were treated with 5-ASA suspension in an antioxidant carrier (Rowasa enema) to give a final concentration of 5-ASA in the media of 2 mg/ml, and untreated cells served as control (C).

- Fig. 6. A: evaluation of MnSOD protein levels in IEC-6 cells by Western blot analysis at 12, 14, and 48 h after treatment with 1 mg/ml 5-ASA. B: densitometry data from the Western blot analyses (n = 3) are plotted as the mean fold induction ± SE relative to control. *P < 0.015.

- Fig. 7. MnSOD protein levels in the distal colonic mucosa and submucosa of rats treated with a 2 ml enema of a 4 mg/60 ml 5-ASA suspension (Rowasa enema; lanes 3, 4, and 5). Untreated animals served as controls (lanes 1 and 2). Densitometry shows that 5-ASA treatment results in a 1.7-fold increase in MnSOD protein levels.

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Plyizes the reaction $2\text{O}_2$ sequence homology with Cu/ZnSOD (22). SOD catalyzes the conversion of two superoxide radicals ($2\text{O}_2^-$) into peroxide and water ($2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$), thus eliminating oxygen free radicals. $\text{H}_2\text{O}_2$ is then inactivated by catalase and glutathione peroxidase to form water. In IBD, the production of oxygen free radicals by cytokines and activated neutrophils may overwhelm the intestinal defense mechanisms. This is particularly relevant since the colonic mucosa, submucosa, and muscularis mucosa have been found to contain low levels of SOD, catalase, and glutathione peroxidase activity (18). Evidence of direct oxidant-induced colonic epithelial cell injury in IBD has been established by examination of the oxidation of GAPDH in freshly isolated colonic epithelial cells (31). Treatment with SOD has been shown to reduce inflammation in acetic acid (13) and trinitrobenzenesulfonic acid (52) models of colitis and in Crohn’s disease (12). Cytoprotective properties of MnSOD have been demonstrated in other tissues as well. Overexpression of MnSOD in the lung in transgenic mice reduces the toxic effects and improved survival in mice exposed to 95% oxygen (50).

The induction of MnSOD mRNA by 5-ASA is dose dependent and within the concentration range of 0.5–3.5 mM interstitial concentrations (15) to 7 mM luminal concentrations (34) that have been documented in patients taking 2–3 g of sulfasalazine orally. Oral 5-ASA is frequently given in doses of 2.4–4.8 g/day (equivalent to 6–12 g of sulfasalazine), and the concentration of 5-ASA in enema form (4 g/60 ml) is 433 mM (19). Consequently, newer forms of 5-ASA delivery provide even higher concentrations of 5-ASA to the colon and result in even higher interstitial and luminal levels of 5-ASA (14). 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 cell line 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 the finding 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-lipoxygenase 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.

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