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5-AMINOSALICYLATE: OXIDATION BY ACTIVATED LEUKOCYTES AND PROTECTION OF CULTURED CELLS FROM OXIDATIVE DAMAGE

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Abstract—It has been postulated that oxygen radicals may play a role in the pathogenesis of inflammatory bowel disease. If so, then a drug like 5-aminosalicylate (5-ASA), which is used to treat such diseases, might work by interacting with oxygen-derived species. We found that activated mononuclear cells and activated granulocytes, as well as the products of the Fenton reaction, transformed [^{14}C]5-ASA to a number of metabolites, among which we have characterized salicylate and gentisate. We also found that the lethal effect on cultured Chinese hamster ovary cells of adding either superoxide radical or hydrogen peroxide, components of the respiratory burst of activated white blood cells, was diminished by the addition of 100 μg/ml (0.65 mM) of 5-ASA. Thus, we have demonstrated that 5-ASA was oxidized by the oxidative burst of white blood cells and that 5-ASA protected cells from damage by oxygen-derived species, two findings which may offer an explanation for the role of 5-ASA in the treatment of inflammatory bowel disease.

The efficacy of sulfasalazine in effecting the remission and prophylaxis of human ulcerative colitis [1] can be attributed to 5-aminosalicylate (5-ASA)† [2–5], a metabolite resulting from the reductive cleavage of sulfasalazine that is mediated by the intestinal flora [6]. Although 5-ASA is effective in clinical trials [2–5], its mechanism of action remains unknown.

An inflammatory reaction, which includes infiltration by polymorphonuclear leukocytes, macrophages and lymphocytes, is a major component of the pathology of ulcerative colitis. It would be expected, therefore, that ulcerated areas of intestine are subject to the "respiratory burst" of phagocytic cells—a reaction that is characterized by the production of such oxygen-derived species as O_2^- H_2O_2, OH· and $^1O_2$ [7]. These species are cytotoxic, break DNA strands, initiate lipid peroxidation, and inactivate enzymes [8–12]. They also react with various phenols and anilines [13]. It seems possible, therefore, that 5-ASA may be effective in ulcerative colitis by means of a cytoprotective effect mediated by its absorption of various oxygen-derived species.

One might expect at least two consequences of such a hypothesis. First, 5-ASA might be oxidized by the respiratory burst and, second, 5-ASA might show the capacity to protect cells from damage by various forms of oxygen-derived species.

In this paper we describe experiments showing that 5-ASA inhibited the lethal effect of either KO_2 or H_2O_2 on Chinese hamster ovary (CHO) cells. Furthermore, we found that 5-ASA was transformed to a variety of metabolites, including salicylate and gentisate (2,5-dihydroxybenzoate), by activated human mononuclear cells and granulocytes, as well as when exposed to such components of the respiratory burst as superoxide, hydroxyl radicals, and hydrogen peroxide [7].

EXPERIMENTAL PROCEDURE

Materials. [^{14}C]5-ASA radiolabeled in the carboxyl position was prepared from [7-^{14}C]salicylate by a modification of the procedure described by Cirstea *et al.* [14]. To 1 ml of a solution of nitrosonium aniline at 0° was added 2 ml of water at 0° in which was dissolved 100 mg of salicylic acid containing 50 μCi of [7-^{14}C]salicylic acid (New England Nuclear, Boston, MA) and 440 mg of anhydrous Na_2CO_3. The mixture was stirred for 15 min at 0°, and then the procedure of Cirstea *et al.* [14] was followed except that the scale was 2% of that described. The resultant [7-^{14}C]5-ASA was twice recrystallized by dissolving it in a minimal amount of water followed by the addition of an equal amount of concentrated HCl. Non-radiolabeled 5-ASA was a gift from Rowell Laboratories, Inc. (Baudette, MN). The purity of both radiolabeled and non-radiolabeled 5-ASA was verified by HPLC. All other chemicals employed were of reagent grade and, together with the enzymes used, were obtained from the Sigma Chemical Co. (St. Louis, MO).

Incubation of 5-ASA with various fractions of human blood cells. Under a protocol approved by the Harvard Human Studies Committee, 70 ml of venous blood was drawn from a healthy human vol-

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† Abbreviations: 5-ASA, 5-aminosalicylate; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; SOD, superoxide dismutase; HEPES, 4(2-hydroxyethyl)-1-piperazine sulfonic acid; and MEM, Eagle's minimum essential medium.

unteer who had been medication-free for at least 3 weeks; the "mononuclear cell fraction" was obtained [15], which when suspended in 10 mM HEPES buffer, pH 7.4, at a concentration of 10^5 cells/ml was found to contain 32% monocytes and 68% lymphocytes. The suspended cells, in 10-ml aliquots, were added to 15 ml polypropylene tubes (Becton Dickenson Laboratories, Oxnard, CA) at room temperature, and the contents of each tube were activated by the addition of 125 μg of cytochalasin B in 10 μl dimethyl sulfoxide (DMSO). The tubes were gently inverted and then allowed to stand at room temperature. After 15 min the chemotactic peptide, formyl-methionyl-leucinyl-phenylalanine (100 μg/10 μl DMSO) was added to each tube. Two minutes later, crystalline [7-^{14}C]5-ASA (10 mg; 10^7 cpm) was added and dissolved by gentle mixing. The reaction vessel, mixed continuously by repeated gentle inversion, was maintained at room temperature for 2 hr. Reaction mixtures that lacked cytochalasin B and formyl-methionyl-leucinyl-phenylalanine served as controls to indicate the need for activation of the white cells in order to transform 5-ASA. Other reaction mixtures contained the complete system described above together with 1000 units/ml of superoxide dismutase (SOD) and 1000 units/ml of catalase in order to assess the importance of oxygen-derived species. In all cases, the reaction was terminated by the addition of 500 μl of concentrated HCl, the samples were clarified by centrifugation (800 *g* for 15 min), and the supernatant solution was analyzed by HPLC as described below.

Similar experiments were also performed on "granulocytes" that were separated from 70 ml of fresh venous blood [15] and suspended in 10 mM HEPES buffer, pH 7.4, at a concentration of 10^5 cells/ml.

Chemical oxidation of 5-ASA. The products of the chemical oxidation of 5-ASA were determined by dissolving 5-ASA (5 mg) in 5 ml of 0.1 M KPO_4 buffer at pH 7.0 and then, with vigorous stirring (Vortex mixer, Fisher Scientific, Pittsburgh, PA), adding either 1 mg of pulverized KO_2 or 120 μg of H_2O_2 and allowing the reaction to continue at room temperature for 2 hr. Untreated 5-ASA in the same buffer served as the control. Reactions were terminated by the addition of 0.2 ml of 10 N HCl, and samples were stored at −15° until analyzed.

Alternatively, 5-ASA was exposed to hydroxyl radicals generated by the Fenton reaction [16]. In this procedure, $FeSO_4 \cdot 7H_2O$ (13.9 mg) was dissolved in 10 ml of 0.1 M KPO_4 buffer, pH 7.2, that also contained 20 mM Na_2EDTA and 44 mM H_2O_2. After stirring this solution for 20 min, 10 mg of crystalline 5-ASA was added and the stirring continued for another 2 hr, during which time 1-ml aliquots were removed periodically for analysis. Similar incubation mixtures contained either SOD (1000 units/ml), catalase (1000 units/ml), or the combination of the two enzymes. All reactions were terminated by the addition of 200 μl of 10 N HCl and then stored at −15° until analyzed.

Analysis of metabolites of 5-ASA. HPLC was performed with a Hewlett–Packard (Palo Alto, CA) model 1080A liquid chromatograph using either a 5 μ C-18 or a C-8 reverse phase column (25 cm × 4.6 mm; Supelco, Bellefonte, PA), held at 25°. The eluting solvent initially was isocratic 5% methanol in 0.1 M KPO_4 buffer, pH 2.0, for 3 min at a flow rate of 1.5 ml/min. A linear gradient was then applied that brought the concentration of methanol to 50% over a period of 20 min. 5-ASA was eluted at 3.0 min. The column was regenerated, first with a linear gradient that reached 5% methanol within 5 min, and then with an additional isocratic elution with 5% methanol for 5 min. The eluate was collected in 1-ml fractions using a FRAC-100 (Pharmacia, Piscataway, NJ) fraction collector, and aliquots (50 μl) were assayed for radioactivity by means of a Packard TriCarb model 4530 (United Technologies, Downers Grove, IL) liquid scintillation spectrophotometer. Fractions with ultraviolet-absorbing (225 nm) peaks that also contained radiolabel were retained, lyophilized and stored at −15° until analyzed further.

Aliquots of such samples (1 mg) were prepared for gas chromatography as their trimethylsilyl derivatives by adding 100 μl Silon BFT (Supelco, Bellefonte, PA) and 50 μl dry pyridine. The mixture was then heated at 80° for 2 hr. Chromatography was performed with a Hewlett–Packard 9890 gas chromatograph equipped with a 30 meter, DB 1701 (J & W Scientific, Rancho Cordova, CA) capillary column; helium was the carrier gas. The column temperature was held initially at 50° for 0.5 min and then raised at a rate of 70°/min to 100°. After 0.5 min at this temperature, the column was brought to 300° at a rate of 8°/min. Mass spectroscopic identification was performed by interfacing the gas chromatograph to a Finnegan MAT 312 mass spectrometer (Varian Instruments, Florham Park, NJ) operated in the electron impact mode.

Cytotoxicity assay. Chinese hamster ovary (CHO-K1) cells (provided by Drs. Terry Woodford and Arthur Pardee of the Dana Farber Cancer Center, Boston, MA) were allowed to grow in 100 mm tissue culture dishes (Corning Glass, Corning, NY) containing 10 ml of Eagle's minimal essential medium (MEM, K.C. Biologicals, Lexena, KS) supplemented with 10% fetal bovine serum, 100 units penicillin and 100 μg streptomycin (complete MEM) at 37° in a humidified incubator containing an atmosphere of 5% CO_2 in air. The cells, when confluent, were detached with a solution that contained 0.2% trypsin and 0.02% EDTA in normal saline (K.C. Biologicals) and collected in a pellet by centrifugation at 800 *g* for 15 min. After resuspension in 20 ml of complete MEM, these cells were used to seed tissue culture plates (300–500 cells/plate). When the cells became adherent, usually within 6 hr, cytotoxicity assays were carried out by a modification of the methods of Cunningham and Lokesh [17] and Andrae *et al.* [18].

For the cytotoxicity assay, the medium of the adherent cells was removed and replaced with 10 ml of MEM, that also contained 100 μl of 5-ASA in DMSO (10 mg/ml), as well as various concentrations of either KO_2 (in DMSO) or H_2O_2. (It was determined previously that 5-ASA at concentrations up to 100 μg/ml has no effect on the viability of these cells under these conditions.) After 3 hr, the medium containing 5-ASA and the various concentrations of oxidants was removed, and the adherent cells were

washed twice with 7 ml of Hanks' balanced buffered saline (K.C. Biologicals). The plates were then replenished with 15 ml of complete MEM, and the cells were allowed to grow at 37° until controls were almost confluent (approximately 7 days). The medium was then removed. The cells, after two washes with 7 ml of Hanks' balanced buffered saline, were detached by treatment with 10 ml of a solution that contained 0.2% trypsin and 0.2% EDTA in normal saline and, after appropriate dilution, quantified using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL, model ZBI). Survival of treated cells was expressed as a percentage of the survival of cells that had not been exposed to either 5-ASA or oxidizing agents. Some cell cultures were treated in medium that contained 1000 units/ml of catalase and 1000 units/ml of SOD to verify the role of oxygen-derived species in the observed cytotoxicity.

RESULTS

Products of the oxidation of 5-ASA. HPLC records of the reaction mixture obtained from the incubation of [7-^{14}C]5-ASA with activated human mononuclear cells revealed the presence of several ultraviolet absorbing peaks (Fig. 1). Of these, two were particularly prominent, one at 3.0 min which represents 5-ASA, the other at 15.5 min. Additional less prominent ultraviolet absorbing peaks were detected at 5.4, 10.2, 10.7, 12.6, 13.5 and 28.4 min. As each of these peaks contained radiolabel, they were considered to be derived from 5-ASA. When the cell preparations were not activated, HPLC records (not shown) demonstrated only one peak eluting at 3 min, which was due to the [^{14}C]5-ASA in the incubation mixture. Furthermore, the addition to the reaction mixture of SOD (1000 units/ml) and catalase (1000 units/ml) also eliminated the appearance in the HPLC record of all peaks except that eluting at 3 min. To purify the metabolites of 5-ASA on a larger scale, 10 ml of the reaction mixture was added to a cation exchange column (1.5 × 15 cm) of AG50W×4 (200 mesh, Bio-Rad Laboratories, Richmond, CA) and eluted with H_2O (70 ml). Under these conditions, 5-ASA and other cations were retained on the column, and HPLC analysis of the water eluate revealed all the metabolites mentioned above except those eluting at 3.0 (5-ASA) and 13.5 min. The compounds in these two peaks were recovered, however, when the column was eluted with 1 N HCl (70 ml).

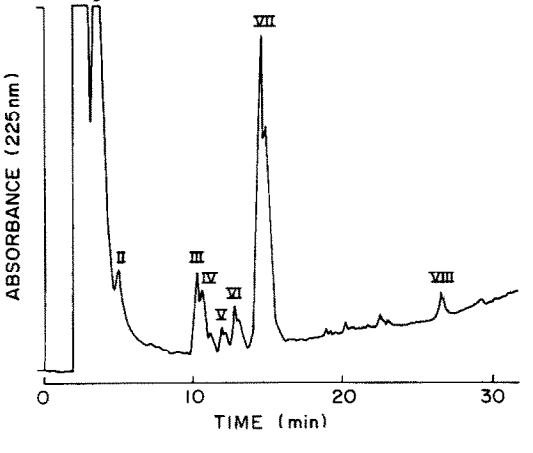

Fig. 1. A representative HLPC chromatogram obtained from the incubation of 5-ASA with activated human mononuclear cells. A 5 μ C-18 column was used.

Because OH· has been found to mediate the hydroxylation or oxidative deamination of various aromatic amines [7, 13], we concentrated our efforts on identifying the anionic compounds. Thus, we found that authentic samples of salicylate and gentisate had retention times, respectively, of 5.4 and 15.5 min, which matched those of two of the compounds that resulted from the incubation of mononuclear cells with 5-ASA; furthermore, the products obtained from the reaction mixture had the same absorption spectra as the authentic samples. The identity of these metabolites was confirmed by mass spectral analysis.

Quantification of these metabolites (Table 1), based on the recovery of radiolabel, was 0.2% for gentisate and 0.14% for salicylate. The other metabolites remain unidentified. By making comparisons of retention times and spectra of the unidentified peaks with those of authentic standards, it was possible to exclude the following compounds as metabolites of 5-ASA; 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,6-dihydroxybenzoic acid, and 2,4,6-trihydroxybenzoic acid.

When 5-ASA was incubated with activated human granulocytes the metabolites shown in Fig. 1 again appeared, and it was again possible to use ultraviolet absorption spectra and HPLC retention time to confirm the presence of salicylate and gentisate. Furthermore, the formation of these products was again eliminated by the addition of SOD (1000 units/ml) and catalase (1000 units/ml). When 5-ASA was oxidized by either KO_2 or H_2O_2, however, salicylate failed to appear. The Fenton reaction, however, resulted in the formation of salicylate, presumably because it produced a higher concentration of OH·.

Use of 5-ASA to protect CHO cells from damage by KO_2 and H_2O_2. In initial experiments, CHO cells were exposed for 3 hr to media that contained various concentrations of 5-ASA up to 400 μg/ml, and it was found that cell viability was unaffected by concentrations of 5-ASA up to 100 μg/ml. Therefore, the protective effect of 5-ASA was examined by adding it to the medium at a concentration of 100 μg/ml. The results (Fig. 2A) indicate that this concentration of 5-ASA diminishes the lethal effects on CHO cells of various concentrations of KO_2 up to 100 μg/ml.

To confirm this effect of 5-ASA in diminishing the lethal effects of KO_2 on CHO cells, six additional preparations of these cells were treated with KO_2 (at concentrations of either 22 or 45 μg/ml) in the presence or absence of 5-ASA (100 μg/ml). These concentrations of KO_2 were selected because they appear, from Fig. 2A, to show the largest protective effect of 100 μg/ml of 5-ASA. Table 2 confirms that the effect of 5-ASA in protecting CHO cells from KO_2 is statistically significant.

Similar experiments showed that 5-ASA protected CHO cells from the lethal effect of added H_2O_2 (Fig.

Table 1. Fate of radiolabeled 5-ASA with activated mononuclear cells*

Retention time (min)	Identity	Radioactivity (cpm/peak)	Recovery of radiolabel (%)
3.0 (I)†	5-ASA	9.15×10^6	91.5
5.4 (II)	Gentisate	19,800	0.2
10.2 (III)	‡	1,190	0.01
10.7 (IV)	‡	950	0.01
12.6 (V)	‡	780	0.008
13.5 (VI)	‡	480	0.005
15.5 (VII)	Salicylate	14,000	0.14
28.4 (VIII)	‡	890	0.009

* Incubation of mononuclear cells with [^{14}C]5-ASA (10 mg, 1.53×10^5 cpm/μmol) was as described in Experimental Procedure.
† Roman numerals refer to peaks shown in Fig. 1.
‡ Not identified.

2B; Table 2). It must be emphasized, however, that the lethal effect on these cells may not be mediated directly by either O_2^- or H_2O_2, but rather by one of several species of oxygen derived from them [7, 19]. We were unable to determine whether SOD or catalase protected the CHO cells from either O_2^- or H_2O_2 because these enzymes themselves appeared to have a toxic effect on the cells.

DISCUSSION

The oxidation of 5-ASA mediated by two preparations of activated white blood cells resulted in the formation of several metabolites, of which two, salicylate and gentisate, have been identified. That these metabolites are formed as the result of the respiratory burst is not unexpected as others have shown that a secondary component of the respiratory burst, OH·, is capable of deaminating 5-ASA and related compounds [13]. However, the observation that 5-ASA afforded protection of CHO cells from such oxygen-derived species as superoxide and hydrogen peroxide suggests that the ability of 5-ASA to absorb reactive oxygen species may help to explain the action of this drug in inflammatory bowel disease.

Whether the ability of 5-ASA to interact with various forms of oxygen-derived species and its ability to protect CHO cells provide a model for the therapeutic effect of 5-ASA in inflammatory bowel disease is difficult to determine. To estimate the extent to which 5-ASA might protect critical sites in susceptible cells from attack by oxygen-derived species would require that one know the concentration of both 5-ASA and the various species of activated oxygen at sites of successfully treated inflammatory bowel disease. In the absence of such data, it is impossible to judge whether the concentrations of 5-ASA used in these experiments are realistic. Nevertheless, the concentration of 5-ASA used in the cytoprotective experiments, 0.65 mM, was at least an order of magnitude lower than the concentration of 5-ASA found in the colons either of patients taking sulfasalazine (10 mM) [20] or guinea pigs administered sulfasalazine to protect them from carrageenan-induced colitis (7.2 mM) [21]. It must also be emphasized that assaying the viability of CHO cells simply provides a convenient measurement of biological damage by various oxygen-derived species and bears no clear relationship to what might occur in inflammatory bowel disease. Such studies, therefore, provide only a proposal for how 5-ASA might work in the treatment of ulcerative colitis.

In terms of this hypothesis, it might be appropriate to ask why salicylate, which is also known to interact with various oxygen-derived species, does not appear to be effective in treating ulcerative colitis. The answer may lie in the pharmacokinetic properties of 5-ASA. As a zwitterion, 5-ASA tends to remain in

Table 2. Use of 5-ASA to protect the viability of CHO cells subjected to either KO_2 or H_2O_2*

Treatment	Concentration (μg/ml)	Viability (%)† Without 5-ASA	With 5-ASA
KO_2	22	46.9 ± 4.6	74.7 ± 7.1‡
KO_2	45	29.1 ± 0.7	51.3 ± 4.9‡
H_2O_2	800	16.4 ± 2.0	57.0 ± 6.3‡
H_2O_2	1200	10.0 ± 1.1	28.5 ± 1.0‡

* The procedures for incubating CHO cells for 3 hr with various concentrations of either KO_2 or H_2O_2 in the presence or absence of 5-ASA (100 μg/ml) are described in Experimental Procedure.
† Expressed as mean ± SD of six experiments.
‡ Results are significantly different ($P < 0.05$) from corresponding cells without 5-ASA as assessed by Student's *t*-test.

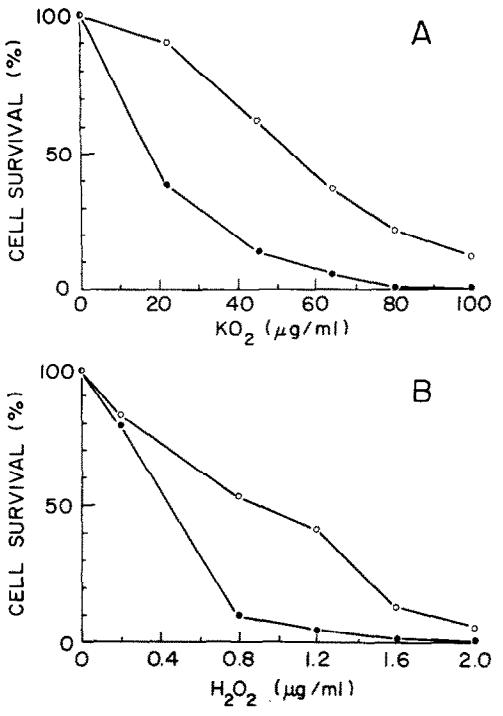

Fig. 2. Protective effect of 5-ASA toward KO_2 (A) and H_2O_2 (B) on CHO cells. Cells were challenged with the indicated concentrations of either KO_2 or H_2O_2 in the presence (○) or absence (●) of 5-ASA (100 μg/ml) as described in Experimental Procedure.

the gastrointestinal tract when released from sulfasalazine [20–22] and hence to maintain high concentrations in proximity to the inflamed mucosa [20, 21]. Although aspirin and other non-steroidal anti-inflammatory drugs can absorb such oxygen-derived species as hydroxyl radicals and therefore prevent their damage to such biological processes as phage replication [23], these drugs are not effective for treating ulcerative colitis, perhaps because they do not reach an adequate concentration at the site of inflammation in the gut.

Our results do not contradict the view that 5-ASA and its prodrug sulfasalazine may control ulcerative colitis by inhibiting synthesis of prostaglandins of the E series [24–26]. Prostaglandin synthesis is inhibited by a variety of scavengers of hydroxyl radicals [27] that include salicylate and gentisate [28]. Indeed, the potency of gentisate as a prostaglandin synthesis inhibitor has led to the suggestion that small amounts of gentisate formed *in vivo* may be responsible for the inhibitory effects of salicylate that are observed *in vivo* [28, 29]. It is also possible that other metabolites formed in the oxidation of 5-ASA, which we have not characterized, may be even more potent inhibitors of prostaglandin synthesis. However, such inhibitors of prostaglandin synthetase are formed in low concentrations and are likely to be too diffusible to achieve local concentrations high enough to exert any significant effect *in vivo*.

It appears, therefore, that 5-ASA may be effective in ulcerative colitis because of two properties: the first, to remain concentrated in the lumen of the bowel [20–22] adjacent to areas of inflammation and, the second, to scavenge oxygen-derived species and so to interrupt the cycle of tissue destruction brought about by the oxidative bursts of the inflammatory reaction. If these two properties are the essential ones, then better drugs might be sought in compounds that are both localized to the colon and have the ability to scavenge reactive oxygen-derived species.

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