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THE PRODUCTION OF AN AMINE-MODIFIED DERIVATIVE OF 5-AMINOSALICYLIC ACID BY ACTIVATED NEUTROPHILS

ROLES FOR MYELOPEROXIDASE AND CHLORIDE IONS

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Abstract—Following incubation with activated neutrophils, two metabolites of 5-aminosalicylic acid (5-ASA) were identified by HPLC. These two metabolites accounted for approximately 60% and 20% of the original 5-ASA. The formation of the major metabolite was prevented by pre-incubation with the peroxidase inhibitor, azide, and reduced by the omission of chloride ions from the incubation medium, or the presence of catalase. A similar product was generated by sodium hypochlorite or myeloperoxidase/H_2O_2, mass spectroscopical analysis being consistent with it being 5-nitroso-salicylate. Our finding suggests that the efficacy of 5-ASA results from its ability to react with and so scavenge hypochlorite ions. The amount of amine-modified 5-ASA in the faecal stream may thus provide an indicator for hypochlorite production in the bowel.

The drug 5-aminosalicylic acid (5-ASA‡) has been shown to be effective in the treatment of inflammatory bowel disease [1–4]. Several groups have demonstrated that this molecule reacts with various oxygen-derived species generated by neutrophils and monocytes *in vitro* [5–7]. Dull *et al.* [5] have demonstrated the generation of small amounts of gentisate and salicylate by monocytes. Aruoma *et al.* [6] have demonstrated that 5-ASA reacts with both hydroxyl radical and hypochlorite. Superoxide generation and oxygen consumption by activated neutrophils are unaffected by the presence of 5-ASA, whereas luminol-dependent chemiluminescence is completely inhibited [7–10]. We have proposed that the luminol-reactive molecule with which 5-ASA reacts is hypochlorite.

In the work reported here, we have identified the nature of the neutrophil-derived products using HPLC separation and mass spectroscopic analysis of the major metabolite of 5-ASA generated under controlled *in vitro* conditions. This metabolite was also generated by hypochlorite or myeloperoxidase plus hydrogen peroxide and was identified as 5-nitroso-salicylate, probably formed by decomposition from a chloramine.

MATERIALS AND METHODS

Reaction of 5-ASA with stimulated neutrophil preparations. Rat peritoneal neutrophils were isolated from caseinate-induced peritoneal fluid as described previously [11]. Cell suspensions (approx. 3×10^7 cells/mL) were suspended in either (i) Krebs medium containing NaCl 120 mM, KCl 4.8 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $CaCl_2$ 1.3 mM, *N*-2-hydroxyethylpiperazone-*N'*-2-ethanesulfonic acid (HEPES) 25 mM, 0.1% bovine serum albumin, pH adjusted to 7.4 with NaOH; or (ii) chloride-free medium containing Na_2SO_4 75 mM, KH_2PO_4 5 mM, $MgSO_4$ 1.2 mM, $Ca(OH)_2$ 1.3 mM, HEPES 25 mM, 0.1% bovine serum albumin, pH 7.4. The cell suspension was incubated with [^{14}C]5-ASA (100 μM) at 37° with gentle mixing in the dark, after stimulation with f-met-leu-phe (1 μM) plus cytochalasin B (5 μg/mL) for up to 2 hr. These conditions were chosen (i) as the optimum for reaction between neutrophil oxidation productions and 5-ASA and (ii) because these neutrophil and 5-ASA concentrations may be similar to those expected in the inflamed gut wall. At the end of the incubation period, the supernatant was treated with HCl in preparation for HPLC analysis.

Separation of metabolites of 5-ASA. Neutrophil derived metabolites of 5-ASA were separated by HPLC (Spectra-Physics SP8750) using a 10 μ C18 reverse phase column (3.8 × 300 mm, Waters μBondapak, Millipore Corp., Milford, MA, U.S.A.). Elution conditions were as follows: (A) 0.1 M K_2HPO_4 pH 2.5 and (B) 50% (v/v) methanol in solution A, using a linear gradient of 99.5% A/0.5% B and reaching 0.5% A plus/99.5% B in 30 min at a flow rate of 1 mL/min. The eluate was collected in 1 mL fractions, absorbance being monitored at 225 nm. Aliquots of the fractions were taken for liquid scintillation counting and fluorescence spectroscopy. The column was regenerated after reversing the gradient and washing with starting conditions for 10 min.

Mass spectroscopic analysis. The major neutrophil-derived metabolite of 5-ASA was isolated for mass spectroscopic analysis by HPLC as before but solutions A and B were changed to the volatile agents, 0.05% TFA and CH_3CN (90%) in 0.05%

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‡ Abbreviations: 5-ASA, 5-aminosalicylic acid; TFA, trifluoacetic acid.

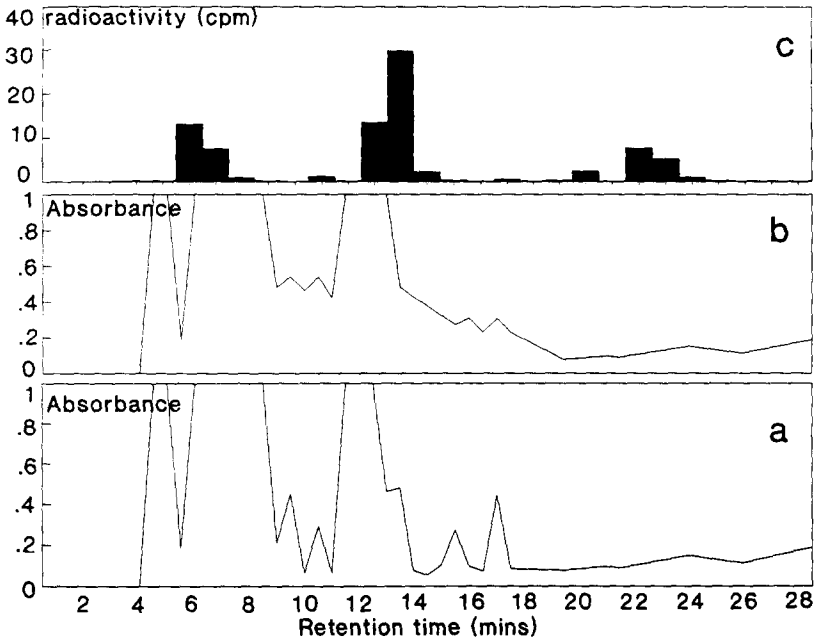

Fig. 1. Presence of non-5-ASA derived peaks in HPLC elution profiles. The supernatants from neutrophils incubated with f-met-leu-phe (1 μM) plus cytochalasin B (5 μg/mL) for 2 hr in the presence (a and c) or absence (b) of [^{14}C]5-ASA were run on HPLC. Absorbance at 225 nm was monitored as fractions were collected from the HPLC column. The diagrams show absorbance profiles (a) in the presence and (b) in the absence of [^{14}C]5-ASA. The histograms (c) show the ^{14}C-radioactivity in each fraction from the same experiment. The first peak represents 5-ASA and the other two peaks are the major and minor metabolites of 5-ASA.

TFA, respectively. These solvents were removed by evaporation before analysis by mass atomic bombardment and positive chemical ionization at the SERC Mass Spectroscopy Service Centre, Dept. of Chemistry, University College of Swansea. Mass spectra were compared with unreacted 5-ASA.

Materials. [^{14}C]5-ASA was prepared by Amersham Radiochemicals (Amersham, U.K.) with a specific activity of 440 MBq/mmol and purity of 95%. Stock [^{14}C]5-ASA was stored at −20°. 5-ASA, gentisate, salicylate, 5-nitro-salicylate, were obtained from Sigma/Aldrich (Poole, U.K.). Myeloperoxidase was obtained from Calbiochem (Novabiochem, Nottingham, U.K.). All other standard reagents were obtained from the Sigma Chemical Co. or BDH (Poole, U.K.).

RESULTS

Two major neutrophil-derived metabolites or 5-ASA detected by HPLC

HPLC analysis of the supernatant from activated neutrophils in the absence of 5-ASA revealed that a number of highly absorbing molecules were generated by the cells (Fig. 1). [^{14}C]5-ASA was, therefore, used as the substrate, and metabolites were identified unequivocally and quantitatively by the measurement of ^{14}C content. After reaction with activated neutrophils two metabolites of [^{14}C]5-ASA were observed, with retention times of 12–13 and 22–24 min (Fig. 1). These peaks accounted for between 20–60 and 2–15% of the total radioactivity, respectively. The appearance of the major metabolite was rapid, 90% of the products appearing during the first 5 min. The major metabolite was weakly fluorescent, having an emission peak at 450 nm (excitation = 340 nm). This fluorescent spectrum was distinct from 5-ASA (500 nm), salicylate (412 nm) and 5-nitrosalicylate (non-fluorescent) but similar to gentisate (453 nm). Whereas the major metabolite was generated in large amounts in all experiments, the quantity of the minor metabolite varied considerably between different cell preparations.

Reaction of 5-ASA with hypochlorite

Incubation of [^{14}C]5-ASA with sodium hypochlorite or a hypochlorite-generating system (myeloperoxidases/hydrogen peroxide) resulted in the production of a number of ^{14}C-containing molecules. At high hypochlorite concentrations (greater than 10 mM), the majority of products had longer retention times (17–28 min) and were non-fluorescent. With milder reaction conditions (either lower hypochlorite concentrations, around 5 mM, or the use of an OCl^--generating system), a compound was generated with a retention time of 12–13 min, which was not significantly different from the major cell-derived metabolite (Fig. 2c). This compound also had a similar fluorescence spectrum to the major neutrophil-derived metabolite. The possibility

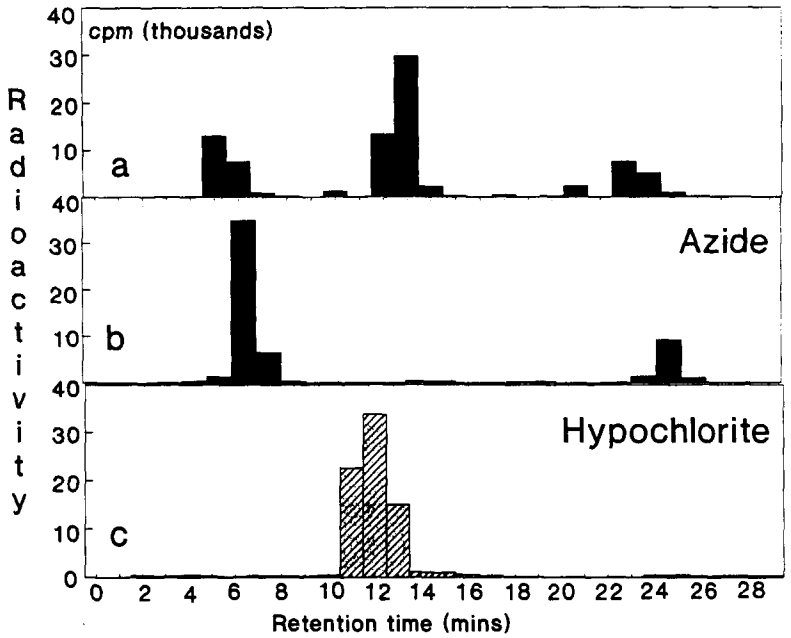

Fig. 2. Production of the major neutrophil-derived metabolite of 5-ASA by hypochlorite. The supernatants from activated neutrophils incubated in the presence of [^{14}C]5-ASA were run on HPLC. The histograms show the ^{14}C-radioactivity in the fractions from (a) untreated neutrophils and (b) pretreated with azide (1 mM). For comparison the profile of 5-ASA (1 mM) treated with NaOCl (5 mM) for 10 min is also shown (c).

Table 1. Neutrophil-derived metabolites of [^{14}C]5-ASA

Condition	Metabolite 1* (%)	Metabolite 2* (%)
Control	100	100
Azide (1 mM)	1.75 ± 0.75	116 ± 43
Cl^--Free medium	30 ± 2	143 ± 16
Catalase	60 ± 8	89 ± 5.5

* Data are expressed as percentage of control for each cell preparation. The data represent seven separate HPLC analyses. Metabolites 1 and 2 were the major and minor metabolites as defined previously.

therefore existed that the major neutrophil-derived metabolite was also generated by hypochlorite.

Dependence of neutrophil-derived metabolite on peroxidase and chloride ions

The possibility that the major neutrophil-derived metabolite was a hypochlorite-derived molecule was tested by determining the effect on the generation of the major metabolite of prevention or reduction in hypochlorite generation by neutrophils. Pretreatment of the cells with the peroxidase inhibitor, azide (1 mM), totally abolished luminol-dependent chemiluminescence and prevented completely the generation of the metabolite (Fig. 2), although the minor metabolite was still generated. Replacing the chloride ions in the medium with sulphate reduced luminol-dependent chemiluminescence by approximately 90% and reduced the appearance of the major metabolite (Table 1). Catalase also reduced its production but to a lesser extent (Table 1), presumably because of its competition with myeloperoxidase for H_2O_2. These observations are therefore consistent with the generation of the major metabolite being dependent upon hypochlorite.

Chemical identification of the major metabolite

Potassium iodide or 2-thiol-2-nitrobenzoic acid [12] has often been used to detect chloramines, although other oxidants also react. Using these molecules, there were three peaks of activity in the HPLC profiles, corresponding to the parent compound and the major and minor metabolites (Fig. 3). Although it was not possible to purify the metabolite from cellular experiments due to the presence of other co-eluting molecules (see Fig. 1), in non-cellular systems the metabolite was isolated with sufficient purity and quantity for mass spectroscopic analysis. Fast atom bombardment produced major ions of molecular fragments with mass 14 daltons larger than equivalent fragments of 5-ASA (Fig. 4), the largest mass being 167 was consistent with the product being 5-nitroso-salicylate (theoretical $M_r = 167.2$).

DISCUSSION

In this paper we have shown that the production by neutrophils of the major metabolite of 5-ASA is dependent upon both peroxidase activity and the presence of chloride ions. This evidence taken together with the mass spectroscopic analysis suggests that the major metabolite of 5-ASA generated by

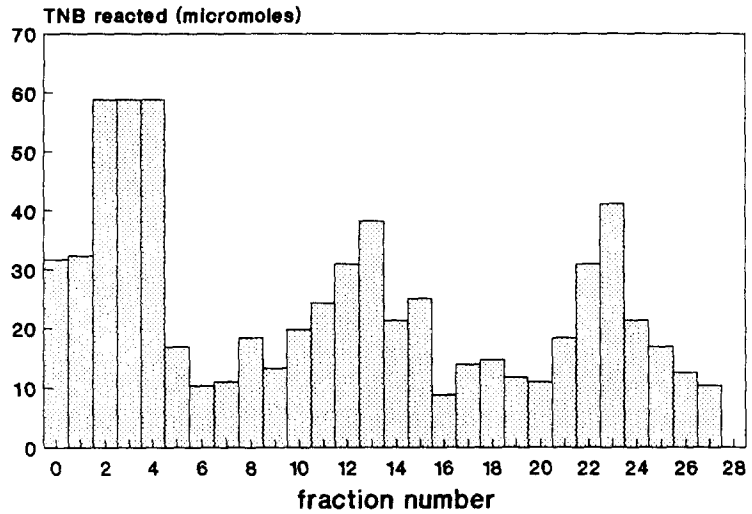

Fig. 3. Reactivity of metabolites with 2-thio-2-nitro-benzoic acid. After reaction of 5-ASA with human myeloperoxidase (0.2 units) and H_2O_2 (10 mM) for 15 min, HPLC was performed and samples of each fraction assayed by addition of excess 2-thio-2-nitro-benzoic acid. Reaction to form the colourless dithionitrobenzoate was determined by measurement of the absorbance at 412 nm and the moles of 2-thio-2-nitro-benzoic acid lost calculated using an absorption coefficient of $1.36 \times 10^4 M^{-1}cm^{-1}$. The three peaks of activity correspond to the unreacted 5-ASA (which control experiments showed gave this reaction) and the two metabolites of 5-ASA.

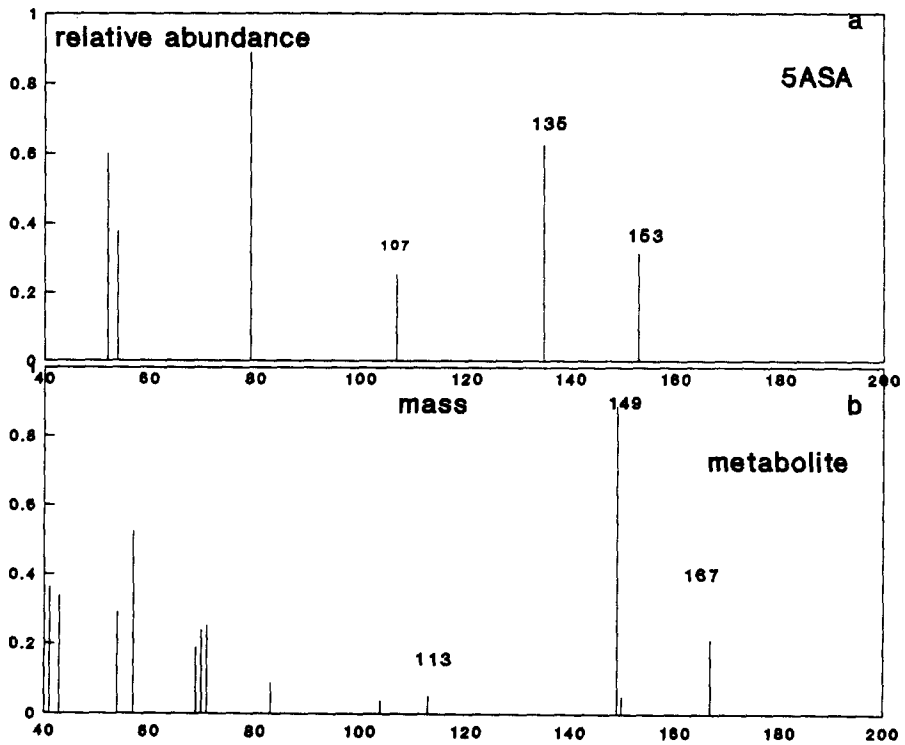

Fig. 4. Mass spectra of 5-ASA and its major peroxidase-generated metabolite. After reaction of 5-ASA with human myeloperoxidase (0.2 units) and H_2O_2 (10 mM) for 15 min, HPLC separation was performed using the volatile solvents TFA and CH_3CH. Fraction 14, containing the major 5-ASA metabolite, was concentrated by evaporation of the solvents and analysed by fast atomic bombardment. Mass spectra for (a) 5-ASA ($M_r = 53$) and (b) the metabolite are shown.

neutrophils is an amine-modified derivative of 5-ASA, 5-nitroso-salicylic acid. We propose that myeloperoxidase catalyses, by a reaction similar to those documented for other amino compounds [12–14] via the production of hypochlorite, the formation of a (mono- or di-) chloramine derivative of 5-ASA, which subsequently decomposes to form the 5-nitroso-salicylate. Hypochlorite has been proposed to mediate tissue damage, being a powerful oxidant which also inhibits α-1-antiproteinase [15, 16] and activates the latent activity of neutrophil collagenase [17, 18]. This hypochlorite scavenging effect of 5-ASA may provide an explanation for its efficacy in inflammatory bowel disease. This hypothesis is strengthened by the lack of efficacy of salicylate in protecting against inflammatory bowel disease and the efficacy of 4-ASA [19–21], indicating that the amino group is the important constituent of these molecules. We therefore conclude that the reaction of the amino group with hypochlorite to form the chloramine (and subsequently the nitroso-compound) is crucial for the therapeutic activity of 5-ASA. We must also conclude that the extracellular production of hypochlorite by inflammatory neutrophils is central to the pathogenesis of inflammatory bowel disease.

Our demonstration that neutrophils produce relatively large amounts of amine-modified 5-ASA is in contrast to the report by Dull *et al.* [5]. These workers reported only the production of small amounts of salicylate and gentisate (0.14 and 0.2%, respectively). Two features may account for this discrepancy. Firstly, we have used neutrophils stimulated to release myeloperoxidase [22], whereas Dull *et al.* [5] looked mainly at monocytes (containing only about one third the peroxidase), stimulated in a manner that would liberate little peroxidase. Secondly, our conditions caused the release of a group of molecules from the activated neutrophils which had similar retention times to those reported for salicylate and gentisate. These cell-derived molecules have been reported by other workers and were mainly chloro-taurine and other chloramines [23]. This may suggest the conditions of our experiments were more favourable for the formation of extracellular chloramines. Unfortunately, the presence of these molecules prevented our purification of the 5-ASA metabolite of interest from cellular experiments.

Some caution must be exercised in the exact chemical identification of the major metabolite identified here. Although reactivity with KI and TNB is often taken as evidence for chloramines, we have found that the reaction products from 5-ASA and H_2O_2 in the absence of chloride ions also react with these agents. The mass spectrum was not consistent with the product being 5-chloroamino-salicylate and the most likely identification was 5-nitroso-salicylate. Further investigation will be required to determine whether the production of the nitroso-compound occurred from decomposition of the chloramine during the cellular experiments or during preparation of the samples for analysis, for example during acidification and evaporation.

It is now important to establish whether the amine-modified derivative of 5-ASA is generated within the inflamed bowel and can be detected in the faecal material. Preliminary studies have found a molecule with the appropriate retention time and fluorescence in the faeces of a patient with active inflammatory bowel receiving 5-ASA and no other mediation. The unequivocal demonstration of the presence of amine-modified 5-ASA will provide evidence for the first time of the production of the extracellular production of hypochlorite in the inflamed bowel, *in vivo*. Furthermore, its detection will enable the course of the inflammatory process to be followed within patients receiving this drug and enable the efficacy of treatments to be monitored.

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