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Cytoprotection against neutrophil derived hypochlorous acid: a potential mechanism for the therapeutic action of 5-aminosalicylic acid in ulcerative colitis

F Dallegri, L Ottonello, A Ballestrero, F Bogliolo, F Ferrando, F Patrone

Abstract

The aim of the present study was to investigate the effects of 5-aminosalicyclic acid (5-ASA) on the cell injury mediated by activated neutrophils. We used a system constituted of neutrophils, triggered with phorbol myristate acetate, and ^{51}Cr-labelled Daudi cells as targets. The results show that 5-ASA is capable of efficiently preventing neutrophil-mediated lysis. 5-ASA was up to 10-fold more effective than taurine, which acts as an hypochlorous acid scavenger. Moreover, 5-ASA was found to compete with taurine for the neutrophil derived hypochlorous acid. The results are consistent with the conclusion that 5-ASA is capable of limiting the neutrophil mediated cell damage by scavenging the generated hypochlorous acid. This may represent a potential mechanism for the therapeutic action of 5-ASA in ulcerative colitis.

The therapeutic benefit of 5-aminosalicylic acid (5-ASA) in ulcerative colitis is well established.[1,2] It appears to act directly on the inflamed mucosa possibly by blocking cyclooxygenase and lipooxygenase metabolite mediated inflammation[2,3] and/or by reducing the local generation of highly reactive oxygen species.[4,5] This latter possibility is particularly attractive because of the neutrophil infiltration of the colonic mucosa, coupled with the well known ability of neutrophils to produce reactive oxygen species.[6] Nevertheless, to our knowledge, the actual capacity of 5-ASA to interfere with the oxidative cell damage mediated by neutrophils has not been directly proved. In the present paper, using a cytotoxicity model generally accepted to study the neutrophil mediated cellular damage (phorbol myristate acetate – triggered neutrophils plus Daudi target cells),[7,8] we provide evidence that 5-ASA is highly effective in limiting the neutrophil cytolytic potential.

First Medical Clinic, University of Genova Medical School, Genova, Italy
F Dallegri
L Ottonello
A Ballestrero
F Bogliolo
F Ferrando
F Patrone

Correspondence to: Dr Franco Dallegri, ISMI – Clinica Medica I, Viale Benedetto XV, no 6, I-16132 Genova, Italy

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Methods

MEDIA AND REAGENTS

Hanks' balanced saline solution with 1 mg/ml glucose and without phenol red (HBSS, Flow Lab, Ltd, Irvine, Scotland) was used as incubation medium. RPMI 1640 and fetal calf serum were purchased from Flow Lab. Taurine ($TauNH_2$), 5-aminosalicylic acid (5-ASA) and Triton X-100 were purchased from Sigma Chemical Co, St Louis, MO. The drug 5-ASA (~99% pure, as assayed by Sigma; lot 15 F-0803) was dissolved at the concentration of 5 mmol/l in 0·9% NaCl (37°C) immediately before each experiment. The solution of the drug was maintained in the dark to avoid 5-ASA oxidation by light. (The assays were carried out in the dark as well.) Heparin (Liquemin) was from Roche, Milan, Italy, and Ficoll-Hypaque from Nyegaard Co, Oslo, Norway. Na_2 (^{51}Cr) O_4 was from the Radiochemical Centre, Amersham, England. Phorbol-12-myristate-13-acetate (PMA, Sigma), stored at −20°C as stock solution of 2 mg/ml in dimethylsulphoxide (C Erba, Milan, Italy) was diluted in medium and used at the final concentration of 10 ng/ml. 5-thio-2-nitrobenzoic acid (Nbs) was prepared by reducing 5-5'-dithiobis (2-nitrobenzoic) acid (Sigma), as described by Aune and Thomas.[9] Hypochlorous acid was generated by adding sodium hypoclorite (NaOCl, BDH Ltd, Pool, UK) into solution buffered at pH 7·4.[10]

NEUTROPHILS

Heparinised (heparin 10 U/ml) venous blood was obtained from healthy male volunteers. Neutrophils were isolated by dextran sedimentation and subsequent centrifugation on a Ficoll-Hypaque density gradient, as previously described.[10] Contaminating erythrocytes were removed by hypotonic lysis.[10] Neutrophils were then washed three times with HBSS and resuspended in HBSS. Final cell suspensions contained 97% or more neutrophils and more than 98% viable cells, as evaluated by the ethidium bromide fluorescein diacetate test.[10]

DAUDI CELLS

The Daudi cell line (B lymphoblasts, kindly supplied by Prof G Damiani, Department of Biochemistry, University of Genova, Italy) was grown in suspension (RPMI-FCS) and subcultured every four to five days.[11] The cells were washed three times with HBSS and resuspended in HBSS before use. Daudi cells were labelled with 100–200 μCi sodium ^{51}Cr-chromate by incubating for one hour at 37°C.[11] After being washed, the cells were resuspended in HBSS.

CYTOLYTIC ASSAY

Daudi cell lysis by neutrophils in the presence of phorbol myristate acetate was measured using a ^{51}Cr release method.[10] The experiments were carried out in duplicate using 2×10^6 neutrophils,

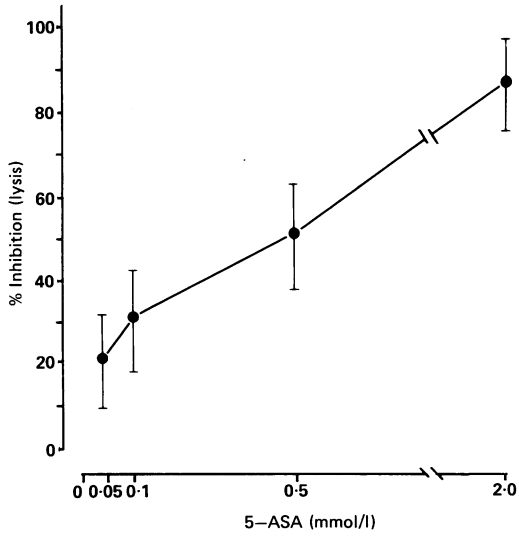

Figure 1: Inhibition of the neutrophil cytolytic activity (ordinate) by 5-ASA. Results are expressed as mean (1) SD of at least three experiments. The % ^{51}Cr release from labelled Daudi cells, incubated with phorbol myristate acetate triggered neutrophils in the absence of 5-ASA, was 27·6 (4·3) (×(1) SD, n=4). The compound 5-ASA had no effect on the ^{51}Cr release from Daudi cells incubated in absence of neutrophils.

5×10^5 Daudi cells, and 10 ng/ml phorbol myristate acetate in a final volume of 1 ml. Tests were done in Falcon Plastic tubes (17×100 mm, Falcon Plastic, Oxnard, Calif) and in a shaking water bath (100 rpm) at 37°C. After incubation for two hours, the ^{51}Cr release from labelled target cells was determined in the cell free supernatants. The percentage of cytolysis (% ^{51}Cr release) was calculated according to the formula (E−S)/(T−S)×100, where E is the cpm released in the presence of effectors, T is the cpm released after lysing target cells with 5% Triton X−100, S is the cpm spontaneously released by target cells in the absence of effectors (in each case ≤10%).

HYPOCHLOROUS ACID ASSAY

The generation of hypoclorous acid by neutrophils in the presence of phorbol myristate acetate was measured by the taurine ($TauNH_2$) trapping technique,[12] as previously described.[10] Briefly, 1×10^6 neutrophils and phorbol myristate acetate 10 ng/ml were incubated (37°C, one hour) in a final volume of 1 ml in the presence of 20 mmol/l $TauNH_2$ (17×100 mm Falcon Plastic tubes, shaking water bath at 100 rpm). After incubation, the amount of TauNHCl, generated by the reaction between hypochlorous acid and $TauNH_2$, (TauNH$_2$+hypochlorous acid→ TauNHCl+$H_2$0) was determined in the cell free supernatants by measuring spectrophotometrically (OD=412 nm, $\varepsilon=1{\cdot}36\times10^4$ mol/l/cm) the TauNHCl mediated oxidation of 5-thio-2-nitrobenzoic acid.[12] A series of experiments was also planned to detect the ability of 5-ASA to limit the formation of Tau NHCl from reagent hypochlorous acid and $TauNH_2$. These experiments were carried out by adding reagent hypochlorous acid (40 μmol/l) to a mixture of $TauNH_2$ (20 mmol/l) and 5-ASA in doses ranging from 0·05 to 1·0 mmol/l (final volume of the reaction=1 ml). After incubation for 10 minutes at 37°C, the amount of the generated TauNHCl was measured as described above.

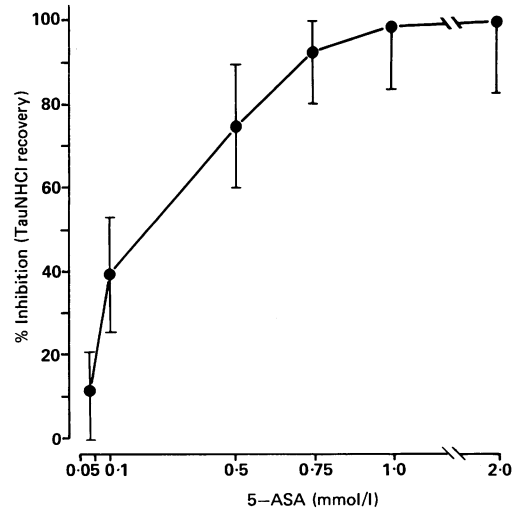

Figure 2: Inhibition of the hypochlorous acid recovery (measured as TauNHCl) from phorbol myristate acetate triggered neutrophils by 5-ASA. Results are expressed as mean (1) SD of at least three experiments. The TauNHCl recovery from neutrophils in the absence of 5-ASA was: 56:88 (4·6) nmol/10^6 neutrophils/1 h (×(1) SD, n=4).

OXYGEN CONSUMPTION ASSAY

The oxygen consumption by neutrophils was measured polarographically using a Clark electrode (Oxygen Monitor, Yellow Springs Instrument Co, Yellow Spring, Ohio) and using the technique described by Metcalf and coworkers.[13] Cell suspensions of 8×10^6 neutrophils in 2 ml HBSS were agitated continuously at 37°C. Oxygen consumption was measured after stimulation with phorbol myristate acetate (10 ng/ml) and the results expressed as nanomoles of oxygen consumed by 4×10^6 neutrophils per minute.

MYELOPEROXIDASE ASSAY

The myeloperoxidase activity released by neutrophils (10^6 cells/ml), incubated (one hour, 37°C) with 10 ng/ml phorbol myristate acetate, was determined in cell free supernatants as previously described.[10] Briefly, the myeloperoxidase assay was done by using 0·167 mg/ml 0-dianisidine (Sigma) and 0·1 mmol/l H_2O_2 in 50 mmol/l phosphate buffer (pH=6). One unit of enzyme activity was defined as that oxidising 1 μmol 0-dianisidine/min/25°C (od=550, ε= 11·3 mmol/l/cm).

Results

As shown in Figure 1, 5-ASA inhibited the neutrophil lytic activity in a dose dependent manner. Moreover, such a neutrophil function was almost completely inhibited by 20 mmol/l taurine ($TauNH_2$) which traps the neutrophil derived hypochlorous acid yielding TauNHCl[12] (% ^{51}Cr release from labelled Daudi cells incubated with phorbol myristate acetate triggered neutrophils: 3·5 (2·7) and 28·8 (3·2) in the presence and absence of 20 mmol/l $TauNH_2$ respectively, ×(1SD), n=3).

As depicted in Figure 2, 5-ASA lowered the recovery of TauNHCl from phorbol myristate acetate-triggered neutrophils incubated in the presence of $TauNH_2$ (20 mmol/l). This suggests that 5-ASA is likely to compete with $TauNH_2$ for the hypochlorous acid generated by neutrophils. Consistent with such a possibility, 5-ASA limited the formation of TauNHCl from reagent hypochlorous acid and $TauNH_2$ in a dose dependent manner (Fig 3). Thus, it appears that 5-ASA inhibits the neutrophil cytolysis

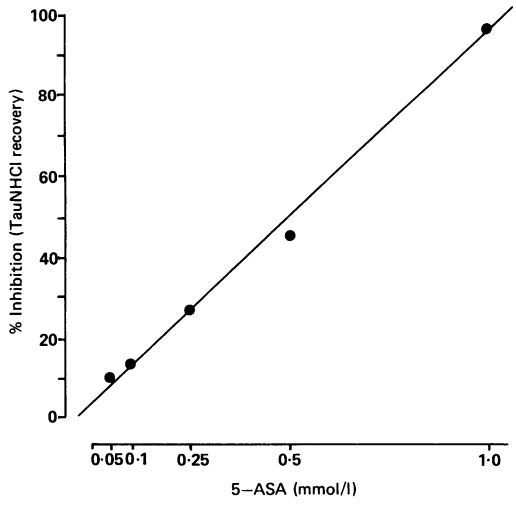

Figure 3: Effect of various doses of 5-ASA on the TauNHCl recovery from a system constituted of reagent hypochlorous acid (40 μmol/l) and $TauNH_2$ (20 mmol/l). Tests were performed in a final volume of 1 ml.

primarily by trapping the generated hypochlorous acid.

The compound 5-ASA gave only slight inhibition of the neutrophil oxygen uptake and myeloperoxidase positive primary granule exocytosis. When used at the concentration of 1 mmol/l, 5-ASA inhibited the neutrophil oxygen consumption by 15–8%. Concentrations of 5-ASA lower than 1 mmol/l were completely ineffective (oxygen consumption by phorbol myristate acetate triggered neutrophils in the absence of the drug: 8·74 (0·70) nmol oxygen per 4×10^6 cells per minute, ×(1SD), n=3). Finally, a slight inhibition (18–6%) of the myeloperoxidase release from neutrophils was only observed using relatively high doses (1 mmol/l) of 5-ASA (myeloperoxidase release from phorbol myristate acetate triggered neutrophils in the absence of the drug: 2·86 (0·32) mU/10^6 cells/1h, ×(1SD) n=3).

Discussion

Our results suggest that 5-ASA is capable of inhibiting the neutrophil cytolytic efficiency by scavenging the generated hypochlorous acid (HOCl). In agreement with reports from other authors,[7,14] the generation of hypochlorous acid by the neutrophil myeloperoxidase pathway ($H_2O_2+Cl^- \xrightarrow{MPO} HOCl+H_2O$) is crucial in promoting the neutrophil-dependent cell damage. In fact, $TauNH_2$, capable of efficiently trapping the generated hypochlorous acid to yield TauNHCl,[12] inhibited the lysis. When compared on a molar basis, 5-ASA was much as 10-fold more effective than $TauNH_2$ in reducing the neutrophil lytic activity. Also, 5-ASA was found to efficiently compete with $TauNH_2$ for reagent or neutrophil derived hypochlorous acid, lowering the production of TauNHCl. Thus, it appears that the reaction between 5-ASA and hypochlorous acid is efficient enough to protect the target cells from the neutrophil delivered hypochlorous acid dependent attack. Consistent with such a conclusion, 5-ASA was found to reduce the lysis of fibroblasts by a neutrophil free myeloperoxidase-H_2O_2-Cl-system,[15] presumably able to produce hypochlorous acid.[14]

The ability of 5-ASA reported here to limit the neutrophillytic efficiency observed at 5-ASA doses well within those detectable in the distal part of the intestinal lumen,[16] raises the possibility that this effect of the drug may contribute to its therapeutic activity in ulcerative colitis. This does not contradict the view that 5-ASA may also act by suppressing the production of prostaglandins or leucotrienes or both,[5,16] although the interference with the arachidonic acid metabolism has been reported to occur at doses of 5-ASA clearly higher than those used in the present set.

Moreover, owing to the wide range of biologic activities exerted by hypochlorous acid,[14] the cytoprotection against neutrophil attack is likely to be only one of the consequence of the hypochlorous acid-5-ASA interaction. In fact, the hypochlorous acid capacity of activating the neutrophil latent collagenase and inactivating proteinase inhibitors[14] suggests that the ability of 5-ASA to scavenge neutrophil derived hypochlorous acid may limit the degraradion of connective tissue components other than the tissue cell damage. Consistent with this view, 5-ASA has been recently shown to limit the inactivation of α_1-antiproteinase by reagent hypochlorous acid.[17]

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1 Habal FM, Greenberg GR. Treatment of ulcerative colitis with oral 5-aminosalycylic acid including patients with adverse reactions to sulfasalazine. *Am J Gastroenterol* 1988; **83**: 15–9.
2 Meyers S. The place of oral 5-aminosalicyclic acid in the therapy of ulcerative colitis. *Am J Gastroenterol* 1988; **83**: 64–7.
3 Craven PA, Pfanstiel J, Saito R, De Rubertis FR. Action of sulfasalazine and 5-aminosalicylic acid as reactive oxygen scavengers in the suppression of bile acid-induced increases in colonic epithelial cell loss and proliferative activity. *Gastroenterology* 1987; **92**: 1996–2008.
4 Del Soldato P, Campieri M, Brignola C, *et al.* A possible mechanism of action of sulfasalazine and 5-aminosalicylic acid in inflammatory bowel diseases: interaction with oxygen free radicals. *Gastroenterology* 1985; **89**: 1215–6.
5 Nielsen OH, Bukhave K, Elmgreen J, Ahnfelt-Ronne I. Inhibition of 5-lipoxygenase pathway of arachidonic acid metabolism in human neutrophils by sulfasalazine and 5-aminosalicylic acid. *Dig Dis Sci* 1987; **32**: 577–82.
6 Henson PM, Johnston RB Jr. Tissue injury in inflammation. Oxidants, proteinases and cationic proteins. *J Clin Invest* 1987; **79**: 669–74.
7 Clark RA. Extracellular effects of the myeloperoxidase-hydrogen peroxide-halide system. In: Weissmann G, ed. *Advances in inflammation research*, Vol 5. New York: Raven Press, 1983: 107–46.
8 Becker EL. The cytotoxic action of neutrophils on mammalian cells in vitro. *Prog Allergy* 1988; **60**: 183–208.
9 Aune TM, Thomas EL. Accumulation of hypothiocyanite ion during peroxidase-catalyzed oxidation of thiocyanate ion. *Eur J Biochem* 1977; **80**: 209–14.
10 Dallegri F, Patrone F, Ballestrero A, Frumento G, Sacchetti C. Inhibition of neutrophil cytolysin production by target cells. *Blood* 1986; **67**: 1265–72.
11 Dallegri F, Patrone F, Frumento G, Ballestrero A, Sacchetti C. Down-regulation of K cell activity by neutrophils. *Blood* 1985; **65**: 571–7.
12 Weiss SJ, Klei R, Slivka A, Wei M. Chlorination of taurine by human neutrophils. Evidence for hypochlorous acid generation. *J Clin Invest* 1982; **70**: 588–607.
13 Metcalf JA, Gallin JI, Nauseef WM, Root RK. *Laboratory manual of neutrophil function*. New York: Raven Press, 1986: 98–100.
14 Test ST, Weiss SJ. The generation and utilization of chlorinated oxidants by human neutrophils. *Adv Free Radical Biol Med* 1986; **2**: 91–116.
15 Molin L, Stendahl O. The effect of sulfasalazine and its active components on human polymorphonuclears leukocyte function in relation to ulcerative colitis. *Acta Med Scand* 1979; **206**: 451–7.
16 Hoult JRS. Pharmacological and biochemical actions of sulphasalazine. *Drugs* 1986; **32** [suppl 1]: 18–26.
17 Aruoma OI, Wasil M, Halliwell B, Hoey BM, Butler J. The scavenging of oxidants by sulphasalazine and its metabolites. A possible contribution to their anti-inflammatory effects? *Biochem Pharmacol* 1987; **21**: 3739–42.