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

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Abstract
The aim of the present study was to investigate the effects of 5-aminosalicylic acid (5-ASA) on the cell injury mediated by activated neutrophils. We used a system constituted of neutrophils, triggered with phorbol myristate acetate, and ^65Cr-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. It appears to act directly on the inflamed mucosa possibly by blocking cyclooxygenase and lipoxygenase metabolite mediated inflammation and/or by reducing the local generation of highly reactive oxygen species. 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. 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), we provide evidence that 5-ASA is highly effective in limiting the neutrophil cytolytic potential.

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 (Taudh), 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. Na2(^51Cr)O4 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. Hypochlorous acid was generated by adding sodium hypochlorite (NaOCl, BDH Ltd, Pool, UK) into solution buffered at pH 7.4.

**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. Contaminating erythrocytes were removed by hypotonic lysis. 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.

**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. The cells were washed three times with HBSS and resuspended in HBSS before use. Daudi cells were labelled with 100–200 μCi sodium ^51Cr-chromate by incubating for one hour at 37°C. 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 ^51Cr release method. The experiments were carried out in duplicate using 2×10^6 neutrophils,
from phorbol myristate
from neutrophils
Cytoprotection

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 % 

\[ 56:88 \pm 4.6 \text{ mmol/l} \times 10^8 \text{ Daudi cells, and } 10 \text{ ng/ml phorbol myristate acetate in a final volume of } 1 \text{ ml. Tests were done in Falcon Plastic tubes (17} \times 100 \text{ mm, Falcon Plastic, Oxnard, Calif) and in a shaking water bath (100 rpm) at } 37^\circ C. \text{ After incubation for two hours, the } ^{56} \text{Cr release from labelled target cells was determined in the cell free supernatants. The percentage of cytosis (% } \] 

\[ = \frac{(E-S)/(T-S)}{\times 100}, \text{ where } E \text{ is the cpm released in the presence of effectors, } T \text{ is the cpm released after lysing target cells with } 5 \% \text{ Triton X} - 100, \text{ and } S \text{ is the cpm spontaneously released by target cells in the absence of effectors (in each case } \pm 10\%). \]

Hypochlorous Acid Test

The generation of hypochlorous acid by neutrophils in the presence of phorbol myristate acetate was measured by the taurine (TauNH2) trapping technique, as previously described. Briefly, 1 \times 10^7 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 TauNH2 (17 \times 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 TauNH2, (TauNH2 + hypochlorous acid → TauNHCl + H2O) was determined in the cell free supernatants by measuring spectrophotometrically (OD=412 nm, ε=1.36 \times 10^5 \text{ mol/l/cm}) the TauNHCl mediated oxidation of 5-thio-2-nitrobenzoic acid. A series of experiments was also planned to determine the ability of 5-ASA to limit the formation of Tau NHCl from reagent hypochlorous acid and TauNH2. These experiments were carried out by adding reagent hypochlorous acid (40 μmol/l) to a mixture of TauNHCl (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.

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. Cell suspensions of 8 \times 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 \times 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. Briefly, the myeloperoxidase assay was done by using 0.167 mg/ml o-dianisidine (Sigma) and 0.1 mmol/l H2O2 in 50 mmol/l phosphate buffer (pH=6). One unit of enzyme activity was defined as that oxidising 1 μmol o-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 (TauNH2) which traps the neutrophil derived hypochlorous acid yielding TauNHCl (% \[ ^{56} \text{Cr release from labelled Daudi cells incubated with phorbol myristate acetate triggered neutrophils: } 3-5 (2.7) \text{ and } 28.8 (3.2) \text{ in the presence and absence of } 20 \text{ mmol/l TauNH2 respectively, } \times (1 SD), n=3). \]

As depicted in Figure 2, 5-ASA lowered the recovery ofTauNHCl from phorbol myristate acetate-triggered neutrophils incubated in the presence of TauNH2 (20 mmol/l). This suggests that 5-ASA is likely to compete with TauNH2 for the hypochlorous acid generated by neutrophils. Consistent with such a possibility, 5-ASA limited the formation of TauNHCl from reagent hypochlorous acid and TauNH2 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 mmol/l) and TauNH2 (20 mmol/l). Tests were performed in a final volume of 1 ml.

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 leukotrienes or both,17 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,1 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 inhibitors18 suggests that the ability of 5-ASA to scavenge neutrophil derived hypochlorous acid may limit the degradation 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 α1-antiproteinase by reagent hypochlorous acid.19

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 the generation of hypochlorous acid by the neutrophil myeloperoxidase pathway (H2O2+Cl−−→HOCl+H2O) is crucial in promoting the neutrophil-dependent cell damage. In fact, TauNH2, capable of efficiently trapping the generated hypochlorous acid to yield TauNHCl,2 inhibited the lysis. When compared on a molar basis, 5-ASA was much as 10-fold more effective than TauNH2 in reducing the neutrophil lytic activity. Also, 5-ASA was found to efficiently compete with TauNH2 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-H2O2-Cl−-system,15 presumably able to produce hypochlorous acid.14

The ability of 5-ASA reported here to limit the neutrophillic efficiency observed at 5-ASA

primary 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 15–18%. Concentrations of 5-ASA lower than 1 mmol/l were completely ineffective (oxygen consumption by phorbol myristate acetate stimulated neutrophils in the absence of the drug: 8.74 (0.70) nmol oxygen per 4×10⁶ 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 stimulated neutrophils in the absence of the drug: 2.86 (0.32) μU/10⁶ cells/l, ×(1SD) n=3).

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